Universidad de León
Departamento de Biología Molecular
Facultad de Ciencias Biológicas y Ambientales
INBIOTEC, Instituto de Biotecnología de León

Ph.D. Thesis

**Proteomics and secretomics of *Penicillium chrysogenum*:
Molecular characterization of proteins relevant for
penicillin biosynthesis**

**Proteómica y secretómica de *Penicillium chrysogenum*:
Caracterización molecular de proteínas importantes
para la biosíntesis de penicilina**

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León, 2010

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تقدیم به مادر فداکارم، همسر مهربانم و همه اعضای خانواده ام

In memory of my honorable father,

In memory of my affable brother,

This thesis is dedicated to my wife, my mother and my family

ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my supervisor, Prof. Juan-Francisco Martín Martín. His wide knowledge and his logical way of thinking have been of great value for me. His understanding, encouraging and personal guidance have provided a perfect basis for the present thesis.

I am deeply grateful to my co-supervisor, Dr. Carlos García-Estrada, for his detailed and constructive comments, and for his fundamental and important support throughout this work. His valuable advice and friendly help have been very helpful for this study.

I wish to express my warm and sincere thanks to Dr. Carlos Barreiro who introduced me to the field of proteomics. His ideals and concepts have had a remarkable influence on my entire career in this field.

During this work I have collaborated with many colleagues for whom I have great regard, and I wish to extend my warmest thanks to Rebeca Dominguez-Santos for her help in this study.

I owe my loving thanks to my darling wife, Zahra Salehi, for her essential assistance in this work as well as helping me to own a happy life in Spain.

I wish to express my love and gratitude to my mother and my family members for their understanding, support and endless love, through the duration of my studies. Without their encouragement and understanding it would have been impossible for me to finish this work.

The financial support of the University of Leon and the institute of biotechnology of Leon (INBIOTEC) are gratefully acknowledged.

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ABBREVIATIONS:

2-DE: two dimensional gel electrophoresis

ACN: acetonitrile

ad7-ACCCA: adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid

CC: Colloidal Coomassie

DTT: 1,4-dithiothreitol

IAT: acyl-coenzyme A:isopenicillin N acyltransferase

IPN: Isopenicillin N

NL: non-linear

PMF: peptide mass fingerprinting

ppm: parts per million

SDS: sodium dodecyl sulphate

TCA: 2,2,2-tri-chloroacetic acid

TFA: trifluoroacetic acid

%Vol: relative volume.

AIMS AND SCOPE

Eighty years after the discovery of penicillin, the interest of this β -lactam antibiotic at industrial level is still remarkable. Despite the good background knowledge, little is known on how *Penicillin chrysogenum* became a good penicillin overproducer, and much of the molecular basis for improved productivity remains obscure. Some light has been shed on this issue after the recent publication of the *P. chrysogenum* genome (van den Berg *et al.*, 2008). These authors reported that transcription of genes involved in biosynthesis of the amino acid precursors for penicillin biosynthesis, as well as of genes encoding microbody proteins was higher in the high-producing strain DS17690. However, full exploitation of *P. chrysogenum* requires the integration of knowledge from other -omics such as proteomics.

Proteomics studies are one of the most powerful methods to evaluate the final result of gene expression and 2-DE has been the technique of choice to obtain a reference global picture of the proteomic and secretomic map. This technique has been successfully applied to other ascomycete fungi, such as *Schizosaccharomyces pombe* (Hwang *et al.*, 2006), some *Aspergilli* (Melin *et al.*, 2002; Kniemeyer *et al.*, 2006; Vödisch *et al.*, 2009) or *Saccharomyces cerevisiae* (Shevchenko *et al.*, 1996; Bruckmann *et al.*, 2009). But only Kiel and coworkers (2009) have performed a proteomics-based inventory of the proteins present in the microbody matrix of *P. chrysogenum*. Therefore, it was necessary to study in depth the cytosolic proteome and secretome of this important fungus.

In this work, we have optimized the methods to extract the *P. chrysogenum* cytosolic proteins (proteome) as well as secreted protein to the culture (secretome). As a result, the cytosolic proteome reference map and secretome of this filamentous fungus has been developed. We have also characterized the relevant modifications produced during the industrial strain improvement program through the proteome analysis and of three *P. chrysogenum* strains: i) the wild-type strain NRRL 1951 isolated in Peoria, IL, ii) the genome reference strain Wisconsin 54-1255 (an improved but still low-producer) and iii) the high-producing strain AS-P-78 developed by Antibioticos S.A. (León, Spain).

CHAPTER 1

INTRODUCTION

1.1. *Penicillium chrysogenum*

Penicillium chrysogenum (Figure 1.1) is a mold that is widely distributed in nature, and is often found living on foods and in indoor environments. From this fungus the antibiotic penicillin is obtained in large scale in industrial fermentators. Penicillin works against gram-positive bacteria, such as *Staphylococcus* and *Streptococcus pneumoniae* by disrupting bacterial cell wall synthesis (Samson *et al.*, 1977). It has rarely been reported as a cause of human disease but the airborne spores of *P. chrysogenum* are important human allergens. Vacuolar and alkaline serine proteases have been identified as the major allergenic proteins (Shen *et al.*, 2003).

Other secondary metabolites of *P. chrysogenum* include various different penicillins, roquefortine C, meleagrin, chrysogenin, xanthocillins, secalonc acids, sorrentanone, sorbicillin, and PR-toxin (De Hoog *et al.*, 2000). *P. chrysogenum* has been used industrially to produce penicillin and xanthocillin X, to treat pulp mill waste, and to produce the enzymes polyamine oxidase, phospho-gluconate dehydrogenase, and glucose oxidase (Raper *et al.*, 1949, De Hoog *et al.*, 2000).

Figure 1.1. *Penicillium chrysogenum*; **A.** The morphology of spores in solid culture, **B.** Microscopic image of conidia and **C.** the scientific classification.

Like the many other species of the genus *Penicillium*, *P. chrysogenum* reproduces non-sexually by forming dry chains of spores (or conidia) from brush-shaped conidiophores. The conidia are typically carried by air currents to new colonisation sites. In *P. chrysogenum*, the conidia are blue to blue-green, and the mold sometimes exudes a yellow pigment. However, *P. chrysogenum* cannot be identified based on color

alone. Observations of morphology and microscopic features are needed to confirm its identity (Raper *et al.*, 1949).

The name *Penicillium* comes from the resemblance of the conidiophores of the fungus to a paintbrush (penicillus is the Latin word for paintbrush). *Penicillium* is a member of the deuteromycetes, fungi with no known sexual state. But recently it has been shown that *P. chrysogenum* has opposite mating types and that both have retained transcriptionally expressed pheromone and pheromone receptor gene required for sexual reproduction (Hoff *et al.*, 2008). Some other species of *Penicillium* have also an additional sexual state in the Ascomycota division of the Eurotiales (Samson *et al.*, 1977).

1.1.1. A brief history of the discovery of penicillin

The discovery of antibiotics is one of the most important milestones of the human story. Penicillin was discovered quite occasionally by Alexander Fleming, who was a Scottish-born microbiologist. He had also done some work during world war I with a bacteriolytic enzyme found in human tears. After the war he had a faculty position at St Mary's Hospital in London. When he went away for a weekend in 1928, neglected to clean up his bacterial experiments with *Staphylococcus aureus*, leaving the dishes out on the lab bench. He returned after a long weekend and noticed that some mold was growing on one of the plates. He also noticed that there was a clearing in the lawn of bacteria around one of the molds. Discovery of penicillin was really a lucky set of coincidences. Fleming tried to repeat the inadvertent experiment, with no success. But his colleague, Ronald Hare, was able to reproduce the results by lowering the temperature. Not all strains of *Penicillium* produce penicillin. The strain that fell onto Fleming's plates was a particularly good producer of penicillin (Raper *et al.*, 1944).

Fleming published his results in 1929 in the British Journal of Experimental Pathology (Fleming, 1929). Fleming and his colleagues did a series of experiments that demonstrated the ability of extracts of the fungus to kill various gram-positive bacteria, including *Staphylococcus pyogenes*, *S. viridans*, *Micrococcus* spp., and a few others. Effectiveness against gram-negative bacteria (such as *Escherichia coli* and *Klebsiella pneumoniae*) was limited with very high concentrations of penicillin needed to kill those organisms. We now know that an “external membrane” protects those gram-

negative bacteria against penicillin. Fleming and his coworkers also performed careful experiments by injecting penicillin with no toxic effects into mice. They did not report on the cure of any bacterial diseases in mice, but speculated that penicillin might have some efficacy against infections by *Staphylococcus* and *Pneumococcus*. They identified the mold as *Penicillium rubrum*, which Charles Thom would identify (many years later) as *Penicillium notatum*. Fleming did not realize the practical importance of his discovery and went on to work on other projects (Fleming *et al.*, 1929, Raper *et al.*, 1944).

Figure 1.2. From left to right; Fleming, Chain y Florey, they won the Medicine and Physiology Nobel Prize in 1945.

Fleming and his group's attempts to purify penicillin were unsuccessful, partly due to the instability and also the low production yield by the fungus. Ten years later, a group of students at the Sir William Dunn School of Pathology of the Oxford University (Howard W. Florey, Ernst B. Chain, Norman Heatley, CM Fletcher, AD Gardner, M.A. Jennings, J. Orr-Ewing, A.G. Saunders and Edward Abraham) resumed studies on penicillin. This group managed to purify a penicillin extract from the original Fleming's strain and confirmed in mice as a chemotherapicaly active agent with no toxicity (Abraham *et al.*, 1941). The next step was to conduct a clinical trial in humans. Since the production of penicillin by *P. notatum* was very low, it took a year to accumulate enough amount of compound for this aim. The success made evident the production of penicillin on a large scale but at this point, the Second World War made it impossible. Further research was continued in the Northern Regional Research

Laboratory (NRRL) in Peoria, Illinois (USA) in 1941. Then, a great cooperation was established between universities and industrial laboratories in England and the United States. They were heavily financed by the U.S. government after the USA joined the war.

For the discovery of penicillin and the development of a method of mass production, Sir Alexander Fleming, Ernst B. Chain and Howard W. Flory won the “Nobel prize” in medicine and physiology in 1945 (Figure 1.2).

1.1.2. The *P. chrysogenum* Industrial strain improvement

Due to the very low amounts of penicillin (1.2 µg/ml) yielded by the original Fleming’s *P. notatum* strain (NRRL1249B21), the isolation of new strains became of paramount importance. The improvement of the original strain occurred in 1943 with the isolation of *P. chrysogenum* NRRL 1951 from a cantaloupe in Peoria, IL, USA (Raper *et al.*, 1944). This microorganism was more suitable than *P. notatum* for penicillin production (60 µg/ml to 150 µg/ml) in submerged cultures for which it was X-ray treated by Dr. Demerec (Carnegie Institution at Cold Spring Harbor, 1955 New York), giving rise to the X-1612 mutant (Figure 1.3). This mutant was able to yield 300 µg/ml of penicillin and was subjected to ultraviolet mutation at the University of Wisconsin. After several rounds of classical mutagenesis, the Q-176 strain, which produces up to 550 µg/ml of penicillin, was obtained. This strain was adopted by industry and is the original ancestor of the Wisconsin line of strains; the low-producing Wisconsin 54-1255 (Elander, 1983) and subsequent strains (Elander, 2002). They gave rise to high penicillin producing strains, such as the P2 strain, derived from the Panlabs in Taiwan (Lein, 1986), the DS04825 strain, obtained at DSM (The Netherlands) (Gouka *et al.*, 1991) or the E1 and the AS-P-78 strains, which were selected at Antibioticos S.A. (León, Spain). Some of these strains are the parents of those currently used for the industrial production of penicillin, which reaches titers of more than 50 mg/ml.

Figure 1.3. Genealogy of some *P. chrysogenum* strains during industrial strain improvement programs. Thin arrows represent either steps of selection (without mutagenic treatment) or X irradiation, UV irradiation or nitrogen mustard treatment. Arrows between strains BL3-D10 and Wis 54-1255 represents a total of nine selection steps. The thick arrows between the Wis 54-1255 strain and the high-producer strains represent several selection steps in different industrial laboratories. The names of the strains used in this work are boxed. Penicillin titers are indicated on the right for some of these strains.

The numerous rounds of mutagenesis undergone by the *P. chrysogenum* strains have induced the production of several modifications and biochemical and genetical analysis have allowed the identification of some of them. It is well known that the homogentisate pathway for the catabolism of phenylacetic acid (the side chain precursor in the biosynthesis of benzylpenicillin) is diminished in the Wis 54-1255 and presumably, in derived strains as well. This is due to modifications in the microsomal cytochrome P450 monooxygenase (encoded by the *phacA* gene) involved in the 2-hydroxylation of phenylacetic acid. *P. chrysogenum* Wis 54-1255 has a L181F mutation

in the *pahA* gene, which leads to a reduced oxidation of phenylacetic acid and to penicillin overproduction (Rodríguez-Sáiz *et al.*, 2001).

Another well-characterized modification resultant from the industrial improvement process is the amplification of the genomic region that includes the three penicillin biosynthetic genes. These genes, *pcbAB*, *pcbC* and *penDE*, are linked in a single cluster located on a DNA region that is amplified in tandem repeats bounded by a conserved hexanucleotide TGTAAG/T in penicillin overproducing strains. The amplifiable unit is either 106.5 or 56.8 kb in length depending on the strains. This region is present as a single copy in the genome of the wild-type NRRL 1951 and Wis 54-1255 strains. However, many of the improved penicillin producers contain several copies of the amplified region, like the AS-P-78 strain, which contains 5 or 6 copies. Another penicillin overproducer strain, *P. chrysogenum* E1, also contains a large number of copies in tandem of a 56.8-kb DNA fragment. Furthermore the deletion mutant *P. chrysogenum* npe10 showed a deletion of 57.9 kb that corresponds exactly to the DNA fragment that is amplified in E1 (Fierro *et al.*, 1995; Newbert *et al.*, 1997).

Sequencing and bioinformatic analysis of the 56.8 kb amplified region revealed a total of 15 ORFs (open reading frames) in Wis 54-1255 (van den Berg *et al.*, 2007), and 16 ORFs in *P. chrysogenum* AS-P-78 (Fierro *et al.*, 2006). Three of them correspond to the known penicillin biosynthetic genes (*pcbAB*, *pcbC*, and *penDE*), surrounded by ORFs with no clear function, since none of these ORFs is related to penicillin precursor formation, or penicillin transport and regulation. But surprisingly, several genes known to play an important role in antibiotic biosynthesis in fungi are not included in this region, like the *phl* gene (encoding the phenylacetyl-CoA ligase) (Lamas-Maceiras *et al.* 2006) or the phosphopantetheinyl transferase (PPTase) encoded by the *ppt* gene (García-Estrada *et al.* 2008a).

Microbodies (peroxisomes), organelles containing the enzymes isopenicillin N acyltransferase and phenylacetyl-CoA ligase involved in the two final steps of the penicillin pathway (Muller *et al.*, 1991; Lamas-Maceiras *et al.*, 2006), are also known to be enhanced in high-producing strains (Muller *et al.*, 1991; van den Berg *et al.*, 2008).

1.2. β -LACTAM ANTIBIOTICS

1.2.1. The structure of β -lactams

β -Lactams, like many other secondary metabolites, have unusual chemical structures. All β -lactams contain a four-membered β -lactam ring closed by an amide bond (Figure 1.4).

Penicillins contain a bicyclic ‘penam’ nucleus formed by fused β -lactam and thiazolidine rings and an acyl side-chain bound to the amino group at C-6. They are produced by a few *Penicillium* and *Aspergillus* species (Aharonowitz *et al.*, 1992). Recently penicillins have been found to be produced by a few other deuteromycetes (Laich *et al.*, 1999; 2002; 2003).

A second β -lactam compound, cephalosporin C, produced by the fungi *Acremonium chrysogenum*, *Paecilomyces persicinus* and *Kallichroma tethys* (Kim *et al.*, 2003) and some other deuteromycetes, contains the cephem nucleus (a sixmembered dihydrothiazine ring fused to the β -lactam ring) (Figure 1.4).

Cephalosporin C has a D- α -aminoadipyl side chain attached to the C-7 amino group, which is identical to that of hydrophilic penicillin N but differs from those of hydrophobic penicillins. Penicillins and cephalosporins are of great clinical interest as inhibitors of peptidoglycan biosynthesis in bacteria.

Two groups of modified cephalosporins, the cephamycins and the cephabacins, are produced respectively by various Gram-positive actinomycetes and some Gram-negative bacteria (Aharonowitz *et al.*, 1992). In the cephamycins, the cephem nucleus contains, in addition to the α -aminoadipyl side chain, a methoxy group at C-7; this group renders the cephamycin structure insensitive to hydrolysis by most β -lactamases (Liras *et al.*, 1998).

All these β -lactam compounds (penicillins, cephalosporins, cephamycins and cephabacins) share a common mode of action and are synthesized from similar precursors by pathways with some steps in common.

Figure 1.4. **A.** Chemical structure of the β -lactam ring in penicillins. **B.** The penicillins (penam nucleus) and cephalosporins (cephem nucleus). Different penicillins contain distinct hydrophobic or hydrophilic side-chains “R”; The R₁ and R₂ groups of cephalosporins or cephamycins are indicated below the structure of cephalosporin. **C.** More examples from penicillins and cephalosporins.

1.2.2. Penicillin, cephalosporin and cephamycin biosynthesis

A brief description of the biochemical pathways leading to classical β -lactam antibiotic biosynthesis is made here. More detailed information on the specific steps are given in several review articles (Aharonowitz *et al.*, 1992; Brakhage, 1998; Demain *et al.*, 1998; Martín *et al.*, 1999, 2010; Liras and Martín, 2009). The biosynthesis of the β -lactam compounds proceeds through a series of sequential reactions including the formation of a linear tripeptide intermediate and cyclization of the tripeptide to form the penam nucleus (so-called ‘early biosynthetic steps’), conversion of the penam to the cephem nucleus by ring expansion (‘intermediate steps’) and ‘late (decoration) steps’ (Figure 1.5) involving modifications of the β -lactam nucleus.

Three amino acids, L- α -aminoadipic acid, L-cysteine and L-valine, are the precursors of the basic structure of all the classical β -lactam antibiotics; L-valine and L-cysteine are common amino acids but L- α -aminoadipic acid is a non-proteinogenic amino acid and is formed by a specific pathway related to lysine biosynthesis. In bacteria-producing β -lactams, lysine is converted into α -aminoadipic acid semialdehyde by lysine-6-aminotransferase (LAT) and this semialdehyde is oxidized to α -aminoadipic acid by a piperidine-6-carboxylic acid dehydrogenase (P6C-DH) (Coque *et al.*, 1991; de la Fuente *et al.*, 1997).

In fungi, α -aminoadipic acid is an intermediate of the lysine biosynthesis pathway. In addition, lysine is catabolized to α -aminoadipic acid in *P. chrysogenum* (i) by an ω -aminotransferase, encoded by the *oat1* gene, which is induced by lysine (Martín de Valmaseda *et al.*, 2005), and (ii) by a reversal of the lysine biosynthesis pathway catalysed by the enzymes saccharopine dehydrogenase/saccharopine reductase (Naranjo *et al.*, 2001; Martín de Valmaseda *et al.*, 2005). Two early enzymatic steps are common to all the classical β -lactam producers, resulting in the formation of isopenicillin N (IPN), the first compound in the pathway with antibiotic activity. The first enzyme of the pathway is the ACV synthetase (ACVS), a non-ribosomal peptide synthetase. The ACV synthetases are very large multifunctional proteins (Mr in the order of 420 kDa) encoded by intron-free genes of 11 kb named *pcbAB* (Díez *et al.*, 1990; Gutiérrez *et al.*, 1991) which occur in the fungal and bacterial penicillin and cephalosporin (and cephamycin) clusters (Figure 1.5).

This enzyme sequentially activates the three substrates with ATP to form aminoacyl-adenylates, binds them to the enzyme as thioesters, epimerizes the L-valine to D-valine, links together the three amino acids to form the peptide δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine (hereafter named ACV) and, finally, releases this tripeptide from the enzyme by the action of an internal thioesterase activity. The ACV synthetases have three well conserved domains that activate each of the three amino acids respectively (Zhang and Demain, 1992; Aharonowitz *et al.*, 1993; Martín, 2000a).

Figure 1.5. Biosynthetic pathways of benzylpenicillin, cephalosporin C, cephamycin C and cephabacins. The L- or D-configuration of the α -aminoadipic side-chains in each molecule is indicated by L or D on carbon 1.

The second enzyme in the pathway is the IPN synthase (IPNS, cyclase) encoded by the *pcbC* gene. The IPN synthases are intermolecular dioxygenases that require Fe^{2+} , molecular oxygen and ascorbate. They remove four hydrogen atoms from the ACV tripeptide forming the bicyclic structure (penam nucleus) of IPN. The cyclase of *P. chrysogenum* has been crystallized showing a bread roll-like structure (Roach *et al.*, 1995; 1997). In addition to the *pcbAB* and *pcbC* genes common to filamentous fungi and bacteria, the producers of hydrophobic penicillins (i.e. *Penicillium*, *Aspergillus*) contain a third gene in the penicillin cluster, named *penDE*, of eukaryotic origin (it contains three introns at difference of *pcbAB* and *pcbC* that are of bacterial origin) which encodes an IPN acyltransferase (IAT). This enzyme hydrolyses the α -minoadipic

side-chain of IPN and introduces an acyl molecule activated as its acyl-CoA derivative to produce hydrophobic penicillins (e.g. benzylpenicillin). This gene is not present in cephalosporin C- or cephamycin-producing microorganisms.

In addition to these key enzymes, other enzymes are also required for penicillin biosynthesis, such as the aryl-CoA ligases, which activate the side-chain aromatic acid (Lamas-Maceiras *et al.*, 2006; Wang *et al.*, 2007) and the phosphopantetheinyl transferase (PPTase) (Baldwin *et al.*, 1991a; Lambalot *et al.*, 1996), which activates the non-ribosomal ACV tripeptide synthase (the first enzyme of the pathway) (García-Estrada *et al.* 2008a).

Isopenicillin N is converted to its D-isomer (penicillin N) in all the cephalosporin and cephamycin producers. Purification of the *A. chrysogenum* so-called ‘IPN epimerase’ proved to be difficult and unreliable. In 2002 a breakthrough in our understanding of cephalosporin formation occurred when it was reported that the epimerization reaction was different in eukaryotic and prokaryotic microorganisms. The epimerization of IPN in *A. chrysogenum* is encoded by two linked genes, *cefD1*–*cefD2*, located in the ‘early’ cephalosporin gene cluster. The first gene, *cefD1*, has four introns and encodes a 71 kDa protein with similarity to fatty acid acyl-CoA synthetases. In bacteria, epimerization of IPN to penicillin N is catalysed by a classical pyridoxal-phosphate-dependent epimerase. The second gene, *cefD2*, contains one intron and encodes a protein homologous to α -methyl-acyl-CoA racemases of eukaryotic origin. Disruption of either of these ORFs results in a lack of cephalosporin C production, loss of IPN epimerase activity and accumulation of IPN in the culture (Ullán *et al.*, 2002a).

The proposed conversion includes three biochemical steps: CefD1 converts IPN into isopenicillinyl N-CoA; then CefD2 isomerizes the compound into penicillinyl N-CoA, which seems to be released from the enzyme by the third enzyme, a thioesterase (see Figure 1.5).

The following step in the cephalosporin/cephamycin pathway is the enzymatic expansion of the five-membered thiazolidine ring of penicillin N to a six-membered dihydrothiazine ring. The enzyme responsible of this important conversion is the deacetoxycephalosporin C (DAOC) synthase. This protein is an intermolecular dioxygenase that requires Fe²⁺, molecular oxygen and α -ketoglutarate to form DAOC and succinic acid. The DAOC synthase does not recognize (or does so very poorly) the

isomer IPN, penicillin G or the deacylated 6-aminopenicillanic acid (6-APA) as substrates (Wu *et al.*, 2005), although it recognizes adipyl- or glutaryl-6-APA derivatives. The DAOC synthase of *Streptomyces clavuligerus* has been crystallized (Öster *et al.*, 2006) and the gene *cefE* has been introduced into *P. chrysogenum*, leading to the production of adipyl-7-aminodeacetoxycephalosporanic acid (ad-7-ADCA) and adipyl-7-aminocephalosporanic acid (ad-7-ACA), (reviewed by Díez *et al.*, 1997). The production of adipyl derivatives requires addition of adipic acid to the fermentation. The DAOC synthase from *A. chrysogenum* is also able to catalyse the next step of the pathway, namely the hydroxylation at C-3-forming deacetylcephalosporin C (DAC), whereas in bacteria there is a separate C-3 hydroxylase encoded by *cefF* that performs this reaction.

The final step in cephalosporin C biosynthesis is the conversion of DAC to cephalosporin C by the DAC-acetyltransferase, which uses acetyl-CoA as donor of the acetyl group. This enzyme encoded by the *cefG* gene has a Mr of 49 kDa and is evolutionary similar to O-acetylhomoserine acetyl transferases (Gutiérrez *et al.*, 1992; Velasco *et al.*, 1999). The *cefG* gene contains two introns and is linked to the *cefEF* gene, but in the opposite orientation (Figure 1.6).

1.2.3. Penicillin biosynthesis genes

While in *A. nidulans* and *P. chrysogenum* the penicillin biosynthesis genes are found in a single cluster, in *A. chrysogenum* the genes involved in cephalosporin biosynthesis are organized in at least two clusters located on different chromosomes. The *pcbAB*, *pcbC*, *cefD1* and *cefD2* genes are linked in the so-called ‘early’ cephalosporin cluster, while the ‘late’ cluster contains the *cefEF* and *cefG* genes (Figure 1.6). These genes are involved in the last two steps, which are specific for cephalosporin C biosynthesis (Gutiérrez *et al.*, 1992). Expression of the cephalosporin biosynthesis genes in *A. chrysogenum* is also controlled by several global regulators including the carbon catabolite repressor CreA (Jekosch and Kück, 2000), the pH regulator PacC (Schmitt *et al.*, 2001; 2004a) and the winged helix transcriptional factor CPCR1 (Schmitt *et al.*, 2004b).

Figure 1.6. A. Penicillin gene clusters in *P. chrysogenum* and *Aspergillus nidulans*. B. Cephalosporin gene clusters in *A. chrysogenum*. The 'early cluster' contains seven genes encoding the biosynthetic steps up to penicillin N and the MFS transporters, and the 'late cluster' contains the two genes converting penicillin N to cephalosporin C (see text).

1.2.4. Expression of the penicillin biosynthesis gene cluster

As indicated before in *P. chrysogenum*, the three 'core' genes responsible for penicillin biosynthesis are clustered with other ORFs forming an amplifiable DNA unit of 56.8 kb (Fierro *et al.*, 2006; van den Berg *et al.*, 2007); this unit is present in several copies in high-penicillin-producing strains (Fierro *et al.*, 1995). The gene *phlA* encoding the phenylacetyl-CoA ligase and the *ppt* gene encoding the PPTase that activates the ACV synthetase are not located in the amplifiable region.

Two of the penicillin biosynthetic genes, *pcbAB* and *pcbC*, are transcribed from divergent (bidirectional) promoter regions (Figure 1.6). The expression of genes from a region containing two divergent promoters has been associated with reorganization of the chromatin structure that allows facilitated interaction of those promoter regions with the RNA polymerase II and the transcriptional factors (García *et al.*, 2004; Ishida *et al.*, 2006; Shwab *et al.*, 2007). These transcriptional factors exert a regulatory role in the biosynthesis of penicillin.

1.2.5. Regulation of the biosynthesis of penicillin

Biosynthesis of hydrophobic penicillins is affected by several factors. Easily utilizable carbon, nitrogen and phosphorous sources have been shown to affect dramatically the production of penicillin. This indicates the existence of complex regulatory processes controlling the synthesis of penicillin (Sánchez *et al.*, 1981; Abraham, 1986; Chang *et al.*, 1990; Aharonowitz *et al.*, 1992; Brakhage and Turner, 1992; 1995; Brakhage *et al.*, 1992; Feng *et al.*, 1994; Brakhage, 1997; 1998).

1.2.5.1. Carbon catabolite regulation

Carbohydrates exert an important role on regulation of the penicillin biosynthetic genes. Glucose, sucrose, maltose, fructose and galactose (these three to a lesser extent) repress the activity of ACV synthetase and IPN synthase but not (or only to a low extent) acyltransferase in *P. chrysogenum* (Revilla *et al.*, 1984, 1986). Glucose represses the expression of the *pcbAB* and *pcbC* gene promoters (Feng *et al.*, 1994). Sucrose and glucose also repress expression of the *pcbC* gene in *A. nidulans* (Espeso and Peñalva, 1992; Brakhage *et al.*, 1992), although other authors reported that these sugars seem not to have significant effect on *pcbC* or *penDE* genes (Brakhage *et al.*, 1992; Litzka *et al.*, 1995). In this microorganism, carbon repression of penicillin genes occurs via an unknown mechanism distinct from the wide-domain carbon regulators CreA, CreB and CreC (Espeso and Peñalva, 1992).

1.2.5.2. Nitrogen source regulation

Ammonium has been shown to be an important repressor of both *pcbAB* and *pcbC* gene promoters in *P. chrysogenum* (Feng *et al.*, 1994). When this compound is used as nitrogen source in both *P. chrysogenum* and *A. nidulans*, production of β -lactam antibiotics decreases (Shen *et al.*, 1984; Sánchez *et al.*, 1988).

Nitrogen regulation in fungi is mediated by GATA-binding factors (Marzluf, 1997) like AreA in *A. nidulans* (Kudla *et al.*, 1990) and its homologue in *P. chrysogenum* NRE (Haas *et al.*, 1995; Haas and Marzluf, 1995). There are six GATA motifs in the *P. chrysogenum pcbAB-pcbC* intergenic region and only one in the same region of *A. nidulans*; the fact that the introduction of the *P. chrysogenum pcbC* promoter region in *A. nidulans* causes that the expression of the *A. nidulans pcbC* gene

becomes sensitive to nitrogen regulation (Kolar *et al.*, 1991), indicates that the same machinery mediating nitrogen repression of penicillin biosynthesis in *P. chrysogenum* is also present in *A. nidulans*.

1.2.5.3. pH regulation

The external pH of the medium has also influence on biosynthesis of hydrophobic penicillins (Shah *et al.*, 1991; Espeso *et al.*, 1993).

The transcriptional activator PacC (Espeso *et al.*, 1993; Tilburn *et al.*, 1995; Suárez and Peñalva, 1996) binds to the intergenic region between the *pcbAB* and *pcbC* genes and activates transcription of the *pcbAB* (Then Bergh and Brakhage, 1998) and *pcbC* (Tilburn *et al.*, 1995) genes at alkaline pH. Putative PacC-binding sites have also been identified in the promoter region of the *penDE* gene, which indicates that external pH also controls the expression of this gene.

1.2.5.4. Regulation by amino acids

Amino acids are important for biosynthesis of penicillin in two different ways; they are used as precursors and they can also play a regulatory role in the β -lactam biosynthesis. The intracellular concentration of L- α -aminoadipate, unlike that of cysteine or valine seems to be a limiting factor for the synthesis of the tripeptide ACV, which also affects the overall penicillin biosynthesis rate in low-producing strains (Friedrich and Demain, 1978; Honlinger and Kubicek, 1989).

It has also been observed that addition of L-lysine to *P. chrysogenum* and *A. nidulans* culture medium gave rise to a decrease in penicillin production (Luengo *et al.*, 1979a; Brakhage and Turner, 1992). Since L- α -aminoadipate is a precursor of L-lysine, it seems that the effect observed by L-lysine is due to a reduction of the L- α -aminoadipate pools. Nevertheless this is not the only regulatory effect of L-lysine on penicillin biosynthesis, since it has been shown that this amino acid represses the expression of *pcbAB* and *pcbC* genes of *A. nidulans* (Brakhage and Turner, 1992).

The effect of other amino acids on penicillin production has also been studied (Then Bergh and Brakhage, 1998). Some amino acids like L-histidine, L-valine, and L-methionine, in addition to L-lysine, have a negative effect on the expression of *pcbAB* and *pcbC* genes in *A. nidulans* because of acidification of the medium. In case of L-

histidine, L-valine the negative effects are probably due to reduced activation by PacC under acidic conditions. Repression by L-methionine of those genes is mediated via a *cis*-regulatory element placed in the intergenic region.

1.2.5.5. Other regulatory elements

The heterotrimeric HAP complex (PENR1) binds the CCAAT box domain, represses *pcbAB* and induces *pcbC* and *pcbDE* gene expression (Brakhage, 1997; 1998).

Another transcriptional activator named PTA1 (Kosalková *et al.*, 2000; 2007) has been also characterized. This factor binds the TTAGTAA heptamer located at the divergent *pcbAB*-*pcbC* promoter region.

However, surprisingly, no penicillin pathway-specific regulatory genes have been found in the amplified region containing the three biosynthetic genes (Fierro *et al.*, 2006; van den Berg *et al.*, 2007), which indicates that penicillin biosynthesis might be controlled directly by global regulators (e.g. CreA, PacC, Nre) rather than by pathway-specific ones. One of these regulators is the LaeA protein, which is a nuclear methyltransferase controlling expression of the penicillin genes in *P. chrysogenum* (Kosalková *et al.*, 2009) and the synthesis of sterigmatocystin, lovastatin, penicillin and pigmentation in several aspergilli (Bok and Keller, 2004).

LaeA also regulates the synthesis of gliotoxin and the virulence of *Aspergillus fumigatus* (Bok *et al.*, 2005; Sugui *et al.*, 2007). The LaeA protein contains a SAM binding site characteristic of methyltransferases and is predicted to function at the level of chromatin modification (Bok and Keller, 2004; Bok *et al.*, 2005; 2006; Keller *et al.*, 2005). It has been proposed that LaeA regulates the gene clusters through heterochromatin repression, perhaps by interacting with methylases or deacetylases that are associated with heterochromatin (Keller *et al.*, 2005; Shwab *et al.*, 2007; Kosalková *et al.*, 2009).

1.2.6. Compartmentalization of intermediates and enzymes

The penicillin biosynthetic pathway occurs in different cellular compartments (Martin *et al.*, 2010), which enables the spatial separation of precursors and enzymes,

and the regulation and optimization of the processes involved. The distinct subcellular organization of penicillin biosynthesis implies transport of enzymes, precursors, intermediates and products through these compartments (Figure 1.7). Therefore, every enzymatic step performed in an organelle has its own optimal environmental conditions.

As indicated before, the penicillin biosynthetic pathway begins with the non-ribosomal condensation of the amino acids L- α -aminoadipate, L-cysteine and L-valine to form the tripeptide ACV. This step is catalysed by the 420 kDa ACV synthetase (Díez *et al.*, 1990; Byford *et al.*, 1997; Martín, 2000a). Initial studies on *P. chrysogenum* ACV synthetase associated this protein to membrane structures that were identified as Golgi-like organelles (Kurylowicz *et al.*, 1987). Additional cell fractionation experiments located ACV synthetase attached to or inside vacuoles (Müller *et al.*, 1991; Lendenfeld *et al.*, 1993). However, the pH for the optimal *in vitro* ACV synthase activity (pH = 8.4, which was higher than that of the vacuolar pH), the co-factor requirement and protease sensitivity indicated that this enzyme is a cytosolic enzyme.

Figure 1.7. Compartmentalization of the penicillin biosynthetic pathway. Schematic representation showing the enzymatic steps and organelles involved in the penicillin biosynthetic pathway. (1) ACV synthetase; (2) IPN synthase; (3) IPN acyltransferase; (4) Phenylacetyl-CoA (aryl-CoA) ligase. α AA, L- α -aminoadipic acid; Cys, L-cysteine; Val, L-valine; PAA, phenylacetic acid; PAA-CoA, phenylacetyl-CoA; PenG, benzylpenicillin.

Cytosolic localization of this enzyme was later confirmed by electron microscopy techniques (van der Lende *et al.*, 2002). The ACV synthetase is synthesized as an inactive apoprotein form that becomes active (holo form) by means of a stand-alone large PPTase, which covalently attaches the 4'-phosphopantetheine moiety derived from coenzyme A (Baldwin *et al.*, 1991a; Lambalot *et al.*, 1996). Therefore, PPTases are necessary for penicillin biosynthesis, as it was initially reported in *A. nidulans* (Lambalot *et al.*, 1996; Keszenman-Pereyra *et al.*, 2003; Marquez- Fernandez *et al.*, 2007) and in *P. chrysogenum* (García-Estrada *et al.*, 2008a). Stand-alone PPTases are likely located in the cytosol because of their role in the activation of ACV synthetase and α -aminoadipate reductase. The latter enzyme that converts α -aminoadipate into α -aminoadipate semialdehyde in the fungal lysine biosynthetic pathway (Casqueiro *et al.*, 1998; Ehmann *et al.*, 1999; Guo *et al.*, 2001) occurs in the cytosol (Bhattacharjee, 1985; Matsuyama *et al.*, 2006). A *P. chrysogenum* mutant defective in the PPTase is a lysine auxotroph due to the lack of activation of the α -aminoadipate reductase (García-Estrada *et al.*, 2008a).

The amino acid precursors of the ACV tripeptide are provided through *de novo* synthesis, although they may also be taken up from the medium; L- α -aminoadipic acid, a non-proteinogenic amino acid, is taken from the culture medium via the acidic amino acid permease and via the general amino acid permease (Trip *et al.*, 2004). Unlike L-cysteine and L-valine, which are final products of primary metabolism, L- α -aminoadipic acid is a key intermediate in the lysine metabolism pathway of fungi (Bhattacharjee, 1985; Bañuelos *et al.*, 1999; 2000; Nishida and Nishiyama, 2000; Zabriskie and Jackson, 2000). Mitochondria are essential organelles for the synthesis of this precursor amino acid, since enzymes responsible for the biosynthesis of α -aminoadipic acid such as homocitrate synthetase and homoisocitrate dehydrogenase are located within the mitochondrial matrix (Jaklitsch and Kubicek, 1990; Kubicek *et al.*, 1990). Once these precursor amino acids are synthesized, they are stored in vacuoles (Lendenfeld *et al.*, 1993). These organelles regulate the levels of L- α -aminoadipate and cysteine, which are toxic at moderate concentrations (Klionsky *et al.*, 1990; Kubicek-Pranz and Kubicek, 1991).

After the formation of ACV in the cytosol, this compound serves as substrate of the 38 kDa IPN synthase, which cyclizes the tripeptide to form IPN, an intermediate

already containing the β -lactam nucleus (Ramos *et al.*, 1985; Carr *et al.*, 1986; Baldwin *et al.*, 1987; 1991b; Roach *et al.*, 1997). The IPN synthase behaves as a soluble enzyme (Abraham *et al.*, 1981), although its activity in cell-free extracts seems to be stimulated by addition of Triton X-100 or sonication (Sawada *et al.*, 1980). This protein colocalizes with ACV synthetase in the cytosol, as it was confirmed by electron microscopy (Müller *et al.*, 1991; Van der Lende *et al.*, 2002).

1.2.6.1. Peroxisomal location of the IATs

Unlike the cephalosporin C biosynthetic pathway, where IPN is epimerized to form penicillin N (see below), in the penicillin pathway the L- α -aminoadipyl side-chain of IPN is exchanged for a more hydrophobic side-chain by the acyl-CoA : IPN acyltransferase, IAT (Álvarez *et al.*, 1987; Barredo *et al.*, 1989; Tobin *et al.*, 1990; Whiteman *et al.*, 1990). The IAT is expressed as a 40 kDa precursor protein (proIAT) which undergoes an autocatalytic selfprocessing between residues Gly-102–Cys-103 in *P. chrysogenum*, thus constituting a heterodimer of two subunits; α (11 kDa, corresponding to the N-terminal fragment) and β (29 kDa, corresponding to the C-terminal region) (Barredo *et al.*, 1989; Veenstra *et al.*, 1989; Tobin *et al.*, 1990; 1993; Whiteman *et al.*, 1990). *In vivo*, IAT catalyses six different reactions (Álvarez *et al.*, 1993) related to the cleavage of IPN (releasing 6-APA) and the replacement of the α -aminoadipyl side-chain of this intermediate by aromatic acids activated in the form of aryl-CoA derivatives (Álvarez *et al.*, 1993). As a consequence of the side-chain substitution, hydrophobic penicillins (benzylpenicillin or phenoxymethylpenicillin), which show a significant increase of antimicrobial activity as compared with that of IPN, are formed. Isopenicillin N acyltransferases from *P. chrysogenum* and *A. nidulans* contain a functional peroxisomal targeting sequence PTS1 at the C-terminal end (ARL and ANI respectively). Electron microscopy immunodetection has shown that the acyltransferase of *P. chrysogenum* is located inside peroxisomes (microbodies) (Müller *et al.*, 1991; 1992; 1995; García-Estrada *et al.*, 2008b). In addition, transport of IAT inside the peroxisomal matrix is not dependent on the processing state of the protein, since the unprocessed proIAT variant IAT^{C103S} is correctly targeted to peroxisomes, although it is not active (García-Estrada *et al.*, 2008b).

Activation of the side-chain precursor (phenylacetic acid for benzylpenicillin, or phenoxyacetic acid for phenoxymethylpenicillin) as a thioester with CoA (to form phenylacetyl-CoA or phenoxymethyl-CoA) is required for penicillin formation (Álvarez *et al.*, 1993). This reaction is catalysed by aryl-CoA ligases. These proteins belong to the acyl adenylate protein families (Turgay *et al.*, 1992), which activate the acyl or aryl acids to acyl-AMP or aryl-AMP, respectively, using ATP. After activation, AMP is released and the carboxyl group is transferred to the thiol group of CoA forming a thioester. Two aryl-CoA ligases involved in the activation of phenylacetic acid have been identified in *P. chrysogenum* (Lamas-Maceiras *et al.*, 2006; Wang *et al.*, 2007). Like IAT enzyme, phenylacetyl-CoA ligases bear a peroxisomal targeting signal on their C-terminus and are localized into microbodies (Gledhill *et al.*, 1997). The localization of IAT and the two phenylacetyl-CoA ligases in peroxisomes has been confirmed recently by physical isolation of those organelles and MS identification of the peroxisomal proteins (Kiel *et al.*, 2009).

The peroxisomal colocalization of IAT and aryl CoA ligases indicates that the last two enzymes of the penicillin pathway form a peroxisomal functional complex, pointing to this organelle as a key compartment of the penicillin biosynthetic pathway. Peroxisomes (microbodies) are organelles that show a diameter of 200–800 nm and are surrounded by a single membrane (Müller *et al.*, 1991). They are involved in a variety of metabolic pathways like β -oxidation of fatty acids (Kionka and Kunau, 1985; Thieringer and Kunau, 1991a, b; Valenciano *et al.*, 1996; 1998; Kiel *et al.*, 2009) or karyogamy (Berteaux-Lecellier *et al.*, 1995).

P. chrysogenum microbody luminal pH has been estimated to be slightly alkaline, in the range of 7.0–7.5 (Van der Lende *et al.*, 2002), a pH range optimal for the aryl-CoA ligase and IAT (Álvarez *et al.*, 1987; 1993; Gledhill *et al.*, 1997). The importance of microbodies in penicillin biosynthesis has been highlighted through the alteration of the number and shape of these organelles as a result of the overexpression of the Pc-Pex11p protein (a peroxin that is involved in microbody abundance); overexpression of this protein led to an increase in the production of penicillin.

This positive effect is likely to be related to an increased transport of penicillin and/or its precursors across the microbody membrane (Kiel *et al.*, 2005), which evidences that transport events through the membrane of this organelle are very

important. Because of the hydrophilic nature of IPN, the uptake of this precursor from cytosol is likely to occur through specific carriers.

An active IPN transport system must be present in the peroxisomal membrane to assure an adequate pool of IPN inside microbodies. The 6-APA is a penicillin precursor that is originally formed into the microbody by the IAT although it is also found extracellularly in the culture broth; it has been shown that this compound is taken up very efficiently through the plasma and peroxisomal membranes, probably by passive diffusion (García-Estrada *et al.*, 2007). The difference in the transport efficiency between IPN and 6-APA may be due to differences in the polarity between these two compounds. The hydrophobic penicillins formed by the IAT might be released from the microbody through simple diffusion, but this hypothesis is very unlikely because the accumulated extracellular level is much higher than the intracellular one.

1.2.6.2. Transport systems for large-scale secretion of penicillin

Penicillin is secreted in very large quantities by the overproducing strains. In fed-batch cultures these industrial strains reach a biomass of about 25–30 g/l and they produce more than 40–50 g/l of penicillin. Since the last steps of penicillin biosynthesis (namely the activation of phenylacetic acid to phenylacetyl-CoA and the transacylation reaction by the IAT) are located in peroxisomes, the final product has to be transported out of the peroxisome, first into the cytoplasm and then into the culture medium.

A significant effort has been made to clone transporters involved in the secretion of penicillin from *P. chrysogenum*. Penicillin secretion is sensitive to verapamil, an antagonist of multidrug transporters (van den Berg *et al.*, 2008), suggesting that secretion is an active process involving this type of transporters. In a patent description, a large number of *P. chrysogenum* ABC transporters were cloned (29 different sequences) based on the conserved motifs of the transporters (van den Berg, 2001; Patent application number WO 2001/32904). Surprisingly, genetic modification of several of these transporters did not affect the secretion of penicillin. The lack of clear involvement of any of these ABC transporters in secretion of penicillin is intriguing and may indicate that secretion of this antibiotic in the overproducing strains does not proceed through the classical ABC pumps, although this might be the case in the wild-type strains; in other words, new secretion pathways may have been implemented in the

penicillin-overproducing strains that were absent (or inefficient) in the low-producing wild-type strains.

After sequencing the *P. chrysogenum* genome, two approaches have been used to investigate if any of those ABC transporters has a role in penicillin secretion, namely (i) transcriptional upregulation following phenylacetic acid addition to the culture and (ii) overexpression in an industrial penicillin-producing strain when compared with the wild-type strain (van den Berg *et al.*, 2008). Transcriptional studies following phenylacetic acid addition revealed two groups of MDR genes that were upregulated. However, many of those genes are probably related to phenylacetate transport and catabolism, which is known to affect penicillin biosynthesis (Rodríguez-Sáiz *et al.*, 2001), rather than with penicillin secretion. The second approach, namely comparison of expression of MDR genes in a laboratory strain and in the industrial producer, revealed that some of the ABC transporters had increased expression in the industrial strain (van den Berg *et al.*, 2008). However, a detailed functional analysis is required to confirm if the disruption or amplification of these genes affect penicillin secretion. A gene encoding an ABC transporter of *A. nidulans* putatively involved in penicillin secretion was reported by Andrade and colleagues (2000). However, a detailed analysis of the reported evidence suggests that this gene is likely involved in nutrient transport, affecting directly or indirectly penicillin biosynthesis.

1.2.6.3. The pexophagy phenomenon in filamentous fungi

In a late stage of the cultures, peroxisomes are known to be integrated into vacuoles by the pexophagy phenomenon. Two types of pexophagy processes are known (Sakai *et al.*, 2006; Kiel and van der Klei, 2009). The so-called macro-autophagy involves the sequestering of portions of the organelles and cytoplasm into vacuoles (Yorimitsu and Klionsky, 2005). In other cases, an autophagy process is involved in the sequestering and incorporation of peroxisomes into vacuoles. This involves several proteins encoded by the autophagy (ATG) genes (Klionsky *et al.*, 2003). The ATG genes were initially discovered in yeasts, but recently have been reported in *Podospora anserina* (Pinan-Lucarré *et al.*, 2003; 2005), *Aspergillus oryzae* (Kikuma *et al.*, 2006), *A. fumigatus* (Richie *et al.*, 2007) and *A. nidulans* (Kiel and van der Klei, 2009).

If the peroxisomes are integrated into vacuoles, the benzylpenicillin formed in peroxisomes would be transferred to vacuoles and might be later secreted out of the cells. Although fusion of the vacuoles to the plasma membrane by an exocytosis process is possible, there is no evidence to support that this might be a major mechanism of penicillin release.

1.3. PROTEOMICS

Proteomics studies are the most powerful methods to evaluate the final result of gene expression and 2-DE has been the technique of choice to obtain a reference global picture of the proteome map.

This technique has been successfully applied to several ascomycete fungi, such as *Schizosaccharomyces pombe* (Hwang *et al.*, 2006), some *Aspergilli* (Melin *et al.*, 2002; Kniemeyer *et al.*, 2006; Vödisch *et al.*, 2009) or *Saccharomyces cerevisiae* (Shevchenko *et al.*, 1996; Bruckmann *et al.*, 2009). However, only Kiel and coworkers (2009) have performed a proteomics based inventory of the proteins present in the microbody matrix in *P. chrysogenum*. Therefore, it is of great interest the optimization and analysis of the *P. chrysogenum* proteome reference map.

2-DE with IPGs (Görg *et al.*, 1998, Görg *et al.*, 2000) combined with protein identification by MS (Aebersold *et al.*, 2003) is currently the workhorse for proteomics. In spite of promising alternative/complementary technologies (*e.g.* multidimensional protein identification technology, stable isotope labelling, protein arrays) (Aebersold *et al.*, 2003, Washburn *et al.*, 2001) that have recently emerged, 2-DE is currently the only technique that can be routinely applied for parallel quantitative expression profiling of large sets of complex protein mixtures such as whole cell lysates.

Whatever technology is used, proteome analysis is technically challenging, because the number of different proteins expressed at a given time under defined biological conditions is likely to be in the range of several thousand for simple prokaryotic organisms and up to at least 10000 in eukaryotic cell extracts. Moreover, current proteomic studies have revealed that the majority of identified proteins are abundant housekeeping proteins, whereas proteins such as receptor molecules that are present in much lower concentrations are usually not detected. Consequently, improved methods for enrichment of low-abundance proteins are required, such as

prefractionation procedures, as well as more sensitive detection and quantitation methods (Görg *et al.*, 2004).

2-DE couples IEF in the first dimension with SDS-PAGE in the second dimension, and enables the separation of complex mixtures of proteins according to *pI*, *Mr*, solubility, and relative abundance (Figure 1.8). Depending on the gel size and pH gradient used, 2-DE can resolve more than 5000 proteins simultaneously (2000 proteins routinely) and can detect 1 ng of protein *per* spot. Furthermore, it delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or post translation modifications (PTM). This is in contrast to LC-MS/MS based methods, which perform analysis on peptides, where *Mr* and *pI* information is lost, and where stable isotope labelling is required for quantitative analysis.

One of the greatest strengths of 2-DE is its capability to study proteins that have undergone some form of PTM (such as phosphorylation, glycosylation or limited proteolysis) and which can, in many instances, be readily located in 2-DE gels as they appear as distinct spot trains in the horizontal and/or vertical axis of the 2-DE gel. In addition, 2-DE not only provides information on protein modifications and/or changes in their expression levels, but also allows the isolation of proteins in µg amounts for further structural analyses by MALDI-TOF MS, ESI-MS or Edman microsequencing (Beranova-Giorgianni *et al.*, 2003, Celis *et al.*, 1999, Rabilloud 2002).

The former limitations of carrier ampholyte (CA) based 2-DE (O'Farrell 1975) with respect to reproducibility, resolution, separation of very acidic and/or very basic proteins and sample loading capacity, have been largely overcome by the introduction of commercial IPGs for the first dimension of 2-DE. Narrow-overlapping pH gradients provide increased resolution ($DpI = 0.001$) (Görg *et al.*, 1985) and detection of low abundance proteins (Westbrook *et al.*, 2001), whereas alkaline proteins up to pH 12 have been separated under steady-state conditions (Drews *et al.*, 2004).

The major steps of the 2-DE-MS workflow include: (i) sample preparation and protein solubilization; (ii) protein separation by 2-DE; (iii) protein detection and quantitation; (iv) computer assisted analysis of 2-DE patterns; (v) protein identification and characterization; (vi) 2-D protein database construction (Dunn *et al.*, 1993).

Figure 1.8. 2-DE couples IEF in the first dimension with SDS-PAGE in the second dimension, and enables the separation of complex mixtures of proteins according to pI , M_r , solubility, and relative abundance.

1.3.1. Sample preparation and protein solubilization

1.3.1.1. Sample preparation

To take advantage of the high resolution 2-DE, proteins of the sample have to be denatured, disaggregated, reduced and solubilized to achieve complete disruption of molecular interactions and to ensure that each spot represents an individual polypeptide. The major problems concerning the visualization of proteins from total cell or tissue extracts lie in the high dynamic range of protein abundance, and the diversity of proteins with respect to M_r , pI and solubility. Although a one-step procedure for protein extraction would be highly desirable with regard to simplicity and reproducibility, there is no single method of sample preparation that can be universally applied to all kinds of samples analyzed by 2-DE (Dunn *et al.*, 1993). Although a large number of standard protocols have been published, these protocols have to be adapted and further optimized for the type of sample (*e.g.* microbial cells or mammalian tissue) to be analyzed, as well as for the proteins of interest (*e.g.* soluble cytosolic or highly insoluble membrane proteins, respectively). Some general recommendations, however, can be made: sample preparation should be as simple as possible to increase reproducibility, and protein modifications during sample preparation must be minimized, because they might result in artifactual spots on 2-D gels. In particular, proteolytic enzymes in the sample must be

inactivated. Samples containing urea must not be heated, to avoid charge heterogeneities caused by carbamylation of the proteins by isocyanate formed in the decomposition of urea (Dunn *et al.*, 1993). The three fundamental steps in sample preparation are cell disruption, inactivation or removal of interfering substances, and solubilization of the proteins (Dunn *et al.*, 2001, Shaw *et al.*, 2003). Cell disruption can be achieved by osmotic lysis, freezethaw cycling, detergent lysis, enzymatic lysis of the cell wall, sonication, grinding with (or without) liquid nitrogen, high pressure (*e.g.* French press), homogenization with glass beads and a bead beater, nitrogen cavitation, or a rotating blade homogenizer. These methods can be used individually or in combination. Typically, microbial cells or plant tissues require rather harsh conditions for the cell lysis due to the robustness of their cell walls, whereas more gentle methods can be applied for mammalian tissues.

Gentle cell disruption procedures (*e.g.* enzymatic lysis) are also required for the preparation of intact organelles (*e.g.* mitochondriae) for subsequent subproteome analysis. During or after cell lysis, interfering compounds such as proteolytic enzymes, salts, lipids, nucleic acids, polysaccharides, plant phenols and/ or highly abundant proteins have to be removed or inactivated. The two most important parameters are salt and proteolysis.

Proteases must be inactivated to prevent protein degradation that otherwise may result in artifactual spots and loss of high *Mr* Proteins. Protease inhibitors are usually added, but they may modify proteins and cause charge artifacts (Dunn *et al.*, 1993). Other remedies are boiling the sample in SDS-buffer (without urea), or inactivating proteases by low pH (*e.g.* precipitating with ice-cold TCA). However, it should be kept in mind that it may be rather difficult to completely inactivate all proteases (Castellanos-Serra *et al.*, 2002). TCA/acetone precipitation is very useful for minimizing protein degradation and removing interfering compounds, such as salt, or polyphenols. Attention has to be paid, however, to protein losses due to incomplete precipitation and/or resolubilization of proteins. Moreover, a completely different set of proteins may be obtained by extraction with lysis buffer depending on whether or not there was a preceding TCA precipitation step. On the other hand, this effect can be used for the enrichment of very alkaline proteins (such as ribosomal or nuclear proteins) from total cell lysates (Görg *et al.*, 2000).

Salt ions may interfere with electrophoretic separation and should be removed if their concentration is too high (100 mM). This is particularly a problem when samples are applied by “in-gel rehydration manner”, whereas higher salt concentrations are better tolerated by cup-loading. In this case, however, rather low voltages (approximately 150 V) have to be applied for several hours; otherwise proteins may precipitate at the site of sample application.

Salt increases the conductivity of the IEF gel, thereby prolonging the time required to reach the steady-state. In extreme cases, IEF may virtually stop due to salt fronts. Salt removal can be achieved by spin dialysis, or precipitation of proteins with TCA or organic solvents (*e.g.* cold acetone). One alternative is the use of 2-D clean-up kits (*e.g.* Amersham Biosciences, Uppsala, Sweden). Another is dilution of the sample below a critical salt concentration followed by application of a larger sample volume onto the IPG gel. The sample is desalted in the gel by applying low voltages (100 V) at the beginning of the run for up to several hours and replacing the filter paper pads beneath the electrodes (where the salt ions have collected) several times (Görg *et al.*, 2000).

High amounts of lipids may interact with membrane proteins and consume detergents. Delipidation of lipid-rich biological material (*e.g.* brain tissues) can be accomplished by extraction with organic solvents (*e.g.* cold ethanol or acetone). However, severe losses in proteins may be experienced, either because certain proteins are soluble in organic solvent, or because the precipitated proteins do not always resolubilize. Alternatively, high-speed centrifugation (Fountoulakis *et al.*, 2004) and subsequent removal of the lipid-layer is employed.

Polysaccharides (especially the charged ones) and nucleic acids can interact with carrier ampholytes and proteins, and give rise to streaky 2-D patterns. Moreover, these macromolecules may also increase the viscosity of the solutions and obstruct the pores of the polyacrylamide gels. Unless present at low concentrations, polysaccharides and nucleic acids should be removed. A common method is precipitation of proteins with acetone or TCA/acetone, but losses in proteins cannot always be avoided due to insufficient resolubilization of the precipitated proteins.

Other recommendations for the removal of nucleic acids are digestion with a mixture of protease-free RNases and DNases, or by ultracentrifugation and addition of a basic polyamine for example spermine (Rabilloud 1999).

Phenols are present in plant materials, especially in plant leaves, and may interact with proteins, giving rise to horizontal streaks in 2-D gel patterns. Polyphenolic compounds can be removed either by binding to polyvinylpolypyrrolidone, or by protein precipitation with TCA and subsequent extraction of phenols with ice-cold acetone (Mechin *et al.*, 2003).

Sometimes, highly abundant proteins present a problem since they impair separation and detection of low-abundance proteins by limiting the amount of these proteins to be loaded onto the 2-D gel and/or by masking them on the 2-D pattern. In particular albumin, which constitutes up to 60% of the bulk protein in plasma, is a major problem (Pieper *et al.*, 2004). There are several albumin removal kits on the market, but due to nonspecific binding, one has to be aware that most of these kits remove proteins other than albumin, too (Simpson 2004).

1.3.1.2. Protein solubilization

After cell disruption and/or removal of interfering compounds, the individual polypeptides must be denatured and reduced to disrupt intra- and intermolecular interactions, and solubilized while maintaining the inherent charge properties. Sample solubilization is usually carried out in a buffer containing chaotropes (*e.g.* urea and/or thiourea), nonionic and/or zwitterionic detergents (*e.g.* Triton X-100 or CHAPS), reducing agents, CA and, depending on the type of sample, protease inhibitors. The most popular sample solubilization buffer is based on O'Farrell's lysis buffer (O'Farrell *et al.*, 1975) and modifications thereof (9 M urea, 2–4% CHAPS, 1% DTT, and 2% v/v CA. Unfortunately, urea lysis buffer is not ideal for the solubilization of all protein classes, particularly for membrane or other highly hydrophobic proteins. Improvement in the solubilization of hydrophobic proteins has come with the use of thiourea (Rabilloud 1998) and new zwitterionic detergents such as sulfobetaines (Luche *et al.*, 2003). Merits and limits of these new detergents, chaotropes, and reducing agents have been reviewed recently (Santoni *et al.*, 2000).

■ Chaotropes

Urea is quite efficient in disrupting hydrogen bonds, leading to protein unfolding and penetration. In contrast, thiourea, introduced by Rabilloud in 2002, is better suited for breaking hydrophobic interactions, but its usefulness is somewhat limited due to its poor solubility in water. However, it is more soluble in concentrated urea solutions. Currently the best solution for solubilization of hydrophobic proteins is a combination of 5–7 M urea and 2 M thiourea, in conjunction with appropriate detergents. The major problem associated with urea in aqueous solutions is that urea exists in equilibrium with ammonium (iso)cyanate, which can react with the α -amino groups of the *N*-terminus and the ϵ -amino groups of lysine residues (Figure 1.9), thereby forming artifacts such as blocking the *N*-terminus and introducing charge heterogeneities (altered *pI*s). To prevent this carbamylation reaction, temperatures above 37 °C have to be avoided under all circumstances and CA (2% v/v), which act as cyanate scavengers, should be included in the urea solution. Given that these precautions have been complied with, it has been demonstrated that protein carbamylation is negligible for a period of at least 24 h, which is sufficiently long for most protein extraction and solubilization protocols (Thoenes *et al.*, 2003). Similar experiments have shown that carbamylation is not a problem during electrophoresis in the presence of urea, even with prolonged run-times, since the urea breakdown products are electrophoretically removed (McCarthy *et al.*, 2003).

Figure 1.9. A. Effect of heat on urea, B. carbamylation

■ Detergents (surfactants)

Detergents are utilized to prevent hydrophobic interactions between the hydrophobic protein domains to avoid loss of proteins due to aggregation and precipitation. Since the anionic detergent SDS is one of the most efficient surfactants,

solubilization of proteins in (boiling) SDS solution has been recommended for protein solubilization (Harder *et al.*, 1999). However, horizontal streaks in the 2-D pattern are observed if samples initially solubilized in 1% SDS are not diluted with at least 4-fold excess of (thiourea/urea) lysis buffer, to displace the anionic detergent SDS from the proteins and to replace it with a nonionic or zwitterionic detergent to decrease the amount of SDS below a critical concentration (0.2%). Additionally, obtaining sufficient dilution may constitute a major problem when micropreparative protein loads in 2-DE are used, since the amount of sample volume that can be applied onto an IPG strip is limited. Therefore, nonionic or zwitterionic detergents are currently favoured for protein solubilization.

The most popular nonionic detergents are NP-40, Triton X-100 and dodecyl maltoside. Regrettably, NP-40 and Triton X-100 are not very effective in solubilizing very hydrophobic membrane proteins. In contrast, zwitterionic detergents such as CHAPS (Figure 1.10) and sulfobetaines (*e.g.* SB 3-10 or ASB 14) perform better, and have been shown to solubilize, in combination with urea and thiourea chaotropes at least several integral membrane proteins (Molloy 2000). More detailed investigations revealed that the efficiency of zwitterionic detergents for solubilizing hydrophobic proteins not only depends on the nature of the protein itself, but also on the presence and nature of other compounds, in particular on the lipid content of the sample (Santoni *et al.*, 1999).

Besides these chaotropes and detergents, organic solvents have also been successfully applied for solubilization and IEF of very hydrophobic proteins (Deshusses *et al.*, 2003). Regardless of recent advances, these studies demonstrated that no single solution exists for the complex solubility problem of membrane proteins. Most membrane proteins cannot be solubilized adequately with a single nonionic or zwitterionic detergent and empirically testing and optimizing the composition of sample solubilization buffer to improve the solubility of membrane proteins still remains important (Luche *et al.*, 2003).

Figure 1.10. Molecular structure of zwitterionic detergents, these detergents such as CHAPS and sulfobetaines (e.g. SB 3-10 or ASB 14) are so effective in solubilizing the hydrophobic proteins.

■ Reducing agents

Reduction and prevention of re-oxidation of disulfide bonds is also a critical step of the sample preparation procedure. Reducing agents are necessary for cleavage of intra- and intermolecular disulfide bonds to achieve complete protein unfolding.

The most commonly used reductants are DTT or dithioerythritol (DTE) which are applied in excess, *i.e.* in concentrations up to 100 mM. Unfortunately, these agents are weak acids with pK values between 8.5 and 9; it means they will ionize at basic pH, and therefore, run short in the alkaline gel area due to migration to the anode during IEF. Moreover, DTT and DTE are not well suited for the reduction and solubilization of proteins which contain high cysteine content, such as wool keratins.

Herbert and coworkers (Herbert *et al.*, 1998) have proposed tributylphosphine (TBP) as an alternative to DTT. TBP is applied in quite low concentrations (2 mM) due to its stoichiometric reaction. However, this reagent has also several disadvantages, the major of which are its low solubility in water and its short half-life.

Moreover, TBP (and its solvent dimethylformamide, respectively) is toxic, volatile, and has a rather irritating odor. Alternatively, tris (2-carboxyethyl) phosphine (TCEP) is used in the saturation labelling procedure in DIGE.

In conclusion, in certain cases TBP and TCEP have several advantages over DTT and DTE, but the choice of reductant is predominantly sample specific (Getz *et al.*, 1999).

1.3.1.3. Prefractionation procedures

Since there is no amplification step for proteins analogous to the PCR method for amplifying nucleic acids, and due to the high dynamic range and diversity of expressed proteins, particularly in eukaryotic tissues, it is often preferable to carry out a prefractionation step to reduce the complexity of the sample, enrich for certain proteins such as low-copy number proteins or alkaline proteins, and to get some information on the topology of the proteins. This can be accomplished by: (i) isolation of specific cell types from a tissue, *e.g.* fluorescence activated cell sorting (FACS), or laser capture microdissection (LCM), (ii) isolation of cell compartments and/or organelles, *e.g.* by sucrose gradient centrifugation, or free-flow electrophoresis, (iii) selective precipitation of certain protein classes (*e.g.* TCA/acetone precipitation for ribosomal proteins), (iv) sequential extraction procedures with increasingly powerful solubilizing buffers, for example, aqueous buffers, organic solvents (*e.g.* ethanol or chloroform/methanol), and detergent-based extraction solutions, (v) chromatographic or electrokinetic separation methods, such as column chromatography, affinity purification, electrophoresis in the liquid phase and/or IEF in granulated gels. The major problem with diseased (*e.g.* cancerous) mammalian tissues is their heterogenous nature. It is, therefore, of utmost importance to obtain targeted populations (*e.g.* tumor cells) from tissue specimens (Hanash *et al.*, 2000). Hence, several microdissection techniques are applied to enrich for targeted cell populations, such as LCM technology that allows the isolation of pure cell populations (Emmert-Buck *et al.*, 1996). Another possibility for recovering specific subpopulations of cells from clinical samples is FACS of antibody-bound cells (Orfao and Ruiz-Arguelles 1996). However, it is not clear to what extent stress is exerted on the cells by this kind of treatment, and whether their protein expression profiles are affected.

Subfractionation of cell components, *e.g.* organelles such as mitochondria and proxisomes by centrifugation in a sucrose density gradient is the most effective method for organelle isolation (Huber *et al.*, 2003). Less common techniques are free-flow electrophoresis (FFE) or immunoaffinity binding methods (Vlkl *et al.*, 1997) but the quality of these preparations is not always sufficient for proteome analysis. Nevertheless Görg and coworkers in 2000 established an improved purification protocol for yeast cell mitochondriae which has been successfully applied for 2-DE/MS analysis

of the yeast mitochondrial proteome. Due to the limitations in sample loading capacity on the first dimension IEF gel, a whole cell lysate may not yield sufficient quantities of lower abundance proteins to be displayed on a 2-DE gel. Precipitation procedures allow increased loading of particular proteins while keeping the total protein load constant. In particular, TCA/acetone precipitation has been found to be very valuable for the enrichment of alkaline proteins such as ribosomal proteins from total cell lysates (Görg *et al.*, 2000). Additional benefits of TCA/acetone precipitation are inactivation of proteases to minimize protein degradation as described above, and removal of interfering compounds. However, attention has to be paid to protein losses due to incomplete precipitation and/or resolubilization of proteins.

1.3.2. Two-dimensional gel electrophoresis with IPG

The prerequisite, but also the challenge, for proteome analysis is to separate proteins from complex biological samples with high reproducibly and high resolution. The first dimension of IPG-Dalt, IEF, is performed in individual, 3 mm wide and up to 24 cm long IPG gel strips cast on GelBond PAGfilm (laboratory-made or commercial Immobiline Dry-Strips). Samples can be applied either by cup-loading or by in-gel rehydration. IPG-IEF has been simplified by use of an integrated system such as the IPGphor (Islam *et al.*, 1998) where rehydration with sample solution and IEF can be performed in a one-step automated procedure. After IEF, the IPG strips are equilibrated with SDS buffer in the presence of urea, glycerol, DTT and iodoacetamide, and applied onto horizontal or vertical SDS gels in the second dimension. After electrophoresis, the separated proteins are visualized by Silver-staining, organic or fluorescent dyes, or autoradiography (or phosphor-imaging) of radiolabelled samples. Limitations (not only of 2-DE, but of almost all current proteome analysis technologies) remain in the field of the analysis of very hydrophobic and/or membrane proteins, as well as in the lack of highly sensitive and reliable techniques for detection and quantitation of low abundant proteins. Yet, the recent introduction of more powerful chaotropes and detergents such as thiourea and sulfobetaines (Chevallet *et al.*, 1998), as well as the advent of sensitive fluorescent dyes, in particular of dual label techniques for the visualization of differentially expressed proteins (Unlü *et al.*, 1997) have contributed to improve the situation considerably.

1.3.2.1. First dimension: Isoelectric focusing with IPGs

Linear or nonlinear (Bjellqvist *et al.*, 1993) wide pH range (*e.g.* IPG 3–12), medium (*e.g.* IPG 4–7), narrow (*e.g.* IPG 4.5–5.5), and/or ultra-narrow (*e.g.* IPG 4.9–5.3) IPGs can be cast in different pH ranges between pH 2.5 and pH 12, as well as in different lengths, usually from 7 to 24 cm (however, IPG strips up to 54 cm long have been applied (Poland *et al.*, 2003)). Besides laboratory-made IPG gels, a variety of commercial IPG dry strips can now be purchased from different suppliers. Ready-made IPG dry strips are increasingly popular due to easier handling, better comparability of results and exchange of data, and have significantly contributed to the widespread application of 2-DE in proteomics (Figure 1.11).

The amount of protein which can be loaded onto a single IPG gel strip for optimum resolution, maximum spot numbers and minimum streaking/background smearing depends on parameters such as the pH gradient used (wide or narrow), separation distance, and protein complexity of the sample. For analytical purposes, typically 100–350 µg of protein can be loaded on an 18 cm long, wide pH range gradient and 500 µg on narrow range IPGs. For micropreparative purposes, five to ten times more protein can be applied. For micropreparative 2-DE, in-gel rehydration is often preferred, but up to several µg of protein may also be applied by cup-loading. Settings are usually limited to 50 mA *per* IPG strip and 150 V to avoid Joule heating, because the conductivity is initially high due to salts. As the run proceeds, the salt ions migrate to the electrodes, resulting in decreased conductivity and allowing high voltages to be applied. Final settings of up to 8000 V are particularly useful for zoom-in gels and alkaline pH gradients. The longer IPG strip, the narrower pH gradient and the more vh (volt-hour) are required to achieve steady state separation for high reproducibility.

Temperature during IEF has an important effect on the resulting 2-DE patterns, since spot positions vary along the pH axis with different applied temperatures. For enhanced reproducibility it is thus extremely important to run the separations at an actively controlled temperature, where 20 °C proved to provide the optimal conditions (Görg *et al.*, 1991).

Figure 1.11. A. Essential equipments for IEF. Ceramic IPG holders for rehydration, B. IPG Phore system and C. ready-made IPG dry strips are available from Amersham Bioscience.

1.3.2.2. Equilibration of IPG gel strips

Before the second-dimension separation, it is essential that the IPG strips are equilibrated to allow the separated proteins to fully interact with SDS. Due to the observation that the focused proteins bind more strongly to the fixed charged groups of the IPG gel matrix than, relatively long equilibration times (10–15 min), as well as urea and glycerol to reduce electroendosmotic effects are required to improve protein transfer from the first to the second dimension (Görg *et al.*, 2004).

The best protocol by far is to incubate the IPG strips for 10–15 min in the buffer containing 50 mM Tris-HCl (pH 8.8), 2% w/v SDS, 1% w/v DTT, 6 M urea and 30% w/v glycerol (Görg *et al.*, 2000). This is followed by a further 10–15 minute equilibration in the same solution containing 4% (w/v) iodoacetamide instead of DTT. The latter step is used to alkylate any free DTT, as otherwise it migrates through the second-dimension SDS-PAGE gel, resulting in an artifact known as pointstreaking that can be observed after Silver-staining (Görg *et al.*, 1987).

More importantly, the iodoacetamide alkylates sulfhydryl groups and prevents their reoxidation; this step is highly recommended for subsequent spot identification by MS (Figure 1.12).

Figure 1.13. DTT (Dithiothreitol) is necessary for cleavage of intra- and intermolecular disulfide bonds to achieve complete protein unfolding, and iodoacetamide alkylates sulfhydryl groups and prevents their reoxidation.

1.3.2.3. Second dimension: SDS-PAGE

Sodiumdodecylsulphate (SDS) is an ionic detergent that dissociates oligomer proteins into their monomers and breaks the hydrophobic bonds and hydrogen bonds between peptides. SDS unfolds the three-dimensional structure of proteins and gives them a negative charge proportional to the length of the polypeptide chain. SDS-PAGE can be performed on horizontal or vertical (Figure 1.13) systems. Horizontal setups (Görg *et al.*, 1999) are ideally suited for ready-made gels (*e.g.* ExcelGel SDS; Amersham Biosciences), whereas vertical systems are preferred for multiple runs in parallel, in particular for large-scale proteome analysis which usually requires simultaneous electrophoresis of batches of second-dimension SDS-PAGE gels for higher throughput and maximal reproducibility (Anderson and Anderson 1978).

The most commonly used buffers for the second dimension of 2-DE are the discontinuous buffer system of Laemmli (Laemmli *et al.*, 1970) and modifications thereof, although for special purposes other buffer systems are employed, such as borate buffers for the separation of highly glycosylated proteins (Patton *et al.*, 1991).

Figure 1.13. The Vertical EttanDalt system is available from Amersham Bioscience for second dimension electrophoresis.

1.3.3. Protein detection and quantitation

After 2-DE, the separated proteins have to be visualized, either by universal or by specific staining methods. Since the concentrations of individual proteins in a single cell differ between six or seven orders of magnitude, ranging from several millions of copies/cell for some highly abundant proteins (*e.g.* glycolytic enzymes) to a few copies/cell for low abundant proteins, these enormous variations in protein concentrations are a major challenge for almost all currently available protein detection methods (Corthals *et al.*, 2000, Wilkins *et al.*, 1998). The most important properties of protein visualization methods are high sensitivity (low detection limit), high linear dynamic range (for quantitative accuracy), reproducibility, and compatibility with post-electrophoretic protein identification procedures, such as MS. Unfortunately, currently no staining method meets all requirements for full proteome analysis.

Universal detection methods of proteins on 2-D gels include staining with anionic dyes (*e.g.* Coomassie Blue), negative staining with metal cations (*e.g.* zinc imidazole), Silver-staining, fluorescence staining or labelling, and radioactive isotopes, using autoradiography, fluorography, or Phosphor-imaging. For most of these staining procedures, the resolved polypeptides have to be fixed (in solutions such as 7% methanol and 7% acetic acid) for at least several hours (but usually overnight) before

staining to remove any compounds (*e.g.* CA, detergents) that might interfere with detection. Specific staining methods for detection of PTM (post translational modifications such as glycosylation, phosphorylation *etc.*) are employed either directly in the 2-DE gel or, more frequently, after transfer (blotting) onto an immobilizing membrane. The blotted proteins can be probed with specific antibodies (*e.g.* against phosphotyrosine residues) or with lectins (against carbohydrate moieties). The protein detection and quantitation methods applied in 2-DE for proteome analysis have been reviewed by Patton in 1991 and Rabilloud in 2002. These methods are, therefore, only briefly discussed in the following sections.

1.3.3.1. Coomassie Brilliant Blue staining

Coomassie Brilliant Blue (CBB) staining methods have found widespread use for the detection of proteins on 2-DE gels, because of their low price, ease of use and compatibility with most subsequent protein analysis and characterization methods such as MS. However, in terms of the requirements for proteome analysis, the principal limitation of CBB stains lies in their insufficient sensitivity, which does not permit the detection of low abundance proteins (the detection limit of CBB stains is in the range of 200–500 ng protein *per* spot). Hence, typically no more than a few hundred protein spots can be visualized on a 2-DE gel, even if milligram amounts of protein had been loaded onto the gel. CBB in colloidal dispersions according to Neuhoff *et al.* (1985) and modifications thereof have been reported to be more sensitive than the classical CBB stain (Candiano *et al.*, 2004), but are still less sensitive than the majority of chemical stains used in 2-DE for proteomics.

1.3.3.2. Reverse staining

Reverse staining exploits the fact that protein-bound metal cations (*e.g.* copper or zinc) are usually less reactive than the free salt in the gel. Thus, the speed of precipitation of free or only weakly bound ions to form an insoluble salt is slower on the sites occupied by proteins than in the protein-free background. This generates transparent protein zones or spots, while the gel background becomes opaque due to the precipitated, insoluble salt.

Compared to the other reverse staining methods, but also CBB, the zinc stain offers some distinct advantages as it can be completed in 15 min for most applications, and is more sensitive than CBB, KCl or copper stains (Adams and Weaver 1990). Hence, zinc or imidazole-zinc stains (Fernandez-Patron *et al.*, 1992) are currently the most sensitive reverse (or negative) staining methods applied in 2-DE. Zinc-imidazole staining is rapid, simple, and sensitive, and has a detection limit of roughly 20–50 ng of protein *per spot*. Moreover, it is compatible with subsequent protein identification by MS, making the stain quite popular for detection of proteins separated on micropreparative 2-DE gels. The major disadvantage of zinc-staining is its rather restricted linear dynamic range, which makes this staining procedure unsuitable for detecting quantitative differences on 2-DE gels.

1.3.3.3. Silver-staining

Silver-staining methods (Oakley *et al.*, 1980, Merril *et al.*, 1981) are far more sensitive than CBB or imidazole-zinc stains (detection limit is as low as 0.1 ng protein/spot). They provide a linear response with over a 10- to 40-fold range in protein concentration, which is slightly worse than with CBB staining. However, Silver-staining methods are far from stoichiometric, and are much less reproducible than CBB stains due to the subjective end-point of the staining procedure which makes them less suitable for quantitative analysis.

Silver staining methods are quite laborious and complex, although some progress with respect to automation has been made (Granier *et al.*, 1986). Silver-staining methods using aldehyde-based fixatives/sensitizers are the most sensitive ones, but prevent subsequent protein analysis (*e.g.* by MS) due to protein cross-linkage. If aldehydes are omitted in the fixative and in the subsequent gel impregnating buffers (except in the developer), microchemical characterization by PMF (peptide mass fingerprinting) is possible, (Mortz *et al.*, 2001), however at the expense of sensitivity.

1.3.3.4. Radiolabelling

Better and more confident results in terms of sensitivity and linear dynamic range of detection are obtained by protein detection methods relying on fluorescent compounds, or by radiolabelling of proteins combined with highly sensitive electronic

detection methods. Prior to the advent of highly sensitive silver staining methods, detection of proteins labelled with radioisotopes was the only method of sensitive detection for proteins separated on 2-DE gels.

Radiolabelling can be accomplished by incorporating radioactive isotopes (*e.g.* ^3H , ^{14}C , ^{32}P , ^{33}P , ^{35}S , ^{125}I , or ^{131}I) into proteins. *In vivo* metabolic radiolabelling of samples by the incorporation of radioactive amino acids (such as (^{35}S)-methionine, (^{14}C)-leucine, and/or (^{32}P)-phosphotyrosine) has been extensively used for proteome analysis of microorganisms and cell culture systems, *e.g.* for studying the stress response of organisms by pulse labelling.

In vitro radiolabelling, *e.g.* of human tissue proteins, is also possible by using iodination with ^{131}I or ^{125}I , however at the risk of formation of artifacts (Dunn *et al.*, 1993).

The radiolabelled 2-DE separated proteins can be detected by autoradiography or fluorography using X-ray films which are exposed to the dried gels and which can be quantified by densitometry (Link 1999).

1.3.3.5. Fluorescent detection and Ettan™ DIGE System

Due to the shortcomings of organic dyes, silver staining or radiolabelling for visualization and quantitation of proteins, fluorescent detection of proteins has increasingly gained popularity for proteome analysis.

Two major approaches for the fluorescent detection of proteins on 2-DE gels are currently practiced. These are: (i) covalent derivatization of proteins with fluorophores prior to IEF, and (ii) post-electrophoretic protein staining by intercalation of fluorophores into the SDS micelles coating the proteins, or by direct electrostatic interaction with the proteins (Patton 2000).

Combining novel proprietary technologies in fluorescence, sample multiplexing and image analysis, Ettan DIGE System is a fully integrated system offering significant benefits over classical 2-D electrophoresis. Ettan DIGE System (Figure 1.14) is the only system to use an internal standard for every spot on every 2-D electrophoresis gel, to give experimental reproducibility and the highest accuracy in protein ratio measurements. The major source of error in a 2-D experiment is gel-to-gel variation. To

eliminate this, an internal pooled standard should be run on all gels within an experiment. Multiplexing is the only way in which an internal standard can be used.

Figure 1.14. The internal standard is labelled with one CyDyeTM DIGE Fluor minimal dyes (e.g. CyTM2) and is run together with experimental and control samples, labelled with other CyDye DIGE Fluor minimal dyes (e.g. Cy3 or Cy5). Every protein from all samples will be represented in the internal standard and each protein can therefore be compared to itself within the internal standard to generate a ratio of relative expression.

With Ettan DIGE System, up to three labeled protein samples can be run on the same 2-D gel simultaneously. One of these, the internal standard, results from the pooling of aliquots of all biological samples in the experiment. This internal standard is labelled with one CyDyeTM DIGE Fluor minimal dyes (e.g. Cy2) and is run together with individual samples, experimental and control, labelled with other CyDye DIGE Fluor minimal dyes (e.g. Cy3 or Cy5). This means that every protein from all samples will be represented in the internal standard and each protein can, therefore, be compared to itself within the internal standard to generate a ratio of relative expression.

The same internal standard is run on all gels within an experimental series thereby creating an intrinsic link across all gels. Normalization of the internal standard across gels allows the ratio of relative expression of the same protein across gels to be compared directly, separating gel-to-gel variation from biological variation.

Even small differences in expression levels can be determined by comparing the ratio obtained from one fluorescent-labeled sample directly with another. As a result, it

is possible to see < 10% differences in protein expression between samples, with > 95% statistical confidence.

1.3.4. Computerized 2D image analysis

One of the key objectives of proteomics is to identify the differential expression between control and experimental samples run on a series of 2-D gels. That is, the protein spots that have been inhibited (disappeared), induced (appeared) or have changed abundance (increased or decreased in size and intensity). Once these gel features have been found, the proteins of interest can be identified using MS. This goal is usually accomplished with the help of computerized image analysis systems (Dowsey *et al.*, 2003). The first step in computerized image analysis of 2-DE protein patterns is capture of the gel images in a digital format. A range of devices, including modified document scanners, laser densitometers, cameras, and fluorescent and phosphor imagers are available for the acquisition of 2-D gel images. The saved images are then subjected to computer assisted image analysis.

The traditional workflow for a 2-DE software package is (i) preprocessing of the gel images, *i.e.* image normalization, cropping and background subtraction; (ii) spot segmentation, detection and expression quantification; (iii) landmarking, *i.e.* an initial user guided pairing of a few spots between the reference and sample gels. The sample gel is then warped to align the landmarks; (iv) matching, *i.e.* automatic pairing of the rest of the spots; (v) identification of differentially expressed spots; (vi) data presentation and interpretation; and (vii) creation of 2-D gel databases (Dowsey *et al.*, 2003).

Currently, several 2-D image analysis software packages are commercially available. These programs have been continuously improved and enhanced over the years in terms of faster matching algorithms with lesser manual intervention, and with focus on automation and better integration of data from various sources. New 2-D software packages have also emerged which offer completely new approaches to image analysis and novel algorithms for more reliable spot detection, quantitation and matching.

Several programs include options such as control of a spot cutting robot, automated import of protein identification results from MS, superior annotation

flexibility (e.g. protein identity, mass spectrum, intensity/quantity, links to the Internet), and/or multichannel image merging of different images to independent color channels for fast image comparison. However, despite these improvements, we have still a long way to totally automatic image analysis systems that do not require user intervention (Dowsey *et al.*, 2003).

Once the 2-D gel database has been established, information stored in it can be exploited by addressing questions such as “Can particular proteins be identified that are associated with a certain metabolic state?”, or “What is the function of a particular protein?”.

Currently, enormous efforts are being undertaken to display and analyze with 2-DE the proteomes from a large number of organisms, ranging from organelles such as mitochondriae, nuclei or ribosomes to simple prokaryotes including *E. coli*, *Bacillus subtilis*, *H. influenzae*, *Mycobacterium tuberculosis* and *Helicobacter pylori*, to single-celled eukaryotes such as the yeast *S. cerevisiae*, to multicellular organisms, e.g. *Caenorhabditis elegans*, plants such as rice (*Oryza sativa*) or *Arabidopsis thaliana*, and mammalian cells and tissues including rat and human heart, mouse and human liver, mouse and human brain, different cancer cell lines, HeLa cells, human fibroblasts, human keratinocytes, rat and human serum *etc.* Most of these and many other studies in progress are summarized at www.expasy.org/ch2D/2dindex.html (“WORLD-2DPAGE Index to 2D PAGE databases”). The Proteomics Standards Initiative aims to define community standards for data representation in proteomics to facilitate data comparison, exchange and verification (<http://psidev.sourceforge.net/>).

1.3.5. Protein identification from 2-D gel spots

MS has become the technique of choice for identification of excised protein spots from 2-D gel as these methods are very sensitive, require small amounts of sample (femtomole concentrations) and have the capacity for high sample throughput (reviewed by Godivac-Zimmermann *et al.*, 2001). Recent advances in MS also allow the investigation of phosphorylation and glycosylation (reviewed by Mann and Jensen 2003).

PMF (peptide mass fingerprinting) is typically the primary tool for protein identification. This technique, which is user-friendly and quite fast, is based on the

finding that a set of peptide masses obtained by MS analysis of a protein digest (usually trypsin) provides a characteristic mass fingerprint of that protein. The protein is then identified by comparison of the experimental mass fingerprint with theoretical peptide masses generated in-silicon using protein and nucleotide sequence databases. This approach proves very effective when trying to identify proteins from species whose genomes are relatively small, completely sequenced, and well annotated, but is not so reliable for organisms whose genomes have not been completed. A second problem is to identify proteins that are extensively post-translationally modified, since the peptides generated from these proteins may not match with the unmodified protein in the database (Graves *et al.*, 2002). A third problem is that PMF does not work very well if several different proteins are present in the same spot.

Figure 1.15. A schematic picture of MALDI-TOF system (left) and an Applied Biosystems/MD SCIEX 4800 MALDI TOF/TOF system (right).

Although search engines such as Mascot and Profound (Zhang *et al.*, 2000) have been developed that enable identification of the correct protein(s) even when the data quality is relatively low or when the sample consists of a simple mixture of proteins, it may prove impossible to identify a protein based on PMF alone. In these cases it is then essential to obtain amino acid sequence information. This is most readily accomplished using either MALDI-MS with chemically assisted fragmentation, or by MS/MS. MS/MS takes advantage of two-stage instruments, MALDI-TOF-TOF-MS/MS (Figure

1.15) or ESI-MS/MS triple-quadrupole, ion-trap, or Q-TOF machines to induce fragmentation of peptide bonds. One approach is to generate a short partial sequence or tag which is used in combination with the mass of the intact parent peptide ion to provide significant additional information for the homology search.

A second approach uses a database searching algorithm SEQUEST to match uninterrupted experimental MS/MS spectra with predicted fragment patterns generated in-silicon from sequences in protein and nucleotide databases. SEQUEST correlates uninterpreted tandem mass spectra of peptides with amino acid sequences from protein and nucleotide databases. It will determine the amino acid sequence and thus the protein(s) and organism(s) that correspond to the mass spectrum being analyzed.

The major drawback of MS/MS based protein identification methods is that the process cannot be readily automated, and that considerable time and expertise are required for interpreting the MS/MS spectra (Zhang *et al.*, 2000).

CHAPTER 2

MATERIALS & METHODS

2.1. MATERIALS

2.1.1. Instruments

The instruments were used for the experiments presented in this study was provided by the following business companies:

GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, USA), (formerly Amersham Pharmacia Biotech):

1. Ettan IPGphor II Isoelectric Focusing Unit
2. Strip Holders for use with Immobiline DryStrip and Ettan IPGphor II Isoelectric Focusing System
3. Immobiline DryStrip gels
4. Ettan DALTsix Separation Unit and Power Supply/Control Unit, 230 VAC
5. Ettan DALT Gel Casting Cassette (hinged cassette)
6. Ettan DALT Blank Cassette Insert
7. Ettan DALTsix Gel Caster, complete, includes 6 filler and 7 separator sheets
8. MultiTemp III Thermostatic Circulator, 230 V
9. ImageScanner II and ImageMaster 2D Platinum software v.5
10. CyDye DIGE Fluor Minimal Dye Labeling Kit (includes Cy2, Cy3, and Cy5)
11. DeCyder 2-D Differential Analysis Software v6.0
12. Hybond nylon filters and autoradiography film Hyperfilm-MP type.
13. Cases for the exposure of autoradiographic films with amplifying screens.
14. Ultraviolet light transilluminator model LKB-Macro Vue.

Beckman Instruments Inc. (Palo Alto, CA, USA): Refrigerated centrifuge model GS-15R.

BioRad Laboratories (Richmond, CA, USA): Protein electrophoresis chamber (model Mini-Protean II and III) and DNA (Subcell equipment).

Buchi Labortechnik AG (Flawil, Switzerland): Vacuum Pumps Model B-169.

Canon Inc. (Japan): Reflex EOS 10D Digital Camera.

Du Pont Instruments (Wilmington, USA): Cases with amplifying screens.

Eppendorf (Hamburg, Germany):

1. Microcentrifuge 5415C and 5417C models and chilled 5415R model.

2. Thermomixer model 5432.

Gallenkamp (Leicester, UK): Orbital Incubators IMR model 250.

Gilson Medical Electronics Inc. (Middletown, WI, USA): automatic pipettes types P2, P20, P200, P1000 and P5000.

Hamilton Co. (Reno, NV, USA): loading syringes for protein electrophoresis gels.

Hitachi Instruments, Inc (Tokyo, Japan): Spectrophotometer Model U-2001.

Maissa (Barcelona, Spain): NYTAL polyamide filters.

Merck (Darmstadt, Germany): Sprayer.

Millipore Corp. (Bedford, MA, USA):

1. Membrane filtration
2. Ultrafiltration units
3. Ultrafree and electrotransferencia PVDF membranes (Immobilon-R).
4. Water filtering equipment including MilliRo MilliQ.

Misonix Inc. (Narmingdale, NJ, USA): Sonicator, Sonicator XL.

Moulinex (Barcelona, Spain): Microwave M41.

New Brunswick Scientific (Edison, NJ, USA): Orbital incubator Innova model 4230.

Olympus Corporation (Shibuya-ku, Japan):

1. BX60 System phases Contrast Microscope with transmitted light bright field.
2. Nomarski differential interference contrast light reflected and fluorescent light reflected.
3. PM20 automatic photomicrography system.

Packard Instrument Comp. (Meriden, USA): Apparatus for electronic autoradiography InstantImagerTM.

Perkin Elmer (Roche Molecular Systems, Inc., Branchburg, NJ, USA):

1. Thermocycler GeneAmp 2400
2. DNA sequencer ABI PRISM 310 Genetic Analyzer.

Q-biogenic (Irvine, CA, USA): Fast-Prep instrument FP 120, Bio 101.

Sartorius GmbH (Weinheim, Germany): Balance precision, models 2442 and 1216MP.

Savant Instruments Inc. (Hicksville, NY, USA): Vacuum centrifuge model SVC-100H Speedvac SC-110.

Selecta (Barcelona, Spain):

1. Autoclave Autester-E model
2. The thermostated baths model 320 OR
3. Incubator models 207, 207, 237 and 372 to stove model for drying oven glass.
4. Vacuum-Term Vacuum model 571.

Sorvall (Newtown, CO, USA): Refrigerated centrifuge, model RC-5B.

Spectrum Medical Industries Inc. (LA, CA, USA): UV light transilluminator Spectroline TR - 302.

Stratagene (La Jolla, CA, USA): DNA UV Stratalinker72400 Fixer.

VirTis (Gardiner, NY, USA): Freeze Top3 Bench model.

Whatman Laboratory Products Inc. (Clifton, NJ, USA): Paper 3MM.

Whatman Biometra7GmbH (Goettingen, Germany): Thermal cycler with gradient temperature TGradient

WTW (Germany): pH meter model Sentix 41.

2.1.2. Chemical reagents for general use

Chemicals for general use were provided by Ambion Inc. (Austin, TX, USA), Apothecon (Princeton, NJ, USA), Oncor Appligene (France), BDH Chemicals (Poole, United Kingdom), BioRad Laboratories (Richmond, CA, USA), Carlo Erba (Milan, Italy), Cayla (Toulouse, France), Difco Laboratories Inc. (Detroit, MI, USA), Chemiker-Biochemiker Fluka (Buchs, Switzerland), GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, USA), Genosys Biotechnologies Inc. (TX, USA), Gibco BRL (Eggenstein, Germany), ICN Biomedicals Inc. (Irvine, CA, USA), Invitrogen Corporation (Carlsbad, CA, USA), Laboratorio Reig Jofre SA (Spain), MBI Fermentas (Vilnius, Lithuania), Merck (Darmstadt, Germany), Millipore (Billerica, USA), New England Biolabs Inc. (Beverly, MA, USA), Oxoid Ltd. (Hampshire, UK), Panreac (Barcelona, Spain), Probus (Barcelona, Spain), Prolabo (FontenayBois, France), Promega Corporation (Madison, WI, USA), Pronadisa (Madrid, Spain), QIAGEN (Hilden, Germany), Roche Diagnostics Corporation (Indianapolis, IN, USA), Schleicher

& Schuell (Germany), Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), Stratagene (La Jolla, CA, USA), Takara Bio Inc. (Shiga, Japan), Whatman7 (United Kingdom)

2.1.3. Specific reagents for molecular biology

2.1.3.1. Specific reagents for Proteomics

Protein Clean up kit (GE Healthcare), Protease inhibitor cocktail Complete™ (Roche), Protein marker, Precision Plus Protein Standards (Bio-Rad), Bradford Protein Assay (Bio-Rad), IPG Buffers (PlusOne, GE Healthcare), Glycine (PlusOne, GE Healthcare), Ammonium persulfate (PlusOne, GE Healthcare), TEMED (PlusOne, GE Healthcare), SDS (PlusOne, GE Healthcare), Thiourea (PlusOne, GE Healthcare), Iodoacetamide (PlusOne, GE Healthcare), Tris (PlusOne, GE Healthcare), Urea (PlusOne, GE Healthcare), CHAPS (PlusOne, GE Healthcare), Dithiothreitol (PlusOne, DTT) (GE Healthcare), Bromophenol Blue (PlusOne, GE Healthcare), Immobiline DryStrip cover fluid (PlusOne, GE Healthcare)

2.1.3.2. Nucleic Acids

The Lambda phage DNA (molecular weight marker in DNA electrophoresis after digestion with restriction endonucleases) was provided by MBI Fermentase trading company. This marker includes eight bands: 23,130, 9416, 6557, 4361, 2322, 2027, 564; 125 bp.

The molecular weight marker 1 Kb (Roche) includes 23 bands: 75, 134, 154, 201, 220, 298, 344, 396, 506, 517, 1018, 1636, 2036, 3054, 4072, 5090, 6108, 7126, 8144, 9162, 10180, 11198 and 12216 bp.

2.1.3.3. Antibiotics

Phleomycin (Cayla Company)

Ampicillin (pharmaceutical preparation Britapen, Beecham SA)

2.1.3.4. Oligonucleotids

The synthetic oligonucleotides were purchased from Sigma-Genosys. Table 2.1 details the the primers and their sequences.

Primer	Sequence
EST- <i>Nco</i> I-F	5'- CAAAAGA <u>ACCATGG</u> ACACTACTTTC - 3'
EST- <i>Stu</i> I-R	5'- CAGCGCCAA <u>AGGCCT</u> CTACTGCTGAC - 3'
EST- <i>Nco</i> I-R	5'- CATTCTCAG <u>CCATGG</u> CATACTGCGCAC - 3'
LOV- <i>Nco</i> I-F	5'- CATACTATCC <u>ACCATGG</u> AGGCAGTCATC - 3'
LOV- <i>Stu</i> I-R	5'- GGAAGTAGTG <u>AGGCCT</u> TTAAGGGGTCTC - 3'
LOV- <i>Nco</i> I-R	5'- GTAGATCAGA <u>ACCCATGG</u> CGCGTCCTTG - 3'
FP-pcbC	5'- GCATGTTGCATCGGGAAATCC - 3'
RP-gpd2	5'- CCTAGGTACAGAAGTCCAATT - 3'
GdhF	5'- GGACTCCCTAATGGATTCCGAG - 3'
TcycR	5'- GAAAAGGGGGACGGATCTCCG - 3'
PenDE-F	5'- ATGCTTCACATCCTCTGTCAAGG - 3'
PenDE-R	5'- TCAAAGCCTGGCGTTGAGCGC - 3'
β -actin-F	5'- CTGGCCGTGATCTGACCGACTAC - 3'
β -actin-R	5'- GGGGGAGCGATGATCTTGACCT - 3'

Table 2.1. Primers used in this study and their sequences. The underlined letters show the restriction site designed for each primer.

2.1.3.5. Enzymes

Restriction enzymes were supplied by New England commercial houses Biolabs Inc., GE-Healthcare, MBI Fermentas and Takara Bio Inc. The enzymes deoxyribonuclease I (DNase I) and ribonuclease A (RNase A), both of bovine pancreas were purchased from Sigma-Aldrich Company, Inc. Alkaline phosphatase from calf intestine (CIAP) and the enzyme DNA ligase of phage T4 were purchased from MBI Fermentas company. The Taq DNA polymerase was purchased from Sigma-Aldrich, Inc., Promega, and Enzyme[®] Platinum pfx DNA polymerase Invitrogen. The egg white lysozyme was supplied by Fluka Chemical-Biochemical Co. The β -glucuronidase from *Helix pomatia* and lytic enzymes of *Trichoderma harzianum*, (for protoplast obtaining) were supplied by Sigma-Aldrich company, Inc.

2.1.3.6. Commercial Systems (Kits)

Several processes described in this chapter were conducted using the following commercial systems (kits):

Micro Plasmid Prep Kit GFXTM (GE Healthcare) for purification and collecting the plasmid DNA for sequencing reactions. This kit allows the extraction of plasmid DNA from 1-3 ml of *E. coli* cultures.

GFXTM PCR DNA and Gel Band Purification (GE Healthcare), was used for isolation, purification and concentration of PCR products and DNA fragments from agarose gels or solutions.

RNAeasyTM Plant Mini Kit (QIAGEN) for obtaining the total RNA from fungi.

Superscript One-StepTM RT-PCR System (Invitrogen), was used to perform RT-PCR reactions.

Oligotex mRNA Mini Kit (QIAGEN). This kit was used for purification of mRNA with polyA tails from a mixture of total RNA.

2.1.3.7. Plasmid vectors

■ pGEM-T Easy

The pGEM-T and pGEM-T Easy Vectors are linearized vectors with a single 3'-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. These vectors are high-copy-number vectors containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α -peptide coding region of the enzyme β -galactosidase. Insertional inactivation of the α -peptide allows identification of recombinants by blue/white screening on indicator plates. Both the pGEM-T and pGEM-T Easy Vectors contain numerous restriction sites within the multiple cloning regions. The pGEM-T Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes *EcoRI*, *BstZI* and *NotI*, providing three single-enzyme digestions for the insert release. The pGEM-T Vector cloning region is flanked by recognition sites for the *BstZI* enzyme. Alternatively, a double-digestion may be used to release the insert from the vector. The pGEM-T and pGEM-T Easy Vector Systems are supplied with 2X Rapid Ligation Buffer. Ligation

reactions using this buffer may be incubated for 1 hour at room temperature. The incubation period may be extended to increase the number of colonies after transformation. Generally, an overnight incubation at 4 °C produces the maximum number of transformants.

■ pJL43RNAi

The pJL43RNAi Plasmid (6007 bp) was used for gene attenuation. The pJL43RNAi plasmid contains a dsRNAs expression cassette (Ullán *et al.* 2008). This cassette includes a single *NcoI* cloning site, the promoter of the *P. chrysogenum pcbC* gene (*ppcbC*) and the promoter of the glyceraldehyde 3-phosphate dehydrogenase gene (*Pgpd*). This vector also has the *Saccharomyces cerevisiae* cytochrome oxidase gene transcriptional terminator (*Tcyc1*). The *ble* gene, that confers resistance to phleomycin, is used as selective marker in the transformations. This gene is under control of the promoter of the *Aspergillus awamori* glutamate dehydrogenase gene (*Pgdh*).

2.1.4. Bacterial strains

■ *E. coli* DH5α (Hanahan, 1983). This strain is used for transformations and amplification of plasmid DNA. It allows obtaining competent cells for high efficiency transformations (up to 5×10^8 transforms/μg of DNA). It has a deletion in the *lacZ* gene of the *lac* operon, which may be supplemented by specific vectors like pBluescript® (Stratagene). This complementation produces a blue color on the colony, caused by the action of the β-galactosidase (encoded by *lacZ*) on the compound X-Gal in the culture.

■ *Micrococcus luteus* ATCC 9341. This strain is sensitive to β-lactam antibiotics, mainly to those with the penam chemical structure. This strain is used as sensor microorganism for assessing the production of IPN and/or benzylpenicillin by the fungi in a bioassay.

2.1.5. Culture Media

■ Power Medium (PW)

PW is a medium for sporulation and maintenance of *P. chrysogenum* which consists of PM1 media (Lopez-Nieto *et al.*, 1985) and Czapek 0.7M KCl.

Sucrose.....	25 g
Lactose	5 g
Peptone.....	2.5 g
Corn steep	0.5 g
KCl.....	26.1 g
NaCl	2 g
NaNO ₃	1.5 g
H ₃ PO ₄	0.25 g
MgSO ₄ •7H ₂ O	0.25 g
PW Salts.....	1 ml
Adjust pH.....	6.75
Distilled water	up to 1 liter
Agar	2.0% (w/v)

For mutated strains lacking pyrG gene, the media contains 10 mg/ml uridine.

■ PW Salts:

KH ₂ PO ₄	6 g
FeSO ₄ •7H ₂ O	1 g
FeCl ₃ •6H ₂ O.....	0.4 g
CuSO ₄ •5H ₂ O.....	0.2 g
Distilled water.....	up to 200 ml

■ Czapek (Cz)

Minimal growth medium is used for microorganisms capable of using NaNO₃ as the sole source of nitrogen (Smith, 1960).

Sucrose.....	30 g
NaNO ₃	2 g
K ₂ HPO ₄	0.5 g
MgSO ₄ •7H ₂ O	0.5 g
FeSO ₄ •7H ₂ O	0.01 g
Distilled water	up to 1 liter
Agar	2.0% (w/v)

When Cz is used as the protoplast regeneration medium an osmotic stabilizer (1 M sorbitol or 0.7 M KCl) is added.

■ **MCIP (complex media for seed culture)**

Corn Steep Solid.....	20 g
Yeast Extract	10 g
CaCO ₃	5 g
Adjust pH (with NaOH)	6.1
Distilled water	up to 900 ml
Aliquot in bottles (90 ml) and sterilize.	

MCIP media for fermentation is prepared by mixing each 90 ml of MCIP with 10 ml of 20% sterile sucrose.

■ **32% Potassium phenylacetate**

Phenylacetic acid.....	32 g
Distilled water	50 ml
Adjust pH (with KOH 40%).....	8.0 - 8.2
Distilled water	up to 100 ml

■ **MCFP (complex media for penicillin production)**

32% Potassium phenylacetate	12.5 ml
Pharmamedia	20 g
CaCO ₃	5 g
(NH ₄) ₂ SO ₄	4 g
Adjust pH with NaOH.....	6.1
Distilled water	up to 900 ml
Aliquot in bottles (90 ml) and sterilize.	

MCFP media for fermentation is prepared by mixing each 90 ml of MCFP with 10 ml of 30% sterile lactose.

■ **MDI (Defined medium for seed culture) (Casqueiro *et al.*, 1999)**

Citric acid	20 g
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Acetic acid	4.76 ml
(NH ₄) ₂ SO ₄	10 g
KH ₂ PO ₄	2 g
MgSO ₄ •7H ₂ O	1 g
FeSO ₄ •7H ₂ O	0.1 g
ZnSO ₄ •7H ₂ O	0.02 g
CuSO ₄ •5H ₂ O	0.02 g
MnSO ₄ •4H ₂ O	0.015 g
CoSO ₄	0.01 g
NaCl	0.002 g
Adjust pH	5.5
Distilled water	up to 1.6 liters

Aliquot in bottles (80 ml) and sterilize.

The defined media for fermentations is prepared by mixing each 80 ml of MDP with 20 ml of 20% sterile glucose.

■ **MDP (Defined medium for penicillin production) (Casqueiro *et al.*, 1999)**

32% Potassium phenylacetate	6.250 ml
Citric acid	20 g
Acetic acid	4.76 ml
(NH ₄) ₂ SO ₄	10 g
KH ₂ PO ₄	2 g
MgSO ₄ •7H ₂ O	1 g
FeSO ₄ •7H ₂ O	0.1 g
ZnSO ₄ •7H ₂ O	0.02 g
CuSO ₄ •5H ₂ O	0.02 g
MnSO ₄ •4H ₂ O	0.015 g
CoSO ₄	0.01 g
NaCl	0.002 g
Adjust pH	5.5
Distilled water	Up to 1.6 liters

Aliquot in bottles (80 ml) and sterilize.

The MDP media for fermentation is prepared by mixing each 80 ml MDP with 10 ml of 10% sucrose + 10 ml of 30% lactose.

■ **PMMY (For germination and growth of *P. chrysogenum* (Anne, 1977))**

Glucose.....	40 g
NaNO ₃	3 g
Yeast Extract	2 g
KCl	0.5 g
MgSO ₄ •7H ₂ O	0.5 g
FeSO ₄ •7H ₂ O	0.01 g
Adjust pH	6.0
Distilled water	up to 1 liter
Aliquot in bottles (100 ml) and sterilize.	

■ **PMMY-PAA (Media for germination, growth and penicillin production)**

32% Potassium phenylacetate	3.125 ml
Glucose.....	40 g
NaNO ₃	3 g
Yeast Extract	2 g
KCl	0.5 g
MgSO ₄ •7H ₂ O	0.5 g
FeSO ₄ •7H ₂ O	0.01 g
Adjust pH	6.0
Distilled water	up to 1 liter
Aliquot in bottles (100 ml) and sterilize.	

■ **LB media (Luria-Bertani for culturing the *E. coli* (Miller, 1972))**

Bacto-tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Agar (Only for solid medium).....	20 g
Distilled water	Up to 1 liter

■ TSB medium

Casein peptone	16 g
Soy peptone.....	3 g
Glucose	2.5 g
NaCl	6 g
K ₂ HPO ₄	2.5 g
Agar (Only for solid medium TSA).....	10 g
Adjust pH.....	7.0
Distilled water	Up to 1 liter

2.2. METHODS

2.2.1. Microorganisms; culture and maintenance

■ *E. coli* cultivation

E. coli cells were grown on LB solid medium plates (2% w/v agar) for about 12 hours at 37 °C. For cultivation in liquid medium, a single colony was inoculated to a 500 ml flask with 100 ml of medium and incubated 16-18 h at 37 °C and 250 rpm. Antibiotic (100 mg/ml ampicillin) was supplemented as selectable marker.

■ *E. coli* maintenance

The conditions for the maintenance of bacterial strains are dependent on the conservation time. For short periods (2-3 weeks), they were kept in the parafilm sealed Petri dishes at 4 °C. In order to preserve them for one year, suspensions were prepared in 20% glycerol and stored at -20 °C. Lyophilization is used for long-time maintenance (several years).

■ *P. chrysogenum* strains; sporulation on solid culture

Conidia from different strains of *P. chrysogenum* were grown on solid Power medium plates and incubated at 28 °C for 5-7 days to achieve complete sporulation.

■ *P. chrysogenum* strains maintenance

P. chrysogenum strains were preserved as conidia. For short periods (maximum a month) conidia were maintained at 4 °C on plates. To preserve them for one year, spore suspensions were prepared with a final concentration of glycerol 20% and stored at -20 °C. For longer periods of time, conidia suspensions were freeze-dried and stored at 4 °C or room temperature.

■ Microorganisms growth determination

To determine the growth of bacteria, the turbidity of the culture was calculated, assessing the absorbance (at 600 nm of OD) in a spectrophotometer.

In filamentous fungi, growth was assessed by calculating the dry weight: Samples were collected (5 ml) from fermentation at different time-points and centrifuged to

collect the mycelium. The mycelia were washed three times with 0.9% NaCl solution. After removing the final wash supernatant, the mycelia were transferred to buckets of aluminum foil. After 2 or 3 days of drying in an oven at 80 °C, the dry weight is calculated subtracting the weight of empty buckets.

■ Fermentation

In this work several types of media were tested to obtain the same growth pattern between different strains and best result in 2-DE gels.

For seed cultures, conidia were scraped from one plate surface in 4 ml of 0.9% NaCl using a sterile inverted Pasteur pipette and inoculated in 100 ml of medium. The seed cultures were shaken for 24 h at 25 °C and 250 rpm in orbital shakers. Then, fermentation was started by adding 5 ml of seed culture to 100 ml of fermentation medium. Flasks were shaken for the desired time under the same conditions.

2.2.2 Proteomics experiments

2.2.2.1. Isolation of the secretome from mycelia

Nytral membranes were used for collecting the mycelia from the medium. Mycelia was washed in another container once with 0.9% NaCl, and twice with ddH₂O and then stored at -80 °C.

The medium containing the secretome (secreted proteins) was immediately centrifuged at 4 °C for 5 min at 2500 rpm to discard the rest of mycelia. The supernatant was then filtrated on ice using the 0.45 µM paper filters and vacuum pump. The filtrated supernatant was mixed with the equal volume of the pre-chilled 20% (w/v) TCA/acetone (trichloroacetic acid in acetone) containing 0.14% (w/v) DTT (dithiothreitol).

The mixture was incubated at -20 °C overnight to precipitate the proteins or stored at -80 °C. Precipitated proteins were separated from supernatant by centrifuging at 4 °C for 5 min at 4500 rpm. The pellet was washed twice with pre-chilled acetone containing 0.07% (w/v) DTT and then twice with 80% (v/v) acetone/MQ-water. The final pellet was resuspended in sample buffer (8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) ampholytes, 25 mM DTT and 0.002% bromophenol blue) and stored at -80 °C.

2.2.2.2. Intracellular protein sample preparation

Frozen mycelia were ground to a fine powder in a pre-chilled mortar in the presence of liquid nitrogen. Different procedures were employed:

■ Direct protein precipitation by Clean up kit (GE Healthcare Bio-Sciences)

About 50 mg of homogenate were directly resuspended in the precipitant solution of the kit. All the steps were followed as instructed by manual guide in the protocol B of the manufacturer's manual. The air-dried pellet was resuspended in 300 µl sample buffer.

Protocol:

All steps should be carried out on ice unless otherwise specified.

- Transfer the protein samples into tubes that can be centrifuged at 13200 rpm. Each tube must have a capacity at least 12-fold greater than the volume of the sample. Use only polypropylene, polyallomer, or glass tubes. The wash buffer used later in the procedure is not compatible with many plastics. This limits the choice of centrifuge tube materials.

- For each volume of sample, add three volumes of precipitant. Mix well by vortex or inversion. Incubate on ice (4-5 °C) for 15 min.

- For each original volume of sample, add three volumes of co-precipitant to the mixture of protein and precipitant. Mix by vortexing briefly.

- Position the tubes in a microcentrifuge with the cap-hinges facing outward. Centrifuge at 13200 rpm for 10 min. Remove the tubes from the microcentrifuge as soon as centrifugation has finished. A pellet should be visible. Proceed rapidly to the next step to avoid resuspension or diffusion of the pellet.

- Remove as much of the supernatant as possible by decanting or careful pipetting. Do not disturb the pellet.

- Carefully position the tubes in the microcentrifuge with the cap-hinges and pellets facing outward. Centrifuge the tubes for at least 1 min to bring any remaining liquid to the bottom of the tubes. Use a pipette to remove the remaining supernatant. There should be no visible liquid remaining in the tubes.

- To each tube, add three-fold to four-fold more co-precipitant than the size of the pellet.
- Carefully reposition the tubes in the microcentrifuge with the cap-hinges facing outward. Centrifuge for 5 min. Use a pipette to remove the supernatant.
- Pipette enough distilled or deionized water on top of each pellet to cover the pellet. Vortex each tube for several seconds. The pellets should disperse, but not dissolve in the water.
- Add 1 ml of wash buffer, prechilled for at least 1 h at -20 °C to each tube. (For an initial sample volume of 0.1-0.3 ml, add 1 ml of wash buffer. However, the volume of wash buffer must be at least 10-fold greater than the distilled/deionized water.
- Add 5 µl wash additive (use only 5 µl wash additive, regardless of the original sample volume). Vortex until the pellet is fully dispersed.

Note: The protein pellet will not dissolve in the wash buffer.

- Incubate the tubes at -20 °C for at least 30 min. Vortex for 20-30 s once every 10 min. At this stage, the tubes can be stored at -20 °C for up to one week with minimal protein degradation or modification.
- Centrifuge the tubes at 13200 rpm for 10 min.
- Carefully remove and discard the supernatant. A white pellet should be visible. Allow the pellet to air dry briefly (no more than 5 min). Do not over-dry the pellet. If it becomes too dry, it will be difficult to resuspend.
- Resuspend each pellet in rehydration solution for first-dimension IEF. The volume of rehydration solution used can be as little as 1/20 of the volume of the original sample. Vortex the tube for 30 s. Incubate at room temperature. Vortex or aspirate and dispense using a pipette to fully dissolve. If the pellet is large or too dry, it may be difficult to resuspend fully. Sonication can speed resuspension.
- Centrifuge the tubes at 13200 rpm for 10 min to remove any insoluble material and to reduce any foam. The supernatant may be loaded directly onto first-dimension IEF or transferred to another tube and stored at -80 °C for later analysis.

■ Tris/DTT/SDS buffer solubilization and precipitation by Clean up kit

A total of 50 mg of homogenate were resuspended in 400 µl solubilising buffer consisting of 1% (w/v) SDS, 25 mM Tris base and 25 mM DTT. The extract was heated

at 95 °C for 5 min and insoluble debris was removed by centrifugation for 10 min at 13200 rpm. The supernatant was transferred to another microcentrifuge tube and three volumes of precipitant of the Clean Up Kit were added. The following steps were performed according to the protocol of the manufacturer's manual. The air-dried pellet was solubilised in 300 µl of sample buffer.

■ Tris/DTT/SDS buffer solubilisation and TCA/acetone precipitation

A total of 50 mg of homogenate were resuspended in 400 µl Tris/DTT/SDS buffer as described above; after heating and centrifugation the supernatant was added to two volumes of pre-chilled 20% (w/v) TCA in acetone containing 0.14% (w/v) DTT and incubated at -20 °C for 2h. After precipitation, the protein pellet was recovered by centrifugation for 10 min at 13200 rpm and washed with acetone containing 0.07 % (w/v) DTT and then with 80% (v/v) acetone in double distilled water. The final air-dried pellet was solubilised in 300 µl of sample buffer.

■ Phosphate solubilisation and TCA/acetone precipitation

A total of 2 g of homogenate was suspended in 10 ml (ratio 0.2 g/ml) of 10 mM potassium-phosphate buffer ($K_2HPO_4:KH_2PO_4$) (pH 7.4) containing 0.07% (w/v) 1,4-dithiothreitol (DTT) and-supplemented with tablets (one per 25 ml of buffer) of the protease inhibitor cocktail Complete™ (Roche). The mixture was stirred at 4 °C for 2 h, then the extract clarified by centrifugation for 10 min at 13200 rpm. Proteins in solution were mixed with 20 ml of 20% (w/v) TCA/acetone containing 0.14% (w/v) DTT and precipitated for 2 h at -20 °C. After precipitation, the protein pellet was recovered by centrifugation for 10 min at 13200 rpm and washed with acetone containing 0.07 DTT and then with 80% (v/v) acetone in double distilled water (Fernández-Acero *et al.*, 2006). The final air-dried pellet was solubilised in 300 µl of sample buffer.

■ TCA/acetone precipitation, phosphate solubilization and clean up purification

The desirable amount of protein extracts from the Phosphate solubilisation procedure was purified using clean up kit and the final air-dried pellet was solubilized in 300 µl of sample buffer.

In order to achieve the best focused first-dimension separation three different sample buffers were tested; these buffers have been also used as the rehydration buffer for IPG dry strips:

Sample buffer I: This buffer includes 8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) ampholytes, 25 mM DTT and 0.002% bromophenol blue.

Sample buffer II: This buffer includes 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) ampholytes, 25 mM DTT, 40 mM Tris and 0.002% bromophenol blue.

Sample buffer III: This buffer is used for the basic ranges (pH 6-9 and 7-10) of IPT dry strips and includes 8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) ampholytes, 25 mM DTT, 2% (v/v) Destreak (Amersham Biosciences) and 0.002% bromophenol blue.

After protein solubilization, extracts were centrifuged two times at 13200 rpm for 15 min to remove any insoluble material (cell debris) at 15 °C (to prevent urea crystallization at low (4 °C) temperatures or protein carbamylation at higher (37 °C) temperatures, respectively). Protein extracts were used immediately, or stored at -80°C for several months. A small amount (30 µl) of sample solution was separated from the protein samples for analytical experiments like determination of protein concentration or trial monodimensional SDS-PAGE analysis.

2.2.2.3. Determination of protein concentration

Protein concentration assessment on a particular solution was carried out by the Bio-Rad Protein Assay, which is based on the method of Bradford. It involves the addition of an acidic dye to protein solution, and subsequent measurement at 595 nm with a spectrophotometer or microplate reader. Comparison to a standard curve provides a relative measurement of protein concentration. The Bio-Rad Protein Assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein (Bradford, 1976).

The maximum absorbance for an acidic solution of Coomassie[®] Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when protein binding occurs (Reisner *et al.*, 1975, Sedmack J. *et al.*, 1963, Fazakes *et al.*, 1977). The Coomassie blue dye binds primarily basic and aromatic amino acid residues, especially arginine (Compton *et al.*, 1985).

The concentration of the sample is determined according to a standard line made with bovine serum albumin (BSA from MBI Fermentas). BSA protein solution with a concentration of 0.25 mg/ml was used to obtain the reference pattern specified in table 2.2.

BSA (mg/ml)	0.0	0.5	1.25	2.5	3.75	5.0	6.25	7.5	8.75	10
BSA solution (μl)	0	2	5	10	15	20	25	30	35	40
MQ water (μl)	800	798	795	790	785	780	775	770	765	760

Table 2.2. BSA protein solutions for the Bradford analysis.

Finally, 1/1000 and 1/2000 dilutions were prepared for each sample in 1.5 ml micro tubes in up to 800 μl of MQ water. Then, 200 μl of Bradford reagent (Bio-Rad Dye Reagent, BioRad) were added.

Solutions were stirred and left for 5 min on the bench. Solutions were stirred again and the measurements were done using absorbance at 595 nm. All experiments were performed in duplicate.

2.2.2.4 SDS-PAGE

Electrophoresis in denaturing polyacrylamide gels (SDS-PAGE) was performed as described by Laemmli (1970). Polyacrylamide gels consist of two phases differing in their characteristics, such as: porosity, pH, ionic strength and in function.

The stacking phase serves to concentrate the sample at the boundary separating the two phases and allow homogeneous proteins entry in the next phase of the gel (Table 2.3).

In turn, the resolving phase (Table 2.4) plays the main role of electrophoresis, separation the proteins according to their molecular mass. Different concentrations of polyacrylamide are used for the separation of proteins different ranges. For example the percentage 7% of resolving is used for separation of proteins with molecular weight between 250 and 50 kDa, 12% for proteins between 100 and 20 kDa and 15% for proteins between between 60 and 10 kDa.

SDS-PAGE (Stacking phase)	
30% Acrylamide/bis-acrylamide	1.3 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml
20% SDS (w/v)	50 µl
10% (w/v) Ammonium persulfate	50 µl
TEMED (N,N,N',N'-Tetramethylethylenediamine)	10 µl
MQ water (up to 10 ml)	6.1 ml

Table 2.3. Composition of the stacking phase of SDS-PAGE (10 ml)

SDS-PAGE (Resolving phase)	7.5 %	12 %	15 %
30% Acrylamide/bis-acrylamide	2.5 ml	4.0 ml	5.0 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	2.5 ml	2.5 ml
20% SDS (w/v)	50 µl	50 µl	50 µl
10% (w/v) Ammonium persulfate	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl
MQ water (up to 10 ml)	4.9 ml	3.4 ml	2.4 ml

Table 2.4. Composition of different concentrations of the resolving phase

Protocol

- Clean and assemble the gel sandwich on a clean surface. Lay the longer rectangular glass plate down first, then place two spacers of equal thickness along the short edges of the rectangular plate.
- Prepare the resolving gel monomer solution by combining all reagents except ammonium persulfate (APS) and TEMED. Degas the solution under vacuum for at least 15 min.
- Place a comb completely into the assembled gel sandwich. Place a mark on the glass plate 1 cm below the teeth of the comb. This will be the level to which the resolving gel is poured. Remove the comb.

- Add APS and TEMED to the degassed monomer solution and pour the resolving solution to the mark, Pour the solution smoothly to prevent it from mixing with air.

- Immediately overlay the monomer solution with MQ-water, water-saturated isobutanol or isopropanol (Isobutanol will chemically attack the acrylic plastic of the sandwich clamp, this will cause crazing of the plastic).

- Allow the gel to polymerize for 45 min to 1 hour. Rinse off the overlay solution completely with distilled water. This is especially important with alcohol overlays. Do not allow alcohols to remain on the gels more than 1 hour, or the top of the gel will dehydrate.

- Prepare the stacking gel monomer solution. Combine all reagents except APS and TEMED and degas under vacuum at least 15 min. Dry the area above the separating gel with filter paper before pouring the stacking gel.

- Add APS and TEMED to the degassed monomer solution and pour the solution. Place a comb in the gel sandwich and tilt it so that the teeth are at a slight (~10°) angle. This will prevent air from being trapped under the comb teeth

- Allow the gel to polymerize 30-45 min. Remove the comb by pulling it straight up slowly and gently. Rinse the wells completely with distilled water or running buffer.

- Place the gel in the electrophoresis tank and fill it by 1X Laemmli SDS electrophoresis buffer¹ and load the desired volume of protein samples as well as protein marker and run the gel.

¹10X Laemmli SDS electrophoresis buffer: 250 mM Tris base, 2 M Glycine, 1% (w/v) SDS

2.2.2.5 Optimized silver staining

Gels were stained according to the following protocol (Table 2.5), where the first fixation may be prolonged up to 3 days if desired. In this protocol by omitting glutardialdehyde from the sensitizer and formaldehyde from the silver nitrate solution, as well as omitting the “preservative step”, the method becomes compatible with mass spectrometry analysis, although sensitivity is reduced. If glutardialdehyde and formaldehyde are to be used, it is added just before staining.

Step	Solutions	Amount	Time
Fixation	Ethanol Acetic acid, glacial MQ-water	200 ml 50 ml up to 500 ml	2 × 60 min
Sensitizing	Ethanol Glutardialdehyde (25% w/v) Sodium thiosulfate (5% w/v) Sodium acetate MQ-water	75 ml 1.25 ml 10 ml 17 g up to 250 ml	60 min
Washing	MQ-water	-	5 × 8 min
Silver reaction	Silver nitrate (2.5% w/v) Formaldehyde (37% w/v) MQ-water	25 ml 100 µl up to 250 ml	60 min
Washing	MQ-water	-	4 × 1 min
Developing	Sodium carbonate Formaldehyde (37%) MQ-water (Stir vigorously)	6.25 g 100 µl up to 250 ml	5 min
Stop	Na ₂ EDTA.H ₂ O MQ-water	3.65 g up to 250 ml	45 min
Washing	MQ-water	-	2 × 30 min
Preservation	Glycerol (87%) OR Ethanol (10% v/v) MQ-water	11.5 ml 25 ml up to 250 ml	20 min

Table 2.5. Optimized silver staining method

The volume of the formaldehyde in the developing solution can be varied from 100 µl up to 250 µl, depending on the amount of protein and the number of spots. Since formaldehyde is consumed in the developing reaction by proteins it is added directly before use.

Developing time is around 5 min but this step may be visually monitored. The gels should be transferred to stop solution when the bands or spots have reached the desired intensity and before the staining background becomes too dark.

After staining the long-term storage of gels is possible in 10% ethanol rather than glycerol, if gels are not being dried down. Glycerol is necessary only if planning to dry down gels. Storage in ethanol allows the gels to be compatible with spot picking/mass spectrometry.

2.2.2.6. 2D Electrophoresis

■ IEF (Iso Electric Focusing) using the Strip Holder for gel rehydration

In this work the Amersham IPGphor fixed-length Strip Holders were used for IEF. They allow IPG strips to be rehydrated and samples loaded in one step before proceeding automatically to perform the IEF separation.

The Amersham IPG strips are 3 mm wide and 0.5 mm thick after rehydration. This protocol applies for both in-gel sample rehydration and sample application after gel rehydration.

Protocol

- Prepare the strip holder(s): Select the strip holder(s) corresponding to the Immobiline DryStrip gel length chosen for the experiment. Handle the ceramic strip holders with care. Clean Strip Holders after each first-dimension IEF run with strip holder cleaning solution, to remove residual protein from the strip holders. Do not let solutions dry in the strip holder. Rinse well with distilled or deionized water. Thoroughly air-dry the strip holders or dry well with a lint-free tissue prior to use. Handle clean strip holders with gloves to avoid contaminating them.
- Apply the rehydration solution: Pipette the appropriate volume of rehydration solution into each strip holder and add the appropriate amount of sample to the rehydration solution.
- Deliver the solution slowly as a stripe of liquid between the two electrodes away from the sample application wells. Remove any large bubbles.
- Position the Immobiline DryStrip gel: Remove the protective cover from the Immobiline DryStrip gel starting at the acidic (+) end. Removal from the acidic end

prevents damage to the basic end of the gel, which is generally softer. Using forceps position the Immobiline DryStrip gel with the gel side down and the anodic (+) end of the strip directed toward the pointed end of the Strip Holder. To help coat the entire strip, gently lift and lower the strip and slide it back and forth along the surface of the solution, tilting the strip holder slightly as required to ensure complete and even wetting. Finally, lower the cathodic end of the Immobiline DryStrip gel into the channel, making sure that the gel contacts the Strip Holder electrodes at each end.

- **Apply Immobiline DryStrip Cover Fluid:** Apply Immobiline DryStrip Cover Fluid to minimize evaporation and thus prevent urea crystallization

- **Place the cover on the strip holder:** Pressure blocks on the underside of the cover ensure that the Immobiline DryStrip gel maintains good contact with the electrodes as the gel swells.

- **Allow the Immobiline DryStrip gel to rehydrate:** Rehydration can proceed on the bench top or on the Ettan IPGphor II platform. Ensure that the Strip Holder is on a level surface. A minimum of 10 h or overnight is required. The rehydration period can be programmed as the first step of an Ettan IPGphor II protocol. This is especially convenient if temperature control during rehydration is a concern.

- **Optional: Apply electrode pads:** For large sample loads and when using narrow-pH-range Immobiline DryStrip gels, better results are obtained by applying damp paper pads. Cut two 3-mm-wide electrode pads from a paper IEF electrode strip or whatman paper. Electrode pads must be damp, not wet. Using forceps lift one end of the rehydrated Immobiline DryStrip gel. Position the electrode pads over the electrode, then lower the gel strip back into place. Additional DryStrip Cover Fluid may need to be added to ensure that the strip is still adequately covered.

- **Ensure that the strip holders are properly positioned on the Ettan IPGphor II platform.** Check that both external electrode contacts on the underside of each strip holder make metal-to-metal contact with the platform.

- **Begin IEF.** As isoelectric focusing proceeds, the bromophenol blue tracking dye migrates toward the anode and leaves the Immobiline DryStrip gel well before focusing is complete. The final step of focusing should be run in volt-hours to ensure reproducibility from run to run.

■ The isoelectric focusing program

Using IPGphor Regular Strip Holder with the 7-cm strips, the maximum voltage was 5000 V and current 50 μ A per strip. The samples were focused up to 20 kVh. The isoelectric focusing program for 7-cm strips in different ranges of pH has been presented in table 2.6.

pH interval	Voltage mode	Voltage(V)	Time (h:min)
3-10 4-7	1. Step and Hold	30	12:00
	2. Step and Hold	60	0:30
	3. Step and Hold	300	0:30
	4. Gradient	1000	0:30
	5. Gradient	5000	0:30
	6. Step and Hold	5000	8:00
	Total	-	22:00

Table 2.6. The isoelectric focusing program for 7-cm strips

Using IPGphor Regular Strip Holder with the 18-cm strips, the maximum voltage was 8000 V and current 50 μ A per strip. The samples were focused up to 60 kVh. The isoelectric focusing program for 18-cm strips in different ranges of pH has been presented in table 2.7.

pH intervals	Voltage mode	Voltage(V)	Time (h:min)
3-10 NL 3-5.6 5.3-6.5 4.5-5.5 4-7 6-9 7-11	1. Step and Hold	30	12:00
	2. Step and Hold	60	1:00
	3. Step and Hold	500	1:00
	4. Step and Hold	1000	1:00
	5. Gradient	8000	0:30
	6. Step and Hold	8000	8:00
	Total	-	22:00

Table 2.7. The isoelectric focusing program for 18-cm strips. NL: Non linear.

■ Preservation of focused Immobiline DryStrip gels

After IEF was complete, the second-dimension separation was immediately started or the Immobiline DryStrip Gels were stored at -80 °C. The DryStrip gels were stored in green-cap tubes (7-cm) or glass tubes (18 cm).

■ Equilibration of Immobiline DryStrip gels

Prior to Immobiline DryStrip gel equilibration the second-dimension vertical gel must be ready for use. Equilibration was carried out in a two-step process.

Protocol

- Prepare an appropriate volume of SDS equilibration buffer¹, then measure into two equal volumes.
- Just prior to use, add DTT to one portion (100 mg per 10 ml) and iodoacetamide to the other (250 mg per 10 ml).
- Place the IPG strips in individual tubes, with the support film toward the tube wall. Add the appropriate (enough to cover the stripe fully) volume of SDS equilibration buffer (+ DTT) to each strip. Cap or seal the tubes with flexible paraffin film and place them on their sides on a rocker for the equilibration process. Equilibrate for 15 min.
- Pour off buffer from above step and add the appropriate volume of SDS equilibration buffer (+Iodoacetamide) to each strip. Again cap or seal the tubes with flexible paraffin film and place them on their sides on a rocker for the equilibration process. Equilibrate for an additional 15 min.

¹SDS equilibration buffer: 6 M Urea, 75 mM Tris-HCl, pH 8.8, 30% (v/v) Glycerol, 2% (w/v) SDS, 0.002% (w/v) Bromophenol blue

■ Second dimension

In this work the second dimension was run by SDS-PAGE in 15% (for the reference map) or in 12.5% (for comparison experiments) polyacrylamide in an Ettan Dalt Six apparatus (GE Healthcare). Vertical second-dimension gels are most conveniently cast several at the same time, in a multiple gel caster.

Protocol

- Assemble the gel caster for the Ettan DALTsix
- Select the gel percentage and calculate the required casting solution volume; the total volume of solution required depends on the gel size, the gel thickness, and the number of gels cast. For six gels 400 ml of solution is required. Table 2.8 gives volumes of gel solution required for 100 ml of gel solution.

Final gel concentration	5%	7.5%	10%	12.5%	15%
Monomer stock solution	16.7 ml	25 ml	33.3 ml	41.7 ml	50 ml
1.5M Tris-HCl, pH 8.8	25 ml	25 ml	25 ml	25 ml	25 ml
10% SDS	2 ml	2 ml	2 ml	2 ml	2 ml
Double distilled water	55.8 ml	47.5 ml	39.2 ml	30.8 ml	22.5 ml
10% Ammonium persulphate	500 µl	500 µl	500 µl	500 µl	500 µl
TEMED	33 µl	33 µl	33 µl	33 µl	33 µl
Total volume	100 ml	100 ml	100 ml	100 ml	100 ml

Table 2.8. Composition of different concentrations of SDS-PAGE

- Make up the gel solution without TEMED or ammonium persulfate. Just before casting the gel, add TEMED and 10% ammonium persulfate. Gently swirl the flask to mix, being careful not to generate bubbles. Immediately pour the gel.
- Fill the gel cassette to 5-10 mm below the top. No stacking gel layer is required.
- Overlay each gel with a layer of MQ-water or isopropanol immediately after pouring to minimize gel exposure to oxygen and to create a flat gel surface.
- After allowing a minimum of 2 h for polymerization, remove the overlay and rinse the gel surface with gel storage solution and replace the isopropanol with an overlay of running buffer.
- Place the IPG strip: Dip the equilibrated IPG strip in the 1X Laemmli SDS electrophoresis buffer¹ to lubricate it. Position the IPG strip between the plates on the surface of the second-dimension gel. With a thin plastic ruler, gently push the IPG strip down so the entire lower edge of the IPG strip is in contact with the top surface of the

gel. Ensure that no air bubbles are trapped between the IPG strip and the slab gel surface or between the gel backing and the glass plate.

- Apply the markers to a paper piece in a volume of 5 µl then place it to the top surface of the gel with forceps. (Precision Plus Protein Standards (Bio-Rad) were used as markers in this study).

- For Ettan DALT electrophoresis units, the lower tank requires 1X Laemmli SDS electrophoresis buffer while the upper chamber requires buffer of a higher concentration (2X). Fill the electrophoresis tank with 5 liter of 1X SDS Laemmli electrophoresis buffer. Turn on the pump and Switch on the MultiTemp III temperature controller and set the desired temperature (20 °C).

- Electrophoresis is performed at constant current in two steps. During the initial migration and stacking period, the current is approximately half of the value required for the separation. In the first step the gels are ran for 45 min on 2-3 W per gel. The second run is done for approximately 3 h on 18-20 W per gel, when the dye front is approximately 1 mm from the bottom of the gel.

¹10X Laemmli SDS electrophoresis buffer: 250 mM Tris base, 2 M Glycine, 1% (w/v) SDS

2.2.2.7. Protein detection and quantitation

After electrophoresis, gels were removed from their cassettes. Each gel was notched or marked at the upper corner nearest the pointed end of the IPG strip to identify the acidic end of the first dimension separation. In this work two main procedures were tested for visualization and detection of protein spots:

■ Silver staining

In order to stain the gels using silver staining, 100 µg of protein samples was loaded for 2-DE. The gels were fixed overnight and staining was done following the optimized protocol that was described before.

■ Blue Silver Staining

A modified Neuhoff's colloidal Coomassie Blue G-250 staining method was used in this work as reported previously by Candiano and coworkers (2004). This method,

dubbed “blue silver”, has considerably higher sensitivity, approaching the one of conventional silver staining. The main modifications, as compared to Neuhoff’s protocol, were: a 20% (w/v) increment in dye concentration (from 0.1% up to 0.12%) and a much higher level of phosphoric acid (from 2% up to 10% (w/v)). The “blue silver” method exhibits a much faster dye uptake (80% during the first hour of coloration, vs. none with a commercial preparation from Sigma). When the dye is ready, gels can be stained overnight after fixing.

Protocol

- To 1/10 of the final volume of solution (200 ml of water) the desired amount of phosphoric acid 85% is added (235 ml), so that, in the final volume, its concentration will be 10%.
- To the above solution, add the required amount of ammonium sulfate (200 g of powder), to obtain a final concentration of 10%.
- When the ammonium sulfate has been dissolved, add enough Coomassie Blue G-250 (2.4 g in powder) to obtain a final concentration of 0.12% (w/v).
- After dissolving all solids, add water to 80% of the final volume.
- To this solution, under stirring, add anhydrous methanol to reach a 20% (v/v) final concentration. This stock dye solution should be kept in a brown bottle and is stable at room temperature for 6 months.
- Fix the gels by shaking them into 350 ml of the fix solution containing 10% (v/v) acetic acid and 40% (v/v) ethanol for 30 min.
- Wash the gels three times for 10 min in 350 ml of MQ-water.
- Add 350 ml of blue silver micellar solution for each gel and shake it overnight.
- Destain the gels to clear the backgrounds, three times for 30 min in 350 ml of MQ-water.

2.2.2.8. Scanning and digitalization

Three gels obtained from three independent cultures (biological replicates) were analyzed in a group to guarantee representative results. 2D images were captured by scanning stained gels using the ImageScanner II (GE Healthcare). The scanner was previously calibrated by using a greyscale marker (Kodak). The results were digitalized

with Labscan 5.00 (v 1.0.8) software (GE Healthcare). These images were performed with resolution of 150 dpi and cropped in the same size and field in the tiff format. This format allows the image to be analyzed by ImageMaster™ 2D Platinum software v 5.0 (GE Healthcare).

■ Analysis of Protein Expression by ImageMaster software

After importing the images into ImageMaster software, automated spot detection was optimized (smooth=2 minimal area=15 saliency=700), then spots were checked manually to eliminate any possible artifacts, such as streaks or background noise.

For proteomic and secretomic reference map the gel with highest number of detected spots was selected to pick and identify all the spots.

For comparison of the pattern of protein expression between different strains or different conditions the gels were grouped first (each three relative gels were added to class), then one of the gels was “set as reference” to be compared to. The patterns of each sample were overlapped using “three landmarks” features, to detect potential differentially expressed proteins. All the gels were selected and auto matched.

Spot normalization is an internal calibration to make the data independent from experimental variations among gels. It was made using relative volumes (%Vol) to quantify and compare the gel spots. The %Vol corresponds to the volume of each spot divided by the total volume of all the spots in the gel. Differentially expressed proteins were marked to be identified. These protein spots were considered when the ratio of the %Vol average (3 gels) between groups was higher than 1.5 and the *p-value* <0.05.

2.2.2.9. In gel protein digestion

The protein spots of interest were manually excised from Colloidal Commassie-stained gels, placed in an Eppendorf tube, and washed twice with ddH₂O. The proteins were digested following the method of Havlis and coworkers (2003).

Protocol

- Dehydrate the spots by adding acetonitrile (AcN) until it covers the band or spot (20 µl) and wait 5 min for dehydration and releasing the dye. Repeat this operation twice to make the gel more or less white.

- Dry in a vacuum centrifuge “Speed-Vac”
- Cover bands with a solution 10 mM DTT in 25 mM ammonium bicarbonate (NH_4HCO_3) and leave it for 30 min at 56 °C then leave at room temperature for 1 min.
- Remove the DTT solution and add AcN for 5 min.
- Replace the AcN by 55 mM Iodoacetamide (10 mg/ml) in 25 mM NH_4HCO_3 and incubate for 15 min in dark.
- Discard the Iodoacetamide solution and incubate for 5 min with AcN, then discard it.
- Add 25 mM NH_4HCO_3 and incubate for 5 min, without removing supernatant add an equal volume of ACN and incubate for 15 min.
- Remove the supernatant. Dry in the “Speed-Vac”.
- Prepare a 12.5 ng/ml trypsin solution in 25 mM NH_4HCO_3 . Keep on ice until use. Add the trypsin solution in a volume that covers the dried spots. Incubate on ice for 45 min.
- Remove the supernatant and add 25 mM NH_4HCO_3 .
- Incubate overnight at 37 °C (or at least 6 hours).
- After digestion, vortex and centrifuge, then pass the supernatant to new tubes.
- Cover the spots with 50% (v/v) AcN / 0.5% (v/v) trifluoroacetic acid (TFA) and sonicate 10 min. Transfer the supernatant to the new tube.
- Cover the gel pieces with AcN for 10 min. Transfer the supernatant to the new tube.
- After collecting all the supernatants, dry the peptide solution in the “Speed-Vac”.
- Dissolve the pellet in 4 μl of 50% AcN and 0.1% TFA solution.

2.2.2.10. Protein Identification by MALDI-TOF MS and MS/MS

One μl from each peptide solution was spotted on a MALDI target plate and air dried for 10 min at room temperature. Subsequently, 0.5 μl of matrix [3 mg/ml of α -cyano-4-hydroxy-trans-cinnamic acid (LaserBio Labs)] diluted (1:1, v/v) in 0.1% TFA-ACN/ H_2O were added to the dried peptide digestion and air dried for another 5 min at room temperature. The samples were analyzed with a 4800 Proteomics Analyzer

MALDI-TOF/TOF mass spectrometer (Applied Biosystems). A 4700 proteomics analyzer calibration mixture (Cal Mix 5; Applied Biosystems) was used as external calibration. All MS spectra were internally calibrated using peptides from the trypsin digestion. The analysis by MALDI-TOF mass spectrometry produced peptide mass fingerprints, and the peptides observed (up to 65 peptides per spot) were collected and represented as a list of monoisotopic molecular weights with a S/N greater than 20. All known contaminant ions (trypsin- and keratin-derived peptides) were excluded for protein database searching and for later MS/MS analysis. Hence, from each MS spectra, the six most intensive precursors with a S/N greater than 20 were selected for MS/MS analyses with CID (atmospheric gas was used) in 2-kV ion reflector mode and precursor mass windows of ± 7 Da. The plate model and default calibration were optimized for the MS/MS spectra processing.

For protein identification, both MS and MS/MS spectra were automatically searched using a local license of Mascot v 2.2 from Matrix Science through the Protein Global Server (GPS) v 3.6 (Applied Biosystems). The search parameters for peptide mass fingerprints and tandem MS spectra obtained were set as follows: i) NCBI nr (2008.11.22 and 2009.11.03 for the intracellular and extracellular proteomes, respectively) sequence databases were used; ii) taxonomy: Total and Fungi; iii) fixed and variable modifications were considered (Cys as S-carbamidomethyl derivative and Met as oxidized methionine); iv) one missed cleavage site was allowed; v) precursor tolerance was 100 ppm and MS/MS fragment tolerance was 0.3 Da; vi) peptide charge: 1+; (vii) the algorithm was set to use trypsin as the enzyme. Protein candidates produced by this combined peptide mass fingerprinting (PMF)/tandem MS search were considered valid when the global Mascot score was greater than 81 (total database) or 69 (fungal database) with a significance level of $p < 0.05$. Additional criteria for confident identification were that the protein match should have at least 15% sequence coverage; for lower coverages, only those proteins with a Mascot ions score above 54 and at least two peptides identified in the tandem MS analysis (with a significance level of $p < 0.05$), were considered valid.

2.2.3. Genetic manipulation

2.2.3.1. Total DNA extraction from *P. chrysogenum* mycelia

Genomic DNA extraction from *P. chrysogenum* was performed using the protocol developed by Fernandez and coworkers (1997).

Protocol

- Inoculate 100 ml of appropriate culture medium (PMMY) with fresh spores from one plate (approximately 10^8 spores) and incubate in a 500 ml flask.
- Shake at 250 rpm and 25 °C in orbital shaker for 24-48 h.
- Collect the mycelia by filtration through a sterile nylon filter (NYTAL) with 30 µm of pore diameter.
- Wash the retained mycelium twice with NaCl 0.9% (w/v), then wash twice with buffer A¹.
- Collect the mycelium and dry it between filter papers and freeze it at -80 °C.
- Grind 1 g of the mycelium in a mortar with liquid nitrogen. Collect the powder in 2 ml microtubes (do not exceed the level of 0.5 ml, which corresponds to approximately 100 mg of powdered mycelium).
- Add 800 µl of breaking buffer². Homogenize the mixture and add 800 µl of neutral phenol.
- Mix gently and incubate the mixture in a water bath at 50 °C for 30 min; shake every 5 min.
- Centrifuge the mixture at 13200 rpm for 10 min at room temperature. Collect the aqueous phase (top) and perform the cleaning process and precipitation as described in the next section.
- Resuspend DNA in 35-50 µl of TE or MQ water.

¹ Buffer A: 50 mM Tris-HCl pH 8, 150 mM NaCl, 100 mM EDTA, pH 8.

² Breaking buffer: 0.2 M Tris-HCl, 10 mM EDTA, 1% SDS, pH 8.2.

2.2.3.2. DNA Cleaning and precipitation

The removal of proteins and other impurities in the DNA solutions was performed by a cleaning process or phenolization followed by precipitation of DNA.

Protocol

- Add DNA solution to the same volume of natural phenol solution¹ and shake vigorously using a vortex. Centrifuge for 5 min at 13200 rpm at room temperature.
- Collect the aqueous (upper phase) and carefully add 0.5 volumes of neutral phenol and 0.5 volumes of CIA².
- Shake vigorously again and centrifuge 5 min at 13200 rpm at room temperature. Repeat the addition of phenol-CIA until the interface is completely free of impurities.
- Add 1 volume of CIA to eliminate traces of phenol. Vortex and centrifuge, collect the aqueous phase at the end of the process.
- Add DNA solution to 0.1 volume 3 M sodium acetate pH 5.2 and 2.5 volumes of absolute ethanol (pre-cooled at -20 °C). Mix using a vortex.
- Keep the mixture for 2 h at -20 °C or 30 min at -80 °C. Centrifuge for 30 min at 13200 rpm and 4 °C. Remove the supernatant.
- Wash the precipitate with 0.5 volumes of 70% ethanol and centrifuge 5 min at 13200 rpm and room temperature. Remove the supernatant and dry the precipitate.
- Resuspend in MQ water and Store at 4 °C or frozen (-20 °C) for long periods.

¹ Phenol neutral: Mix by stirring 3 volumes of acidic phenol with 1 volume of 1M Tris-HCl, pH=8 and 8-hydroxyquinoline (50 mg per 100 ml of acidic phenol, reducing compound that prevents oxidation). Let it stand until the phases are separated and store at 4 °C.

Acidic Phenol: Mix by stirring strongly 500 ml of crystalline phenol with 500 ml of distilled water. Let it to stand up to separate the two phases and remove the upper phase (aqueous).

² CIA: Chloroform 24 volumes, 1 volume isoamyl alcohol.

³ TE: Tris-HCl 10 mM EDTA 1 mM, pH 8.0.

2.2.3.3. Total DNA extraction from *P. chrysogenum* conidia

The following protocol was used to extract the DNA directly from spores very quickly.

Protocol:

- Collect the spores from a PW plate using 2 ml of 0.9% NaCl.

- Transfer the spores to a micro tube, centrifuge for 1 min at 13200 rpm.
- Discard the supernatant completely and add 300 µl of spore breaking buffer¹.
- Add 50-100 µg of glass beads (425-600 µm), vortex for 15-20 sec and put on ice. Repeat this step 3 times.
- Add 150 µl of 3 M sodium acetate (pH=5.2), mix well and incubate at -20 °C for 10 min
- Centrifuge for 5 min at 13200 rpm.
- Transfer the supernatant (450 µl) to a new tube, add the same volume of Isopropanol and incubate for 10 min at room temperature.
- Centrifuge at 13200 rpm 10 min, discard the supernatant.
- Wash the pellet with 70% ethanol (200 µl), discard the supernatant completely.
- Dry the DNA pellet and dissolve it in 30 µl of MQ water.

¹ Spore breaking buffer: 1% SDS, 200 mM Tris-HCl, 100 mM EDTA, pH=8

2.2.3.4. Selective removal of nucleic acids

The RNA or DNA purification processes typically involve DNA or RNA contamination, respectively. To remove one of the nucleic acids selectively, RNase and DNase (Stock: 10 mg/ml in 10 mM Tris-HCl, 15 mM NaCl, pH 7.5) enzymes were used. Both enzymes were used at the same final concentration of 10 mg/ml, and the reaction was incubated at 37 °C for 90 min.

2.2.3.5. Determination of nucleic acids concentration

The nucleic acid concentration was determined spectrophotometrically by measuring the absorbance at 260 nm. For double-stranded DNA, it was estimated that each unit of optical density is equivalent to 50 mg/ml of DNA and for single-stranded RNA, each unit of optical density is equivalent to 40 mg/ml of RNA. The wavelength of side chains of aromatic amino acids absorbance is 280 nm, so the nucleic acid purity regarding protein contamination was estimated using the absorbance ratio 260/280 (a sample is considered pure when the ratio is between 1.8 and 2 (Sambrook and Russell, 2001)).

2.2.3.6. PCR, Principles and procedure

PCR is used to amplify a specific region of a DNA strand (the DNA target). A basic PCR set up requires several components and reagents (Sambrook *et al.*, 2001). These components include:

- *DNA template* that contains the DNA region (target) to be amplified.
- Two *primers* that are complementary to each of the sense and anti-sense strands of the DNA target.
- *Taq polymerase* or another DNA polymerase.
- *Deoxynucleoside triphosphates* (dNTPs), the building blocks from which the DNA polymerases synthesizes a new DNA strand.
- *Buffer solution*, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- *Divalent cations*, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis
- *Monovalent cation* potassium ions.

The PCR usually involves a series of 20 to 40 cycles; each cycle typically consists of 2-3 discrete temperature steps. Most commonly PCR is carried out with cycles that have three temperature steps.

Initialization step: This step consists of heating the reaction to a temperature of 94-96 °C which is held for 1-9 min. It is only required for DNA polymerases that require heat activation by hot-start PCR.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94-98 °C for 20-30 seconds.

Annealing step: The reaction temperature is lowered to 50-65 °C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the T_m of the primers used.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; commonly a temperature of 72 °C is used with the enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs in 5' to 3' direction.

Final elongation: This single step is occasionally performed at a temperature of 70-74 °C for 5-15 min after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold: This step at 4-15 °C for an indefinite time may be employed for short-term storage of the reaction.

The times and temperatures for PCR reaction are essentially dependent on various factors such as G+C content of DNA, G+C content of oligonucleotides, amplified fragment length, speed of processing of DNA polymerase, template DNA abundance, etc. In general terms, these times and temperatures are reflected in Table 2.9.

Step		Temperature	Time	Cycles
Initial Denaturation		94-96 °C	4-6 min	1
Amplification	Denaturation	94-96 °C	30-60 s	25-35
	Annealing	Dependent on primers	30- 45 s	
	Extension	72 °C	30-120 s	
Final Extension		72 °C	10 min	1

Table 2.9. Typical reaction times and temperatures for a PCR reaction.

Protocol

- Make a list of the premix (amounts of primers, buffer, dNTPs, enzyme, additions (BSA, DMSO)). Run always a blank and one positive control. For x DNA samples calculate the amount for X+3 reactions (pos. and neg. control, +1 to compensate for small pipetting errors).

Template DNA 1-10 ng

Primers (18-30 bp) 0.1- 0.6 mM

MgCl₂ 1-5 mM

dNTPs 50-500 mM

10X Buffer..... 1X

DNA polymerase..... 0.5 - 2.5 U

MQ-Water Complete volume

- Vortex and centrifuge, place the samples in the thermocycler and start the PCR.

2.2.3.7. Agarose gel electrophoresis

Nucleic acids will migrate towards the positive electrode, which is usually colored in red. The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes. Bromophenol blue and xylene cyanol dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 400 and 4000 bp, respectively.

When adequate migration has occurred, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. To visualize DNA or RNA, the gel is placed on an ultraviolet transilluminator. Since DNA will diffuse within the gel over time, examination or photography should take place shortly after cessation of electrophoresis.

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight.

Circular forms of DNA migrate in agarose distinctly differently from linear DNAs of the same mass. Typically, uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized (In a 1% agarose gel the observed size of circular DNA should be multiply in 0.6). Additionally, most preparations of uncut plasmid contain at least two topologically-different forms of DNA, corresponding to supercoiled forms and nicked circles.

Higher concentrations of agarose, facilitates separation of small DNAs, while low agarose concentrations allows better resolution of larger DNAs. As the voltage applied to a gel is increased, larger fragments migrate proportionally faster comparing with small fragments. For that reason, the best resolution of fragments larger than about 2 kb is attained by applying no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).

Protocol

- Dissolve by heating agarose in TAE buffer¹ (1X), after cooling the solution to about 60 °C, pour into a casting tray containing a sample comb and allowed to solidify. Remove the comb.

- Mix the DNA samples with 1/6 of final volume from loading buffer² and load into the sample wells. Load 5 µl of the DNA marker in the first well.

- Develop horizontal electrophoresis in TAE buffer (1X) by applying a potential difference of 1-5 v/cm. The current can be confirmed by observing bubbles coming off the electrodes.

- Stain DNA by immersing the gel for 20-30 min in the ethidium bromide solution³.

- Visualize in a UV transilluminator and take a photograph.

¹ 50X TAE buffer: 2 M Tris base, 5.7% (v/v) glacial acetic acid, 50 mM EDTA pH=8.

² concentrated loading buffer (6X): 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene-cyanol, 50% (v/v) glycerol, 0.1M EDTA, 0.1% SDS, 10 mM Tris-HCl.

³ Ethidium bromide solution (EtBr): Prepare a stock solution 10 mg/ml in water and store at 4 °C protected from light. For a liter of distilled water add 50 ml of the concentrated solution.

2.2.3.8. DNA extraction from agarose gels

To perform the extraction of a desired band of DNA from agarose gels the Gel Band Purification Kit (GE Healthcare) was used following the manufacturer's recommendations.

Protocol

- Separate the DNA by electrophoresis and cut the band of interest from the gel. Place the agarose fragment in a 1.5 ml microcentrifuge with known weight. After weighing the whole tube-agarose deduct the weight of the agarose.

- Add 10 µl of capture buffer per 10 mg of agarose. Mix by vortex vigorously and incubate at 60 °C until complete dissolution of agarose.

- Transfer the sample to a GFX column and incubate 1 min at room temperature. Centrifuge for 30 s at 13200 rpm.

- Discard the supernatant and add 500 µl of wash buffer. Centrifuge for 30 s at 13200 rpm.

- Transfer the column to a new tube and add 15 to 50 µl of MQ-water, incubate for 1 min at room temperature.
- Centrifuge for 1 min at 13200 rpm to recover the purified DNA.

2.2.3.9. Hydrolysis by restriction endonucleases

Restriction enzymes catalyze the internal phosphodiester links hydrolysis in both strands of a double-stranded DNA molecule, mainly by specific sites (targets or restriction sites). Every enzyme has its optimal conditions of temperature, pH, salt concentration and presence of co-factors to perform their activity. These conditions vary according to the enzyme.

Restriction enzymes were used following the supplier's indications and the following recommendations:

- Always add less than 1/10 volume of enzyme respecting to the final volume of reaction in order to sufficiently dilute the glycerol present in the enzyme preservation solutions, otherwise it can inhibit the enzyme activity.
- If the DNA is suspended in TE, set the reaction in a volume that exceeds 10 times the volume of DNA. So TE cannot change the characteristics of the reaction buffer.

Protocol:

- Mix in a microtube:

Digestion buffer ¹ (10X)	2
DNA.....	200-500 ng
RNase A (1 µg/ml).....	2
Restriction enzyme(s)	1.2 U/mg DNA (1-2 µl)
MQ water	Up to 20 µl
- Incubate the reaction at the proper temperature of the enzyme during 1 to 4 h.
- Analyze by agarose gel electrophoresis.

¹ This buffer maintains a constant pH for optimal enzymatic reaction (usually Tris-HCl, pH 7.5-8), it requires Mg²⁺ for endonucleases, reducing agents (usually DTT) and salts.

2.2.3.10. Dephosphorylation of 5' ends of DNA fragments

Alkaline phosphatase hydrolyzes the 5' phosphate group of DNA and prevents the formation of phosphodiester bonds between the two ends of the molecule. This type of treatment is applied to prevent the religation of linearized vectors.

Protocol

- Dissolve the DNA in a volume of 40 µl of water. Mix with 5 µl phosphates buffer¹ (10X).
- Heat the DNA for 5 min at 65 °C.
- Cool the mixture 4 or 5 min and add 5 µl of enzyme (5 U/µl).
- Incubate at 37 °C for 30 min (optionally the enzyme can be renewed and incubated for 30 min once more).
- Inactivate the enzyme by maintaining the reaction at 65 °C for 15 min, clean by phenolization and precipitate the DNA.

¹ Phosphatase buffer (10X): 0.5 M Tris-HCl, 1 mM EDTA, pH=8.

2.2.3.11. Ligation of DNA fragments

The T4 phage DNA ligase is an enzyme that catalyzes the formation of phosphodiester bonds between the 3'-hydroxyl and 5'-phosphate of dsDNA, requiring Mg²⁺ and ATP as cofactors.

Protocol

- Run the vector and the insert on an agarose gel along with DNA ladder.
- Quantitate the amount of vector and insert present.
- Mix 50 ng of vector DNA and equimolar amount or more (3 times) from the insert DNA in a microtube.
- Add 1 µl of ligase buffer¹ (10X), mix well and add 0.1 to 1 µl of enzyme (5 U/µl) then adjust the final volume (10 µl) with distilled water.
- In the case of DNA fragments with cohesive ends incubated 2-3 h at 22 °C. For blunt ends incubated 12 h at 14-16 °C.
- Inactivate the ligation by heating at 65 °C for 10 min (optional).

- Transform into an appropriate strain of *E. coli*.

¹ ligase buffer (10X): 0.5 M Tris-HCl, 0.1 M MgCl₂, 0.2 M DTT, 10 mM dATP, bovine serum albumin 500 µg/µl, pH 7.8.

2.2.3.12. Transformation of *E. coli*

Bacteria transformation may be referred to as a stable genetic change by the uptake of naked DNA and competence refers to the state of being able to take up exogenous DNA from the environment.

■ Competent cells

The availability of bacteria that facilitates the passage of naked DNA molecules through the external barriers of the cell is known as “competition” and presenting cells are called “competent cells”. Achieving this state of competence in *E. coli* is based on the rubidium chloride method (Hanahan, 1983, 1985) with high transformation efficiency (up to 10⁸ transforms/µg DNA).

Protocol

- Grow the 5-10 ml overnight culture of *E. coli* in LB.
- Use small culture to inoculate 500 ml culture. Grow at 37 °C to OD₅₉₅ of 0.5.
- Cool for 15 min on ice and transfer to two 250 ml centrifuge bottles. Centrifuge 10 min at 4000 rpm in GSA rotor to pellet cells and pour off supernatant.
- Resuspend pellets in 30 ml total of Tfb I¹ by pipetting up and down until all clumps have been resuspended. Combine and put on ice for 15 min.
- Centrifuge 5 min at 4000 rpm, with cells on ice as much as possible, pour out supernatant, and then use pipet to remove all residual fluid.
- Resuspend the bacterial pellet in 10 ml Tfb II² on ice and place aliquots in crushed-up dry ice.
- Take pre-cooled microcentrifuge tubes out of -80 °C freezer with open lids in an ice bucket containing wet ice. Dispense 100 µl of cell suspension into each tube, seal the tubes and place them in liquid nitrogen. Once all 10 ml of competent cells are dispensed collect tubes and store them in a box with no dividers.

- Measure the efficiency of the competent cells by transforming a standard amount of commercial plasmid. Express efficiency as colony forming units per microgram of DNA [cfu/ug].

¹ Tfb I: 100mM Rubidium Chloride, 50mM Manganese Chloride, 30mM Potassium, 10mM Calcium Chloride, 15% w/v. Adjust pH to 5.8 and the final volume to 1 liter. Sterilize by filtration.

² Tfb II: 10mM MOPS, 10mM Rubidium Chloride, 75mM Calcium Chloride, 15% w/v Glycerol, Adjust the volume to 500 ml. Sterilize by filtration.

■ Transformation

In this work the method of Hanahan (1983) was used for transformation. This method is practically universal for the various strains of *E. coli*, it can be used to transform competent cells prepared by any method.

Protocol

- Thaw competent cells on ice maintaining in the tube.
- Add the DNA (up to a maximum of 10 µl) to 100 µl of competent cells (when thawed) and keep the mixture on ice for 20 min.
- Apply a heat shock at 42 °C for 45 s and cool immediately on ice for 3 min.
- Add 600 µl of LB and incubate the cells for 1 h with shaking (250 rpm) at 37 °C to allow the expression of the antibiotic resistance gene.
- Culture the cell aliquots on solid LB Petri dishes (100 µl on one and the rest on the other) supplemented with the selection antibiotic to allow the growth of only transformed clones.

2.2.3.13. Isolation of plasmid DNA from *E. coli*

For the isolation of plasmid DNA from *E. coli* (minipreparation) two methods was used.

■ Holmes-Quigley method

This method was used for analysis of large numbers of samples (Holmes and Quigley, 1981).

Protocol

- Take a colony with a sterile toothpick, scratch a replicate on a new plate then inoculate the toothpick in a 1.5 ml microtube with 1 ml of LB medium with appropriate antibiotic according to the plasmid of interest. Incubate for 6 to 12 h at 37 °C stirring at 250 rpm.

- Precipitate the cells by centrifugation at 13200 rpm for 1-5 min. Meanwhile, mix 50 µg of lysozyme into 10 ml of STET¹.

- Add 300 µl of the mixture to each microtube. Resuspend the pellet by shaking vigorously and boil for 45 s.

- Centrifuge immediately for 15 min at 13200 rpm and remove the pellet with a sterile toothpick (precipitated chromosomal DNA, cellular debris and proteins).

- Add 300 µl of isopropanol and 30 µl of 3 M sodium acetate pH=5.2.

- Mix, leave 10 min at room temperature and centrifuge 10 min at 13200 rpm.

- Discard the supernatant, wash the precipitate with 70% ethanol and let air dry.

- Resuspend in 30-50 µl of TE or MQ water.

¹ STET: 8% (w/v) sucrose, 0.5% (v/v) Triton X-100, 50 mM EDTA, 10 mM Tris-HCl, pH=8.0.

■ Minipreparation method in 10 min

This method described by Zhou and coworkers (1990), was used for rapid analysis of large number of clones.

Protocol

- Inoculate a colony of *E. coli* in a sterile eppendorf tube with 0.8 ml of LB medium with the appropriate antibiotic according to the plasmid of interest. The tube is incubated at least 6 h at 37 °C and 250 rpm.

- Centrifuge the cells at room temperature at 13200 rpm for 30 s.

- Discard the supernatant remaining approximately 50-100 µl of it. Resuspend the cells in that volume by vortexing.

- Add 300 µl of TENS¹ and mix briefly by vortexing. Leave at room temperature until the mixture acquires a high viscosity (1 to 2 min)².

- Add 150 µl of 3 M sodium acetate pH=5.2, and mixed briefly by vortex.
- Centrifuge the mixture for 3 min at 13200 rpm to sediment cell debris. Transfer supernatant to a new tube.
- Add 900 µl of absolute ethanol. Mix and centrifuge at 13200 rpm for 5 min, to precipitate the plasmid DNA.
- Remove the supernatant and wash the pellet with 70% ethanol. Dry the pellet and resuspend in 30-50 µl of TE or MQ water.

¹ TENS: 0.1 N NaOH, 0.5% SDS. Prepare the solution just prior to use in TE.

² If it takes more than 10 min (due to high number of tubes to be analyzed), transfer the tubes to ice to inhibit the degradation of chromosomal DNA and the resulting plasmid DNA contamination.

2.2.3.14. DNA sequencing: ABI Prism sequencing system

The sequencing process was performed by the dideoxynucleotide method described by Sanger and coworkers (1977). This system has been used in a non-radioactive automated sequencing machine ABI Prism 3130 Genetic Analyzer (Applied Biosystems) by the technical sequencing service of INBIOTEC.

2.2.3.15. Transformation of filamentous fungi

Selection markers that used for obtaining *Penicillium* transformed strains in this work can be divided into two groups:

■ Complementation of auxotrophy

The main selection marker was the complementation of the uracil auxotrophy (mutant strain for pyrG gene). This technique has been the most widely used method for *P. chrysogenum* transformation due to the high efficiency.

■ Acquisition of resistance to the external agent

The best marker for this type of selection is the phleomycin antibiotic resistance. Phleomycin is a glycopeptide antibiotic of the bleomycin family, isolated from a mutant strain of *Streptomyces verticillus*. It binds and intercalates DNA thus destroys the integrity of the double helix. Phleomycin is active against most bacteria, filamentous

fungi, yeast, plant and animal cells. Although the bleomycin antibiotics perturb plasma membranes, their activity is generally believed to be related to their ability to bind DNA by intercalation of their planar bithiazole-containing moiety. The DNA is degraded by the metal ion chelating portion of the molecule which forms an active complex with iron II and molecular oxygen.

The resistance to this antibiotic results from physical blockage of the molecules of phleomycin by the *ble* gene product. This gene has been found in Gram-positive bacteria (McKenzie *et al.*, 1986) in Gram-negative bacteria (Genilloud *et al.*, 1984, Collins and Hall, 1985) and phleomycin producer actinomycete *Streptoalloteicus hindustanus* (Drocourt *et al.*, 1990).

■ Isolation of *P. chrysogenum* protoplasts

The following protocol for the isolation of the protoplasts from *P. chrysogenum* Wis 54-1255 strain and derivatives is based on the procedures described by Cantoral and coworkers (1987) and Wang and coworkers (1999).

Protocol

- Inoculate 100 ml media PMMY¹ with fresh conidia from a Petri dish with PW medium, incubated at 28 °C for 5-7 days (depending on the strain).
- Incubate in orbital agitation at 250 rpm and 28 °C for 18-24 h (adequate time for growth of hyphae for an average length equivalent to about 10-15 times the diameter of the conidia)².
- Collect the mycelia by filtration through a sterile nylon filter (NYTAL) 30 µm pore size. Wash twice with wash solution³.
- Take 1 g of the mycelia from the filter, transfer to a 10 ml tube and resuspend in 10 ml TPP buffer⁴.
- In another 10 ml tube, prepare 10 ml of 10-14 mg/ml lytic enzyme (Sigma-Aldrich Chemical Co) in TPP, Sterilize this solution by filtration through a 0.22 µm millipore filter.
- Mix the solutions of the two tubes in a sterile 250 ml flat flask (final concentration of lytic enzymes is 5-7 mg/ml). Incubate the mixture at 28 °C with orbital agitation of 70-80 rpm for 2-4 h⁵.

- Once the majority of protoplasts are released, filter the suspension through a sterile nylon filter (NYTAL) 30 µm.
- Collect the effluent into two sterile 10 ml tubes. Centrifuge at 2100 rpm for 7 min.
- Resuspend in 5 ml of KCM buffer⁶ and wash twice in this buffer by centrifugation at 2100 rpm for 5 min.
- Perform a count of the number of total obtained protoplasts using a Thoma chamber.
- Centrifuge at 2100 rpm for 5 min. Resuspend in KCM buffer at a final concentration of protoplasts $(1-2) \times 10^8$ protoplasts/ml. Keep on ice until transformation.

¹ In the case of using an auxotrophic strain, supplement the medium with the appropriate additive.

² Successively check the culture samples under a microscope. The Wisconsin 54-1255 strain between 18-22 hours and the strains derived from it, between 20-26 hours.

³ Washing buffer: NaCl 0.9% (w/v) Triton 0.01% (v/v).

⁴ TPP buffer: 50 mM potassium phosphate buffer, 0.7 M KCl, pH=5.80.

⁵ Verify the release of protoplasts, each 45 min under the microscope.

⁶ KCM buffer: 0.7 M KCl, CaCl₂ 50 mM, 10 mM MES, pH=5.80.

■ Protoplast Transformation

The selection of transforms is performed in minimal culture media; in this work the Czapek medium used was. The media should keep osmotic stabilizer to prevent the collapse of the protoplasts: sucrose, NaCl, KCl, glycerol, sorbitol or inositol can be used. Working with resistance to antibiotics, the minimal media should be supplemented with selection agent and an osmotic stabilizer that is compatible with it. In our case Czapek medium supplemented with phleomycin at a final concentration of 30 mg/ml and sorbitol (1 M) was used as the stabilizer. During the transformation positive and negative controls should always be made to verify the sensitivity to the selection agent and the absence of revertant.

Protocol

- In a 10 ml tube, make the following mixture and mix very gently with a pipette:

Protoplast solution (1×10^8 p/ml)	100 μ l
PCM buffer ¹	10 μ l
DNA solution (1 to 2 μ g/ μ l)	up to 10 μ l
Aurintricarboxylic acid (ATA) 0.1 M	10 μ l

• Prepare the same in another tube but without adding DNA, it is a control reaction for determination of regeneration capacity of protoplasts, as well as the appearance of reverting colonies.

• Incubate the mixtures on ice for 20 min.

• Add 500 μ l of PCM buffer and mix very gently. Incubate at room temperature for another 20 min.

• Add 600 μ l of KCM buffer² to dilute the polyethylene glycol in PCM buffer.

• Collect 300 μ l of the transformation reaction³, the selection agent⁴ and the molten Cz-Sorbitol medium (at a temperature of 48-50 °C) in a new 10 ml tube. Mix gently and pour on 10 ml base medium⁵ that is previously prepared in the Petri dish. Let it to be solidified.

• Incubate at 28 °C for 4-7 days until the appearance of the colonies.

• Pass the colonies to new Cz (without Sorbitol) plates and culture for 4-7 days to discard the false positives. Repeat this passage and in the final passage use the PW medium.

¹ PCM Buffer: 50% (v/v) Polyethylene glycol 6000, 50 mM CaCl₂, 10 mM MES, pH=5.80.

² KCM buffer (0.7 M KCl, 50 mM CaCl₂, 10 mM MES, pH=5.8) is used whenever dilutions are required to be made. In the case of control reaction, serial decimal dilutions are made to estimate the percentage of regeneration.

³ The transformation reaction is divided into several tubes to be cultured in several Petri dishes or diluted in decimal series, according to the number of transforms expected.

⁴ In the case of complementation of auxotrophy.

⁵ The composition of the culture medium for the base and the second layer are the same.

2.2.3.16. DNA hybridization

The nucleic acid fragment used as probe in hybridization processes should be labeled to allow the subsequent detection. Radioactive and nonradioactive labeling techniques are available and the use of each method depends on the required threshold of sensitivity.

In this study the nonradioactive labeling system was used. Digoxigenin (Roche) is a hapten, a small molecule with high immunogenicity used in many molecular biology applications. Other popular haptens include DNP (dinitrophenol), biotin, and fluorescein. In general, antibodies generated against haptens have higher affinities for their targets than other antibodies, so haptens are conjugated to other biological molecules as all-purpose immuno-tags. This is because anti-hapten antibodies have high affinities and are readily available, while antibodies may not be commercially available for exotic biological targets.

■ Preparing the DNA probes

Digoxigenin is attached to nucleotide triphosphate dUTP by an ester bond which could be eliminated under alkaline conditions; it facilitates the subsequent reuse of filters. DIG-labeled DNA probes are generated according to the method of random primed labeling, which is based on the hybridization of random oligonucleotides to the denatured DNA template. The complementary DNA strand is synthesized by klenow enzyme, which uses the 3'OH termini of the random oligonucleotides as primers and a mixture of deoxyribonucleotides containing DIG-11-dUTP, alkali-labile for elongation. This results in incorporation of digoxigenin into the newly synthesized DNA. This density of haptens in the DNA provides a high sensitivity in the subsequent immunodetection by anti-digoxigenin conjugated with the alkaline phosphatase enzyme.

Protocol

- Take 0.5-1.5 µg of the linear DNA fragment in a maximum volume of 15 µl.
- Denature the DNA by boiling for 10 min and transfer to ice for 3 to 5 min.
- Mix 15 µl of DNA solution with 2 µl of Hexanucleotide Mix (10X), 2 µl of DIG-DNA labling mix (10X) and 1 µl of Klenow enzyme.

- Centrifuge to collect the sample at the bottom of the microtube. Incubate the mixture at 37 °C at least 60 min preferably between 16 and 24 h.
- Stop the reaction by freezing directly or by adding 2 µl of 0.25 mM EDTA, pH=8.0 and keep frozen until use (optional).

Note: according to the manufacturer with this method of labeling, from 1 µg DNA up to 2-3 µg probe will be obtained after 24 hours.

■ DNA transfer from agarose gels to nylon filters (Southern blotting)

DNA transfer to a solid support is called Southern blotting (Southern, 1975). This technique is based on the fragmentation, *in situ* DNA denaturation and transfer as single-stranded fragments to the membrane followed by subsequent attachment to it by UV light. Traditionally, the fragments of DNA were transferred to nylon filters (Hybond-NTM GE Healthcare) by capillary, but currently, this tends to be replaced by the transfer method using vacuum system (VacuGene XL, GE Healthcare).

Protocol

- Separate the digested DNA by electrophoresis, stain by ethidium bromide and take a photograph.
- Cut a nylon filter 1 cm per side larger than the size of the gel and moisten with SSC (2X). Deposit it in the transfer unit, assembled according the manufacturer's instructions.
- To place the gel on the plastic window over the filter, start with the top of the gel with wells and let it go smoothly and progressively make contact. Check the absence of bubbles under the gel.
- Turn on the vacuum pump (50 mbar).
- Cover the entire surface of the gel with depurination¹ solution for 15-20 min (in this time the front-migration of xylene cyanol should change from blue to yellow). Remove the solution.
- Cover the gel surface with denaturing² solution for 15-20 min (the xylene cyanol recovers the original blue color). Remove the solution.
- Cover the surface of the gel with neutralization³ solution for 20-25 min.

- Remove the neutralization solution and cover the surface of the gel with SSC (20X)⁴ for 30-60 min.
- Remove all liquid and mark the location of the wells of the gel on the filter (with a blue pen ballpoint), before removing it.
- Shut the vacuum pump down.
- Remove the gel and check the efficiency of the process by ethidium bromide staining.
- Place the filter on Whatman 3MM (or normal) paper and fix the DNA by applying the UV (using the UV-Stratalinker 2400, Stratagene).
- Wash the membrane in SSC (2X) and let it dry. These filters can be stored at 4 °C wrapped in Whatman 3 MM paper and aluminium foil for several months.

¹ Depurination solution: 0.25 M HCl.

² Denaturation solution: 1.5 M NaCl, 0.5 N NaOH.

³ Neutralization solution: 1.5 M NaCl, 0.05 M Tris-HCl, 1 mM EDTA, pH=7.2.

⁴ SSC 20X: 3 M NaCl, 0.3 M sodium citrate, pH=7.0.

■ DNA Hybridization with probe and detection

There are four steps in the entire process of hybridization: prehybridization, hybridization, washing and detection.

The prehybridization aims to block the active sites of the membrane that nucleic acids have not joined during the transfer and balance it for the next step.

The hybridization step allows the binding of the labeled probe to the fixed DNA on membrane. The specificity of this binding depends on the conditions during the incubation (hybridization temperature and the concentration of salts and detergents in the hybridization buffer) and subsequent washes. The general annealing temperature is 68 °C but if the solution contains 40% (v/v) formamide this temperature is 42 °C.

The washing steps allow the removal of nonspecific binding that may have occurred between the probe and DNA. Decreasing of nonspecific binding during the washings is achieved by decreasing the salt concentration, increasing the concentration of detergents and increasing the temperature and washing time.

The detection step is the visualization of the binding between probe and nucleic acid fragments.

Protocol

- Place the membrane into a plastic bag and add 10-20 ml of prehybridization solution¹. Seal the bag and incubate at the appropriate temperature (42 °C) for 1 to 4 h.
- Boil the labeled probe for 10 min to denature DNA and cool quickly on ice.
- Mix the denaturized probe with 10-20 ml of prehybridization solution (5-25 ng/ml) to prepare the hybridization solution.
- Remove prehybridization solution, add hybridization solution and incubate at the annealing temperature for at least 8 h.
- Collect the hybridization solution in a tube for reuse and store it at -20 °C.
- Wash the membrane twice with the washing solution I² for 15 min.
- Wash the membrane twice with wash solution II³ for 25 min at 68 °C.
- Block the membrane by shaking within buffer II⁴ for 30 min.
- Replace the buffer II by the buffer II/antibody⁶ and shake at least for 0.5-3 h.
- Remove the antibody solution and wash the membrane with buffer I for 15 min.
- Repeat the wash, replace the buffer I by buffer III⁷ and equilibrate for 1 min.
- Replace the buffer III with buffer IV⁸ and wait until the bands appear.
- Stop the reaction in TE buffer for 2 min, dry the membrane and keep in dark.

¹ Hybridization Solution: 5X SSC, 1-2% (w/v) blocking reagent (Blocking Reagent, Roche); 0.1% (w/v) lauroilsarcosin, 0.02% (w/v) SDS, 40% (v/v) formamide.

² Washing solution I: 2X SSC, 0.1% (w/v) SDS

³ Washing solution II: 0.1X SSC, 0.1% (w/v) SDS.

⁴ Buffer I (100 mM maleic acid, 150 mM NaCl, pH=7.5) + 0.3% (v/v) Tween-20.

⁵ Buffer II: Buffer I plus 1% of blocking reagent (Blocking Reagent, Roche).

⁶ Anti-digoxigenin conjugated with alkaline phosphatase in buffer II (1:10000).

⁷ Buffer III: 0.1 M Tris-HCl pH=9.5, 0.1 M NaCl, 50 mM MgCl₂ (Prepared 1 M Tris-HCl pH=9.5 (10X) and 1 M NaCl, 500 mM MgCl₂.6H₂O (10X) are adjusted to 1X)

⁸ Buffer IV: 45 µl of NBT stock solution plus 35 µl BCIP stock solution per 10 ml of Buffer III; NBT stock solution: 18.75 mg/ml Nitro blue tetrazolium chloride in 67% (v/v) DMSO, BCIP stock solution: 9.4 mg/ml 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt in 67% (v/v) DMSO.

2.2.4. Transcriptomic analysis

2.2.4.1. Total RNA extraction from *P. chrysogenum* mycelia

The presence of RNases is the greatest difficulty for obtaining RNA, RNases are very active enzymes and do not need cofactors for their activity. Any method valid for the extraction of RNA must be quick and conducted in a solution containing an agent inactivating the activity of RNase [phenol, diethylpyrocarbonate (0.1% (v/v) DEPC) or guanidine isothiocyanate]. Breaking cells in the presence of liquid nitrogen or by heating at 65 °C avoids the RNase activity. Regardless the method it is very important to wear gloves along the whole process and clean the equipments with chloroform (and then with ethanol to remove traces of chloroform). The plastic material must be sterilized (preferably twice) in an autoclave.

RNA stabilization is an absolute prerequisite for reliable gene expression analysis. Immediate stabilization of RNA in biological samples is necessary because, directly after harvesting the samples, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation as well as to transcriptional induction. Such changes need to be avoided for all reliable quantitative gene expression analyses.

In this work extraxtion of total RNA from mycelia was carried out using the RNeasy Plant Mini Kit (Qiagen). This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate containing buffer (Buffer RLT and Buffer RLC, which contain guanidine thiocyanate and guanidine hydrochloride, respectively), which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to a RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30-100 µl RNase-free water. Generally, DNase digestion is not required with RNeasy Kits since RNeasy silicamembrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundance target). In these cases,

residual DNA can be removed by optional on-column DNase digestion. The DNase is efficiently removed in subsequent wash steps. Alternatively, residual DNA can be removed by a DNase digestion after RNA purification.

Protocol

- Collect the mycelium from liquid medium by filtration through the sterile 30 micron pore nylon filter and wash with 0.9% NaCl. Remove the excess moisture with filter paper and immediately freeze in liquid N₂. The mycelium can be stored at -80 °C until RNA extraction.
- Add 10 µl β-Mercaptoethanol (β-ME) per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. RLT containing β-ME can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I solution by adding 40 µl of DNase I stock solution to 280 µl of RDD buffer.
- Take up to 100 mg of ground mycelia and add 700 µl of RLT containing β-ME and vortex vigorously. A short 1-3 min incubation at 56 °C may help to disrupt the mycelia.
- Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Carefully transfer the supernatant of the flow-through to a new microcentrifuge tube.
- Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting.
- Transfer the sample (up to 700 µl), including any precipitate that may have formed, to an RNeasy spin column (pink), centrifuge for 15 s at 10000 rpm and discard the flow-through. Reuse the collection tube for the rest of the sample.
- Add 40-80 µl of DNase solution and incubate for 25 min at 30 °C. Centrifuge for 15 s and repeat this step. Discard the flow-through.
- Centrifuge for 15 s add 700 µl Buffer RW1. Leave on the bench for 5 min centrifuge for 15 s to wash the spin column membrane. Discard the flow-through.
- Add 500 µl Buffer RPE to the RNeasy spin column.

- Centrifuge for 2 min. Discard the flow-through and repeat this step.
- Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30-50 µl RNase-free water directly on the spin column membrane. Centrifuge for 2 min, discard the column and store the RNA solution at -80 °C.

2.2.4.2. RNA electrophoresis on denaturing gels

Separation of RNA fragments was carried out by electrophoresis in denaturing agarose gels following basically, the method described by Sambrook and Russell (2001). In RNA denaturing gel electrophoresis formaldehyde is added as a denaturant agent to prevent the formation of secondary structures in RNA.

Protocol

- Wash all the material for electrophoresis with H₂O₂ for 5-10 min and then with water-DEPC.
- Prepare 100 ml of gel mix: 1.5 g of agarose, 5 ml of 20X MOPS¹ and 80 ml of DEPC treated water.
- Heat the mixture until the agarose is completely dissolved. Allow it to cool (50-55 °C) and add 15 ml of 37% formaldehyde (The final composition of the gel is 1.5% agarose, 1X MOPS and 6% formaldehyde).
- Mix and pour on the electrophoresis tray and allow it to solidify.
- Place the gel inside the electrophoresis tank containing the MOPS¹ buffer (1X).
- Before running the samples, they should be heated at 65 °C for 5-10 min and immediately cooled on ice for 5 min. The samples are prepared as described below:

RNA	10-20 µg
Formamide	5 µl
Formaldehyde 37%	2 µl
MOPS ¹ (1X)	1 µl
Loading dye ² (10X)	1 µl
DEPC-water	20 µl

¹ MOPS (20X): 0.8 M 3-(N-morpholin) propanosulfonic acid (MOPS), 0.2 M Sodium acetate, 0.04 M EDTA; adjust pH to 7 with 1 N NaOH, in DEPC treated water (0.1%). Shake and sterilized in an autoclave.

² Loading dye (10X): 50% glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene-cyanol, 1 mM EDTA; in DEPC-water.

2.2.4.3. RT-PCR (Reverse Transcription)

In this work RT-PCR for amplification of cDNA from mRNAs was carried out using the SuperScript[®] One-Step RT-PCR with Platinum[®] *Taq* System. This system is designed for the convenient, sensitive, and reproducible detection and analysis of RNA molecules by RT-PCR. Both cDNA synthesis and PCR are performed in a single tube using genespecific primers and target RNAs from either total RNA or mRNA. RT/Platinum[®] *Taq* mix is a mixture of recombinant *Taq* DNA polymerase complexed with proprietary antibody that inhibits polymerase activity and SuperScript[®] II Reverse Transcriptase. The activity of *Taq* DNA polymerase is blocked at ambient temperatures but is regained after the denaturation step in PCR cycling at 94 °C.

Protocol

- Program the thermal cycler for 1 cycle of 45-55 °C for 15-30 min and 94 °C for 2 min, 35-40 cycles of 94 °C for 15 s, 55-60 °C for 30 s, 68-72 °C for 1 min/kb and 1 cycle of 72 °C for 5-10 min.

- Add the following to the microcentrifuge tubes placed on ice.

2X Reaction Mix.....	25 µl
Template RNA.....	200 ng
Sense Primer (10 µM).....	1 µl
Anti-sense Primer (10 µM).....	1 µl
RT/Platinum [®] <i>Taq</i> Mix.....	1 µl .
Autoclaved distilled water	up to 50 µl .

Note: Absence of genomic DNA in RNA samples can be verified by substitution of RT/Platinum[®] *Taq* Mix by 2 units of Platinum[®] *Taq* DNA polymerase in the reaction.

- Mix, centrifuge briefly and analyze the amplification product by electrophoresis.

2.2.5. Assessment and quantification of penicillin

The production of penicillin from *P. chrysogenum* fermentations was measured by bioassay. *Micrococcus luteus* ATCC9341 was used as a penicillin-sensitive organism; it was grown in TSB medium until it reached an OD₆₀₀ of 2.0. The presence of antibiotic in the medium inhibits the growth of this microorganism creating an inhibition halo whose diameter is proportional to the antibiotic concentration. The use of standard control samples with different concentrations of commercial penicillin allows the development of a pattern line related to the concentration of penicillin and the diameter of the inhibition halo parameters. Unknown concentration of penicillin in a sample can be estimated by interpolation in the pattern line.

Protocol

- Inoculate 1% of *M. luteus* culture in molten 1% TSA medium, mix and dispense into Petri dishes with 15 cm in diameter (40 ml per plate).
- When the medium is solidified drill 6-8 holes with 5 mm of diameter and remove the block of agar with a sterile toothpick.
- Add 50 µl of each sample into each bioassay well.
- Incubate at 4 °C for 2 h to allow the diffusion of the antibiotic in the medium.
- Incubate at 30 °C for 15 h to allow the growth of the test microorganism.

CHAPTER 3

RESULTS & DISCUSSIONS

Full exploitation of *P. chrysogenum* requires the integration of knowledge from several “omic” sciences, such as proteomics, in order to decipher the molecular basis for the improved productivity that has made this fungus such a good penicillin overproducer. In this work, we have optimized a method to obtain the *P. chrysogenum* cytosolic and extracellular proteins. As a result, the cytosolic and secretome reference maps for this filamentous fungus have been developed. In addition, we have characterized the relevant modifications produced during the industrial strain improvement program through the proteome analysis of three *P. chrysogenum* strains: the wild-type strain NRRL 1951 isolated in Peoria, IL, the genome reference strain Wis 54-1255 (an improved but still low-producer) and the high-producer strain AS-P-78 developed by Antibioticos S.A. (Spain).

3.1. THE INTRACELLULAR PROTEOME

3.1.1. Optimization of 2-DE for *P. chrysogenum*

In order to obtain soluble protein extracts optimal for 2-DE analysis, two approaches were followed: i) a protocol based on the Clean Up Kit (GE Healthcare) extraction described by Kniemeyer and coworkers (2006), and ii) the procedure based on phosphate buffer solubilization and protein precipitation (with either TCA or acetone) described by Fernández-Acero and coworkers (2006) for *Botrytis cinerea*. In addition, silver or CC staining conditions were analyzed.

Good results were obtained using the Clean Up Kit protocol, but some streaking was detected under silver staining conditions (Figure 3.1.A). The streaks were almost fully avoided by using the phosphate buffer procedure combined with TCA precipitation (Figure 3.1.C and D).

The acetone precipitation showed the worst results (Figure 3.1.B). In addition to the phosphate buffer and TCA precipitation, the CC “Blue Silver” stain achieved the best staining results (Figure 3.1.D).

Protein extracts of *P. chrysogenum* AS-P-78 needed an extra cleaning step by using the Clean Up Kit before IEF to avoid the streaking in the acidic sector of the gel.

Figure 3.1. Optimization of the protein extraction protocol from mycelia of *P. chrysogenum*. **A.** Extraction and purification of protein samples with “Clean Up Kit” (GE Healthcare) (based on Kniemeyer *et al.*, 2006) and silver stained. **B.** C, D extraction are based on the protocol described by Fernández-Acero *et al.* (2006) by using mortar gridding and phosphate buffer. **B.** Acetone precipitation and silver stain; **C.** TCA/Acetone precipitation and silver stain; **D.** TCA/Acetone precipitation and Colloidal Coomassie stain (Candiano *et al.*, 2004). Gels were done in a Hoeffer system (GE Healthcare) as follows: 13-cm IPG strips (GE Healthcare) with non-linear pH 3-10 gradient and SDS-PAGE (12.5%).

3.1.2. The intracellular reference map analysis

After optimization of the protein extraction protocol, a global proteome analysis of *P. chrysogenum* Wis 54-1255 was performed.

This strain was selected because it was the reference strain for the genome sequencing project (van den Berg *et al.*, 2008).

The global reference map of this fungus was obtained using wide range IPG strips (pH 3-10, NL) and 15% SDS-PAGE gels, which allowed a wide overview of the *P. chrysogenum* soluble protein distribution. Spots were visualized with “Blue Silver” staining, which allowed the detection of 976 protein spots by digital image analysis (Figure 3.2).

Approximately 92% of the 976 spots detected were located between 10 kDa and 100 kDa in a pH range from 3 to 10 (Figure 3.2). Stained spots were excised from the gel, digested as indicated in Experimental Procedures and analyzed by peptide mass fingerprinting (PMF) and tandem MS.

From the total 976 spots detected, 128 spots could not be identified. The remaining 848 spots represented 950 correctly identified proteins (average error of 12 ppm) since some spots contained more than one protein (Supplementary Table 1 in the appendix section).

Almost all the significant MASCOT searches provided an exact match with proteins inferred from the *P. chrysogenum* Wis 54-1255 genome, which indicates high accuracy of the genome annotation process. From the 950 proteins identified, 211 proteins showed a total of 612 isoforms (Supplementary Table 2 in the appendix section).

The final number of different identified proteins was 549. Some examples of the proteins showing a high number of isoforms were a probable flavohemoglobin Fhp (Pc12g14620; 14 isoforms), a probable catalase R (Pc16g11860; 12 isoforms), a glucosamine-6-phosphate deaminase-like protein (Pc22g10040; 10 isoforms), a probable endonuclease SclI 75 kDa subunit Ens1 (Pc22g19990; 8 isoforms) and a hypothetical trunk lateral cell specific gene HrTLC1 (Pc18g00980; 8 isoforms).

The ten spots showing more intensity (% Vol) represented 13.01% of the total spot volume, where spots 352 and 364, corresponding to a probable flavohemoglobin Fhp (Pc12g14620), constituted the 4.63% of the total % volume.

The reference map with a direct link to the identified proteins can be accessed through the URL:

<http://isa.uniovi.es/~cuadrado/varios/RMTS.html>

Figure 3.2. Reference map of the cytosolic *P. chrysogenum* proteome. Proteins were separated by 2-DE using 18-cm wide-range IPG strips (pH 3-10 NL) and 15% SDS-PAGE gels and stained with CC following the “Blue Silver” staining method. A total amount of 976 spots were resolved in this range and were analyzed by PMF and tandem mass spectrometry (see Supplementary Table 1 in the appendix section). Molecular weights are shown on the left. Assigned numbers over each spot correlate with those shown in Supplimentary Table 1.

3.1.3. Functional classification of proteins

The 950 proteins identified (549 different proteins and isoforms) were functionally classified according to their biological roles using the KEGG database (<http://www.genome.jp/kegg/>) and FunCat classification system (Ruepp *et al.*, 2004). As shown in Figure 3.3 and Supplementary Table 1 (see appendix section), 415 proteins were involved in energy formation and metabolism (43.68%); 103 proteins in folding, modification and targeting of proteins (10.84%); 76 proteins in cell rescue, defense and virulence (8.00%); 68 proteins in transcription and translation processes (7.16%) and 149 proteins (15.68%) in other different cellular processes. We also identified 139 proteins of unknown function (e.g. hypothetical proteins) (14.64%).

Figure 3.3. The 950 identified proteins (549 different proteins and isoforms) were classified according to their biological function.

It is well defined that there are three proteins directly involved in the penicillin biosynthetic pathway: α -aminoadipyl-cysteinyl-valine (ACV) synthetase, IPN synthase and IAT (for review see Martn *et al.*, 2010). The first enzyme in the pathway (ACV synthetase) is a non-ribosomal peptide synthetase with an estimated mass of 425 kDa, so its detection was not possible by 2-DE. The other enzymes of the pathway were identified in the reference map (Figure 3.2 and Supplementary Table 1 in the appendix section). The IPN synthase was present in spot 427 (Pc21g21380), whereas the IAT

was found in spot 647 (Pc21g21370). Only the 29-kDa β -subunit of this enzyme could be identified in agreement with previous results (García-Estrada *et al.*, 2008b) (see Discussion). One of the putative orthologs of the bacterial IPN epimerase, CefD (Kovacevic *et al.*, 1990), described in the *P. chrysogenum* genome (Pc12g11540) was identified in the reference map with two isoforms (spots 247 and 250); this protein probably functions as an aminotransferase as it was previously suggested (van den Berg *et al.*, 2008). A probable 7- α -cephem-methoxylase subunit CmcJ was identified in spots 399 and 404 (Pc13g04680). This enzyme is present in cephamycin-producing organisms, such as *Amycolatopsis lactamdurans*, and together with CmcI, is involved in the synthesis of 7 α -methoxylated cephems (Coque *et al.*, 1995). Since the *P. chrysogenum* genome only contains genes encoding putative CmcJ subunits of this enzyme and this microorganism does not produce cephalosporins, it is difficult to assign a specific role in β -lactam biosynthesis to this protein.

Enzymes involved in the biosynthesis of other secondary metabolites were also found (Figure 3.2 and Supplementary Table 1 in the appendix section). Lovastatin, a fungal secondary metabolite capable of lowering the cholesterol level in blood, is synthesized by two polyketide synthases and several accessory enzymes, including an enoylreductase LovC (Kennedy *et al.*, 1999). Six spots including putative enoylreductases of the lovastatin pathway (spots 450 and 461 -Pc20g05830- and spots 456, 471, 474 and 476 -Pc12g14200-), were identified in the reference map. One of the enzymes involved in the biosynthesis of phenazines (chorismic acid derivatives with antibiotic properties) is an oxidoreductase (PhzF) (Parsons *et al.*, 2004), which is identified in spot 490 (Pc13g06860).

Versicolorin A is a key intermediate in the biosynthesis of sterigmatocystin and aflatoxins. Biosynthesis of aflatoxins involves a set of proteins (Ehrlich *et al.*, 2005), including a versicolorin reductase VerA (Keller *et al.*, 1994; 1995), which is present in spot 684 (Pc22g06690). A protein with weak similarity to isoflavone reductase (Pc18g00110) is included in spot 465. Isoflavone reductase was identified as a key enzyme involved in the late part of the medicarpin biosynthetic pathway, which is the major isoflavonoid phytoalexin found in alfalfa (*Medicago sativa*) responding to fungal pathogen attack (Dixon and Steele, 1999).

3.1.4. Protein differences during the early improvement

In order to find the changes introduced during the early strain improvement program, the wild-type strain NRRL 1951 was compared to the improved, but still low producer Wis 54-1255. Analysis of the 2-DE gels identified 45 spots that were overrepresented in the NRRL 1951 strain (including 38 different proteins with isoforms), whereas in the Wis 54-1255, 29 spots (27 different proteins and isoforms) had higher representation compared to the wild-type strain (Figure 3.4 and 3.5). Tables 3.1 and 3.2 display the findings summarized below.

3.1.4.1. Carbohydrate metabolism and energy

Some of the changes in the improved strain Wis 54-1255 with respect to the wild-type NRRL 1951 affect carbohydrate metabolism. A probable β -glucosidase 1 (spot N1) is 6-fold overrepresented in the wild-type strain. This enzyme is involved in cellulose degradation (Kawaguchi *et al.*, 1996), which indicates the ability of the NRRL 1951 to utilize cellulose as carbon source. In addition, a probable sorbitol utilization protein Sou2 (spot N8) is 2.56 fold more represented in the NRRL 1951 strain, indicating the capacity of this fungus to use sorbitol (a sugar alcohol found in stone fruits) as carbon source.

The wild-type strain contains three spots (N16, N20 and N21) that include isoforms of a probable aldehyde dehydrogenase, which are absent in the Wis 54-1255 strain. Another protein that is only present in the wild-type strain (spot N23), is a putative NADP(+) coupled glycerol dehydrogenase proposed to be involved in an alternative pathway for glycerol catabolism (Costenoble *et al.*, 2000); it shows a high similarity to a glycerol dehydrogenase Gcy1 from *A. fumigatus* (92% homology, 87% identity). One of the enzymes of the Leloir pathway for galactose metabolism, a probable UDP-glucose 4-epimerase, is also overrepresented in the wild-type strain (spot N19). On the other hand, the expression of a variety of proteins is increased in the improved penicillin producer Wis 54-1255. This is the case for spot Ws17, containing a probable polygalacturonase for pectate and other galacturonans degradation, which is overrepresented in the Wis 54-1255 strain. Taken together these results suggest that the wild-type NRRL 1951 strain has a capability to use a variety of carbon sources that has been restricted (or enhanced in the case of polygalacturonase) in Wis 54-1255.

Figure 3.4. Comparison of the intracellular proteomes of the wild-type NRRL 1951 and the Wis 54-1255 strains. The designation “N” is used for those spots overrepresented in the NRRL 1951 strain and “Ws” is used for those spots overrepresented in the Wis 54-1255 strain. The spots differentially represented in each strain are numbered and correspond to those proteins listed in Table 3.1 (for the NRRL 1951 strain) and Table 3.2 (for the Wis 54-1255 strain).

Figure 3.5. Close-up view of the spots differentially represented in either the NRRL 1951 or the Wis 54-1255 strains. Enlargement of gel portions containing the spots overrepresented in the gels of Figure 3.4. The designation “N” is used for those spots overrepresented in the NRRL 1951 strain and “Ws” is used for those spots overrepresented in the Wis 54-1255 strain. The number of those spots differentially represented is indicated and corresponds to those present in Table 3.1 (for the NRRL 1951 strain) and Table 3.2 (for the Wis 54-1255 strain).

Table 3.1. Proteins overrepresented in the NRRL 1951 strain compared to the Wisconsin 54-1255 strain. Fold change and p-value are indicated for those proteins present in both strains. Otherwise, non-available (N/A) is shown.

Spot	ORF name	Accession No.	Title	Theoretical		Estimated		peptides identified	Un-matched peptides	Score	Percent coverage (%)	Fold change	p-value	Function
				Mass (kDa)	pI	Mass (kDa)	pI							
METABOLISM AND ENERGY														
N1	Pc18g01940	gi 211586771	strong similarity to beta-glucosidase 1 bg11 - <i>Aspergillus aculeatus</i>	95	4.8	129	4.7	29	34	614	33	6.70	5.38E-04	Carbohydrate Metabolism
N2	Pc20g09560	gi 211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakiense</i>	66	5.2	76	5.3	23	35	518	54	4.22	9.85E-04	Carbohydrate Metabolism
N3	Pc20g09560	gi 211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakiense</i>	66	5.2	75	5.3	22	36	529	41	5.29	7.91E-05	Carbohydrate Metabolism
N4	Pc13g05320	gi 211583586	strong similarity to hypothetical cystathione beta-synthase cysB - <i>Dicystosellum discordeum</i>	56	5.9	54	6.8	32	33	727	66	3.33	1.12E-04	Amino Acid Metabolism
N5	Pc21g12790	gi 211590017	strong similarity to UTP-glucose-1-phosphate uridylyltransferase Ugp1 - <i>Saccharomyces cerevisiae</i>	58	6.4	54	7.9	33	25	1030	73	5.96	2.06E-03	General Metabolism
N6	Pc06g01770	gi 211581395	strong similarity to adenylosuccinate synthase ade2p - <i>Schizosaccharomyces pombe</i>	47	6.4	40	7.9	16	49	126	43	3.42	9.01E-05	Purine Metabolism
N7	Pc12g04750	gi 211581963	strong similarity to malate dehydrogenase precursor MDH - <i>Mus musculus</i>	34	5.4	33	5.9	27	36	1060	92	2.80	1.02E-03	Precursor of MDH. Carbohydrate Metabolism. TCA cycle
N8	Pc12g00830	gi 211581603	strong similarity to sorbitol utilization protein sou2 - <i>Candida albicans</i>	29	5.3	27	5.6	20	38	625	79	2.56	2.22E-04	Carbohydrate Metabolism. (Related to short-chain alcohol dehydrogenases)
N9	Pc16g01840	gi 211585181	strong similarity to guanylate kinase Guk1 - <i>Saccharomyces cerevisiae</i>	22	6.2	21	7.0	15	50	380	70	2.70	2.77E-04	Purin nucleotide / nucleoside / nucleobase Metabolism
N10	Pc21g05830	gi 211589339	weak similarity to zeaxanthin epoxidase AB A2 - <i>Nicotiana plumbaginifolia</i>	45	5.5	43	5.8	24	25	381	65	11.93	7.05E-04	Isoprenoid Metabolism
N11	Pc20g06330	gi 211587897	strong similarity to NADPH-dependent beta-ketoacyl reductase RhlG - <i>Pseudomonas aeruginosa</i>	32	6.1	31	7.0	8	28	125	41	8.72	2.07E-03	Lipid Metabolism
N12	Pc13g08270	gi 211583872	adenylylsulfate kinase AAA81521- <i>Penicillium chrysogenum</i>	24	6.0	27	6.9	11	35	125	59	8.20	4.41E-03	Sulfur Metabolism
N13	Pc12g13740	gi 211582800	strong similarity to catalase/peroxidase like protein An01g01830 - <i>Aspergillus niger</i>	82	5.9	80	7.0	36	26	911	52	N/A	N/A	Amino Acid Metabolism
N14	Pc22g00290	gi 211591091	strong similarity to ATP-hydrolyzing 5-oxoprolinase - <i>Rattus norvegicus</i>	139	5.8	131	6.6	36	24	349	36	N/A	N/A	Amino Acid Metabolism
N15	Pc20g11520	gi 211588373	strong similarity to methylmalonate-semialdehyde dehydrogenase MMSDH - <i>Rattus norvegicus</i>	56	6.7	53	8.1	18	32	300	47	N/A	N/A	Amino Acid (Valine) and Pyrimidine nucleotide / nucleoside / nucleobase Metabolism

Table 3.1 continued

N16	Pc06g00180	gj 211581247	strong similarity to aldehyde dehydrogenase aldA - <i>Aspergillus niger</i>	54	5.9	53	6.4	13	52	167	44	N/A	N/A	Ethanol Metabolism
N17	Pc13g03600	gj 211583417	strong similarity to thiamin-phosphate pyrophosphorylase/hydroxyethylthiazole kinase Thi6 - <i>Saccharomyces cerevisiae</i>	54	6.0	52	6.9	16	39	230	44	N/A	N/A	Metabolism of vitamins, cofactors, and prosthetic groups
N18	Pc18g01450	gj 211586723	strong similarity to 3-hydroxy-3-methylglutaryl-CoA synthase Erg13 - <i>Saccharomyces cerevisiae</i>	51	5.7	49	6.1	13	33	174	35	N/A	N/A	Isoprenoid Metabolism
N19	Pc21g10370	gj 211589783	strong similarity to UDP-glucose 4-epimerase Gal10 - <i>Saccharomyces cerevisiae</i>	41	5.7	38	6.8	19	44	787	63	2.45	1.02E-04	Carbohydrate Metabolism (Leloir pathway)
N20	Pc06g00180	gj 211581247	strong similarity to aldehyde dehydrogenase aldA - <i>Aspergillus niger</i>	54	5.9	40	5.8	8	28	209	24	N/A	N/A	Ethanol Metabolism
N21	Pc06g00180	gj 211581247	strong similarity to aldehyde dehydrogenase aldA - <i>Aspergillus niger</i>	54	5.9	39	6.2	12	22	345	35	N/A	N/A	Ethanol Metabolism
N22	Pc12g06380	gj 211582002	strong similarity to coproporphyrinogen oxidase III Hem13 - <i>Saccharomyces cerevisiae</i>	50	7.2	39	6.7	18	46	293	37	2.09	8.30E-03	Metabolism of vitamins, cofactors, and prosthetic groups. Secondary metabolism (metabolism of porphyrins)
N23	Pc16g05650	gj 211585527	strong similarity to aldoketo reductase Gcy1 - <i>Saccharomyces cerevisiae</i>	33	6.2	32	7.5	18	47	442	68	N/A	N/A	Glycerol Metabolism
N24	Pc08g00080	gj 211581481	weak similarity to scytalone dehydratase - <i>Pyricularia oryzae</i>	17	6.1	15	7.0	8	57	310	60	N/A	N/A	Secondary Metabolism (Metabolism of Melanins)
N25	Pc12g05820	gj 211582045	strong similarity to esterase D ESD - <i>Homo sapiens</i>	31	5.9	31	6.9	17	44	687	82	2.17	2.27E-04	Lipid Metabolism
RESPONSE TO STIMULI														
N26	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	77	5.9	17	40	265	26	N/A	N/A	Cell rescue, defense and virulence
N27	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	72	6.0	18	39	225	29	N/A	N/A	Cell rescue, defense and virulence
N28	Pc13g02920	gj 211583350	strong similarity to host infection protein cap20 - <i>Colletotrichum gloeosporioides</i>	21	7.0	19	8.4	14	42	407	64	N/A	N/A	Cell rescue, defense and virulence
N29	Pc22g02940	gj 211591355	thioredoxin reductase TrxB - <i>Penicillium chrysogenum</i>	36	5.1	36	5.2	21	41	717	77	1.92	1.31E-03	Cell rescue, defense and virulence. Component of the thioredoxin-thioredoxin reductase system
N30	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	75	5.8	11	15	335	19	5.50	3.09E-02	Cell rescue, defense and virulence.
INFORMATION PATHWAYS														
N16	Pc20g00490	gj 211587347	strong similarity to cytosolic serine-tRNA ligase Ses1 - <i>Saccharomyces cerevisiae</i>	54	5.6	53	6.4	20	45	318	44	N/A	N/A	Protein synthesis
N31	Pc22g19990	gj 211592918	strong similarity to endonuclease Scl 75 kDa subunit Ens1 - <i>Saccharomyces cerevisiae</i>	73	5.6	71	5.3	30	32	877	51	6.70	1.44E-04	Protein fate. Protein folding, modification and destination. (Putative function as hsp70)

Table 3.1 continued

N32	Pc18g05190	gj211587079	strong similarity to cyclophilin-like peptidyl prolyl cis-trans isomerase cypA - <i>Aspergillus niger</i>	19	8.6	17	8.1	12	47	248	64	10.49	1,11E-03	Protein fate. Protein folding, modification and destination
N33	Pc18g04820	gj211587046	weak similarity to single-stranded nucleic acid binding protein CBP - <i>Mus musculus</i>	40	5.6	40	6.4	11	26	194	38	N/A	N/A	Transcription
N34	Pc21g06220	gj211589378	strong similarity to rho GDP dissociation inhibitor Rdi1 - <i>Saccharomyces cerevisiae</i>	22	5.2	22	5.8	5	29	139	28	N/A	N/A	Protein with binding function or cofactor requirement. involved in the localization and regulation of Cdc42p, which is a small rho-like GTPase, essential for establishment and maintenance of cell polarity
N35	Pc18g05190	gj211587079	strong similarity to cyclophilin-like peptidyl prolyl cis-trans isomerase cypA - <i>Aspergillus niger</i>	19	8.6	17	8.6	13	48	379	62	1,70	2,16E-03	Protein fate. Protein folding, modification and destination
N36	Pc22g10220	gj211592047	strong similarity to drak-type molecular chaperone Ssb2 - <i>Saccharomyces cerevisiae</i>	67	5.3	67	5.2	30	32	797	62	N/A	N/A	Protein fate. Protein folding, modification and destination
DEVELOPMENTAL PROCESSES														
N37	Pc13g04170	gj211583473	similarity to cell polarity protein tea1p - <i>Schizosaccharomyces pombe</i>	36	5.2	37	5.3	23	35	658	85	1,81	2,13E-02	Cell type differentiation. Microtubule end-associated factor. Cell polarity
MISCELLANEOUS														
N38	Pc13g13470	gj211584381	strong similarity to tubulin beta chain beta-tubulin like protein An08g03190 - <i>Aspergillus niger</i>	50	4.9	51	5.3	19	36	456	42	3,20	2,93E-03	Subcellular localization. Microtubules
UNKNOWN														
N6	Pc22g14810	gj211592419	strong similarity to hypothetical oxidoreductase CAG46711.1 - <i>Schizosaccharomyces pombe</i>	40	6.4	40	7.9	27	37	713	80	3,42	9,01E-05	unknown
N39	Pc22g13590	gj211592302	strong similarity to hypothetical protein - <i>Gloeobacter violaceus</i>	70	5.9	67	7.1	29	34	581	49	4,05	5,89E-03	unknown
N40	Pc23g00350	gj211904221	hypothetical protein BAC82546- <i>Penicillium chrysogenum</i>	23	6.1	21	7.6	12	50	692	56	5,01	1,67E-04	unknown
N41	Pc20g09550	gj211588185	similarity to hypothetical protein PA4204 - <i>Pseudomonas aeruginosa</i>	41	5.4	45	5.6	15	46	601	50	N/A	N/A	unknown
N42	Pc13g05520	gj211583606	strong similarity to hypothetical protein config_1_100_scaffold_7.fta_30wg - <i>Aspergillus nidulans</i>	44	10.1	30	5.7	12	42	141	23	N/A	N/A	unknown
N43	Pc23g00350	gj211904221	hypothetical protein BAC82546- <i>Penicillium chrysogenum</i>	23	6.1	22	7.1	13	50	598	49	N/A	N/A	unknown
N44	Pc23g00350	gj21190422	hypothetical protein BAC82546- <i>Penicillium chrysogenum</i>	23	6.1	22	7.4	14	25	584	58	N/A	N/A	unknown
N45	Pc23g00350	gj211904221	hypothetical protein BAC82546- <i>Penicillium chrysogenum</i>	23	6.1	21	7.6	13	49	694	49	N/A	N/A	unknown

Table 3.2: Proteins overrepresented in the Wisconsin 54-1255 strain compared to the NRRL 1951 strain. Fold change and p-value are indicated for those proteins present in both strains. Otherwise, non-available (N/A) is shown.

Spot	ORF name	Accession No.	Title	Theoretical		Estimated		peptides identified	Un-Matched peptides	Score	Percent coverage (%)	Fold change	p-value	Function
				Mass (kDa)	pI	Mass (kDa)	pI							
METABOLISM AND ENERGY														
Ws1	Pc21g20480	gi 211590746	strong similarity to ATP citrate lyase ACL1-Sordaria macrospora	72	7.6	41	8.2	20	41	566	35	4.73	1.40E-02	Carbohydrate and Lipid Metabolism
Ws2	Pc20g05830	gi 211587847	strong similarity to enoyl reductase of the lovastatin biosynthesis lovC - Aspergillus terreus	37	6.2	36	7.2	19	44	617	74	2.61	1.60E-03	Secondary Metabolism
Ws3	Pc14g01270	gi 211584742	strong similarity to hypothetical 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase BH2000 -Baillus halodurans	31	6.3	31	7.3	20	43	740	75	3.74	2.22E-04	Carbohydrate Metabolism
Ws4	Pc18g01220	gi 211586700	strong similarity to fructose-bisphosphate aldolase Fba1 - Saccharomyces cerevisiae	39	5.4	29	5.5	14	50	595	42	3.06	2.75E-05	Carbohydrate Metabolism. Glycolysis
Ws5	Pc06g01770	gi 211581395	strong similarity to adenylosuccinate synthase ade2p - Schizosaccharomyces pombe	47	6.4	29	7.3	15	35	215	41	2.36	2.52E-04	Purin nucleotide / nucleoside / nucleobase Metabolism
Ws6	Pc21g14890	gi 211590213	cysteine synthase BAC82550-Penicillium chrysogenum	40	8.6	37	8.4	23	39	921	74	2.14	1.25E-03	Amino Acid Metabolism. Cysteine biosynthetic process from serine
Ws7	Pc22g16150	gi 211592548	strong similarity to prostaglandin f(2alpha) synthase TbPGFS - Trypanosoma brucei	31	6.1	31	7.1	14	51	349	65	1.99	9.71E-03	Metabolism of Eicosanoids
Ws8	Pc22g24360	gi 211593341	strong similarity to vacuolar ATPase subunit E Vma-4 - Neurospora crassa	26	6.1	28	6.8	12	37	352	51	1.84	5.08E-03	Energy
Ws9	Pc20g01960	gi 211587490	strong similarity to formyltetrahydrofolate deformylase purU - Corynebacterium sp.	32	6.7	28	8.0	9	41	199	52	3.97	2.47E-02	Purin nucleotide / nucleoside / nucleobase Metabolism
Ws10	Pc12g05480	gi 211582012	similarity to core protein II of ubiquinol-cytochrome c reductase CAA42214.1 - Bos primigenius taurus	48	8.9	28	9.2	22	43	627	52	2.34	2.57E-03	Metabolism and Energy. Oxidative phosphorylation
Ws11	Pc22g21270	gi 211593041	strong similarity to mitochondrial enoyl-CoA hydratase ECHS1 - Rattus norvegicus	32	8.8	26	8.3	19	43	294	58	4.26	8.58E-03	Lipid Metabolism
Ws12	Pc21g05750	gi 211589332	strong similarity to fatty acid regulation protein like protein An14g07060 - Aspergillus niger	24	5.3	23	5.2	11	48	505	57	4.21	2.02E-02	Lipid Metabolism
Ws13	Pc22g11710	gi 211592134	strong similarity to alpha subunit E1 of the pyruvate dehydrogenase complex Pda1 - Saccharomyces cerevisiae	45	8.5	43	6.7	22	38	583	53	N/A	N/A	Energy. Pyruvate dehydrogenase complex
Ws14	Pc22g10010	gi 211592027	strong similarity to N-acetylglucosamine-6-phosphate deacetylase CanAG2 - Candida albicans	46	5.8	43	6.7	23	37	1050	72	N/A	N/A	Carbohydrate Metabolism
Ws15	Pc12g03090	gi 211581814	strong similarity to soluble cytoplasmic fumarate reductase YEL047c - Saccharomyces cerevisiae	67	6.2	22	8.7	15	47	246	26	2.71	1.47E-02	Carbohydrate Metabolism

Table 3.2 continued

Ws16	Pc20g01610	gj211587455	strong similarity to mitochondrial malate dehydrogenase Mdh1 - <i>Saccharomyces cerevisiae</i>	36	8.4	32	7.4	19	46	1460	62	1.74	7.83E-03	Carbohydrate Metabolism, TCA cycle
Ws17	Pc22g02090	gj211592946	strong similarity to polygalacturonase pgal - <i>Aspergillus niger</i>	39	5.5	41	5.4	16	36	882	65	3.84	1.49E-02	Carbohydrate Metabolism
INFORMATION PATHWAYS														
Ws18	Pc22g19990	gj211592918	strong similarity to endonuclease Scl 75 kDa subunit Ens1 - <i>Saccharomyces cerevisiae</i>	73	5.6	31	4.3	14	33	222	26	7.26	6.11E-04	Protein fate, Protein folding, modification and destination. (Putative function as hsp70)
Ws19	Pc13g09680	gj211584011	strong similarity to aspartyl proteinase candidapepsin - <i>Candida albicans</i>	44	5.1	37	4.2	13	48	660	44	1.51	3.85E-04	Protein fate, Protein folding, modification and destination
Ws20	Pc22g11240	gj211592088	strong similarity to heat shock protein 70 hsp70 - <i>Ajeilomyces capsulatus</i>	70	5.0	32	4.2	13	50	192	29	2.83	1.05E-02	Protein fate, Protein folding, modification and destination
Ws21	Pc12g10670	gj211582507	strong similarity to dnaK-type molecular chaperone b1pA - <i>Aspergillus niger</i>	74	4.8	29	3.8	9	32	248	14	3.12	2.69E-03	Protein fate, Protein folding, modification and destination
Ws22	Pc22g10220	gj211592047	strong similarity to dnaK-type molecular chaperone Ssb2 - <i>Saccharomyces cerevisiae</i>	67	5.3	46	5.3	20	41	803	42	1.99	1.82E-02	Protein fate, Protein folding, modification and destination
Ws23	Pc20g04810	gj211587757	strong similarity to estrogen receptor-binding cyclophilin cypD - <i>Bos primigenius taurus</i>	41	5.9	40	6.6	17	48	359	60	2.87	5.60E-03	Protein fate, Protein folding, modification and destination. (cypD accelerates folding of proteins by acting as a PPIase)
Ws24	Pc22g13950	gj211592336	strong similarity to vacuolar aminopeptidase Ysd - <i>Saccharomyces cerevisiae</i>	56	5.7	22	8.9	12	36	227	24	1.84	8.14E-02	Protein fate, Protein folding, modification and destination
Ws25	Pc22g13950	gj211592336	strong similarity to vacuolar aminopeptidase Ysd - <i>Saccharomyces cerevisiae</i>	56	5.7	22	9.0	12	47	194	24	10.25	4.26E-04	Protein fate, Protein folding, modification and destination
Ws26	Pc20g09400	gj211588171	strong similarity to dipeptidyl-peptidase V DPP V - <i>Aspergillus fumigatus</i>	79	5.6	21	8.8	15	49	273	23	2.87	4.38E-03	Protein fate, Protein folding, modification and destination
Ws27	Pc20g09400	gj211588171	strong similarity to dipeptidyl-peptidase V DPP V - <i>Aspergillus fumigatus</i>	79	5.6	21	8.7	17	48	369	23	2.65	1.84E-04	Protein fate, Protein folding, modification and destination
DEVELOPMENTAL PROCESSES														
Ws28	Pc22g03880	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanoyltransferase gel3 - <i>Aspergillus fumigatus</i>	58	4.9	76	4.4	18	44	625	37	1.91	7.30E-02	Biogenesis of cellular components. Required for cell wall assembly and morphogenesis
UNKNOWN														
Ws29	Pc22g17870	gj211592713	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>	41	4.6	59	4.0	18	28	807	50	2.29	1.63E-03	unknown

It is noteworthy that some proteins related to glycolysis and energy formation are overrepresented in the Wis 54-1255 strain. This is the case of spots Ws1 (probable ATP citrate lyase involved in the formation of cytosolic acetyl-CoA), Ws4 (probable fructose-bisphosphate aldolase), Ws10 (probable core protein II of the ubiquinol cytochrome C reductase), Ws13 (probable alpha subunit E1 of the pyruvate dehydrogenase complex) or Ws15 (probable soluble cytoplasmic fumarate reductase likely involved in anaerobic respiration). It is also remarkable that the probable malate dehydrogenase is overrepresented as the precursor protein in the NRRL 1951 strain (spot N7), whereas in the Wis 54-1255 strain the mature form of this protein is predominant (spot Ws16).

3.1.4.2. Penicillin biosynthetic precursors

The non-ribosomal condensation of the three precursor amino acids (L- α -aminoadipic acid, L-cysteine and L-valine) is the initial step of the penicillin biosynthesis. Some proteins involved in metabolism of amino acid precursors for penicillin biosynthesis are differentially represented in these two strains. Spot N4, which contains a hypothetical cystathione beta synthase involved in the biosynthesis of cystathionine from cysteine, is overrepresented in the NRRL 1951 strain. Only the wild-type strain shows a probable methylmalonate semialdehyde dehydrogenase (spot N15), which is involved in valine catabolism (Goodwin *et al.*, 1989; Zhang *et al.*, 1996) and may result in a reduced valine availability for penicillin biosynthesis in the wild-type as compared to the Wis 54-1255 strain. Viceversa, spot Ws6 (including a cysteine synthase) is 2.14-fold overrepresented in the Wis 54-1255 strain. These findings suggest a reduction in cysteine and valine catabolism and explain the enhancement of their biosynthesis during the early improvement program.

3.1.4.3. Pigmentation and secondary metabolism

It is noteworthy that in the wild-type strain analysis a variety of proteins involved in pigment formation were identified, in agreement with the known ability of this strain to synthesize increased amount of pigment. A putative zeaxanthin epoxidase, which catalyses the conversion of zeaxanthin to violaxanthin (natural xanthophyll pigment with an orange color), is 11-fold overrepresented in the wild-type strain (spot N10). In

addition, a putative scytalone dehydratase involved in the biosynthesis of melanin is only found in the wild-type strain (spot N24). This pigment has been reported to protect hyphae and spores from UV light, free radicals and desiccation and also serves as a virulence factor (Gómez and Nosanchuk, 2003).

Other proteins involved in secondary metabolism pathways overrepresented in the wild-type strain are a probable 3-hydroxy-3-methylglutaryl-CoA synthase (spot N18) and a probable coproporphyrinogen oxidase III (spot N22). The first enzyme is involved in the formation of hydroxymethylglutaryl-CoA for terpenoid synthesis, whereas the second one is involved in the porphyrins metabolism.

In general, the improved penicillin producer Wis 54-1255 strain contains a lower amount of proteins involved in the biosynthesis of other secondary metabolites. However, some examples of a positive correlation may occur, e.g. spot Ws2 is 2.61-fold overrepresented compared to the wild-type strain. This spot corresponds to a probable enoylreductase of the lovastatin pathway, which is involved in the biosynthesis of the lovastatin precursor dihydromonacolin N (see Discussion).

3.1.4.4. Virulence and defense mechanisms

Several proteins related to host-infection and virulence that are abundant in the wild-type strain are underrepresented in the Wis 54-1255 strain. A probable glucose oxidase (spots N2 and N3), involved in oxidation of D-glucose to D-glucono-1,5-lactone (further hydrolyzed to gluconic acid), is highly overrepresented in the wild-type strain. This enzyme is involved in virulence, since gluconic acid and glucose oxidase have been associated with the pathogenicity of *Penicillium expansum* in apples (Hadas *et al.*, 2007). Accordingly, spot N28, which includes a probable host-infection protein Cap20, is only present in the wild-type strain. This protein plays a significant role in the fungal virulence on avocado and tomato fruits (Hwang *et al.*, 1995). Proteins involved in redox metabolism are predominant in the wild-type strain. This strain shows three isoforms of the catalase R (spots N26, N27 and N30). Two of them are not detected in the Wis 54-1255 strain (spots N26 and N27) and the third one (spot N30) is 5.5-fold underrepresented in the Wis 54-1255 strain. The thioredoxin reductase (spot N29) is also underrepresented in the Wis 54-1255 as compared to the wild-type strain (1.92 times).

3.1.4.5. Protein targeting and processing

A global view of the enzymes related to protein fate pathways, shows that proteinases/peptidases are overrepresented in the improved penicillin producer Wis 54-1255 strain (spots Ws19, Ws24, Ws25, Ws26 and Ws27). Two of these spots (Ws26 and Ws27), contain isoforms of the same protein (Pc20g09400), which is a probable dipeptidyl-peptidase V. This protein was initially described in *A. fumigatus* and was the first report of a secreted dipeptidyl-peptidase (Beauvais *et al.*, 1997), which is identical to one of the two major antigens used for the diagnosis of aspergillosis. A probable cyclophilin-like peptidyl prolyl cis-trans isomerase CypA (spots N32 and N35) is overrepresented in the wild-type strain.

3.1.5. Protein differences leading to industrial producers

In order to assess some of the modifications occurred during the development of the high-producer AS-P-78 from Wis 54-1255, extracted proteins from these two strains were compared to each other as indicated before. Analysis of the 2-DE gels identified 43 spots (including 41 different proteins and 2 isoforms) that were underrepresented in the AS-P-78 strain, whereas 52 spots (including 37 different proteins and isoforms) had higher representation in the AS-P-78 strain (Figure 3.6 and 3.7). The findings summarized below are detailed in Tables 3.3 and 3.4.

3.1.5.1 Carbohydrate metabolism and energy

Three isoforms of a probable ribose-5-phosphate isomerase RpiB (A13, A14 and A16) were only found in the AS-P-78 strain. This protein is involved in the non-oxidative pentose phosphate pathway. Connected to this pathway is the hypothetical protein included in spot A44, which is 3.82-fold overrepresented in the high-producer strain. This protein shares a high similarity to a transketolase from *Laccaria bicolor* (78% homology, 65% identity). This enzyme connects the pentose phosphate pathway to glycolysis. Interestingly, spot A2, identified as a probable polygalacturonase (Pc22g20290), is 1.94-fold overrepresented in the high producing strain. As indicated above, the same polygalacturonase (Ws17) was 3.84-fold overrepresented in the Wis 54-1255 with respect to the wild-type strain. This enzyme is in charge of pectate degradation and is involved in starch and sucrose metabolism.

Figure 3.6. Comparison of the intracellular proteomes of the Wis 54-1255 and the high producer AS-P-78 strains. The name “W” is used for those spots overrepresented in the Wis 54-1255 strain, whereas “A” is used for those spots overrepresented in the AS-P-78 strain. The number of each differentially represented spot is indicated and corresponds to those in Table 3.3 (for the Wis 54-1255 strain) and Table 3.4 (for the AS-P-78 strain).

Figure 3.7. Close-up view of the spots differentially represented in either the Wis 54-1255 or the AS-P-78 strains. Enlargement of gel portions containing the spots overrepresented in the gels of Figure 3.6. The designation "W" is used for those spots overrepresented in the Wis 54-1255 strain and "A" is used for those spots overrepresented in the AS-P-78 strain. The number of each differentially represented spot is indicated and corresponds to those present in Table 3.3 (for the Wis 54-1255 strain) and Table 3.4 (for the AS-P-78 strain).

Table 3.3 . Proteins overrepresented in the Wisconsin 54-1255 strain compared to the AS-P-78 strain. Fold change and p-value are indicated for those proteins present in both strains. Otherwise, non-available (N/A) is shown.

Spot	ORF name	Accession No.	Title	Theoretical		Estimated		peptides identified	Un-matched peptides	Score	Percent coverage (%)	Fold change	p-value	Function
				Mass (kDa)	pI	Mass (kDa)	pI							
METABOLISM AND ENERGY														
W1	Pc22g02000	gi211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	86	6.0	88	6.6	30	16	695	45	2.19	5.15E-03	Carbohydrate metabolism. TCA cycle
W2	Pc20g07230	gi211587983	strong similarity to inorganic pyrophosphatase <i>lpp1</i> - <i>Saccharomyces cerevisiae</i>	33	5.1	32	5.1	19	39	429	75	2.64	3.19E-03	Phosphate metabolism
W3	Pc22g11710	gi211592134	strong similarity to alpha subunit E1 of the pyruvate dehydrogenase complex Pda1 - <i>Saccharomyces cerevisiae</i>	45	8.5	44	6.5	25	26	648	56	2.39	3.92E-04	Energy. Pyruvate dehydrogenase complex
W4	Pc16g08460	gi211585789	strong similarity to sorbitol dehydrogenase <i>gufB</i> - <i>Bacillus subtilis</i>	37	5.9	37	7.3	20	22	597	67	6.26	1.18E-02	Carbohydrate metabolism
W5	Pc20g09560	gi211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakiense</i>	66	5.2	85	5.4	21	37	484	43	2.00	9.33E-03	Carbohydrate metabolism
W6	Pc22g10020	gi211592028	strong similarity to alcohol dehydrogenase <i>alkJ</i> - <i>Pseudomonas oleovorans</i>	60	5.9	69	6.1	20	12	410	38	4.53	3.97E-04	Ethanol metabolism
W7	Pc22g22710	gi211593182	strong similarity to dihydroxy-acid dehydratase <i>liv3</i> - <i>Saccharomyces cerevisiae</i>	65	6.2	68	5.9	13	20	268	24	3.54	1.75E-03	Amino Acid metabolism
W8	Pc21g11950	gi211589937	strong similarity to UDP-N-acetylglucosamine pyrophosphorylase CaUAP1 - <i>Candida albicans</i>	56	5.4	62	5.6	26	21	540	55	2.18	4.87E-03	General Metabolism
W9	Pc22g19100	gi211592830	strong similarity to 5-aminoimidazole-4-carboxamide ribotide transformylase Ade17 - <i>Saccharomyces cerevisiae</i>	65	5.9	59	6.1	23	7	464	48	2.07	5.61E-03	Metabolism of vitamins, cofactors and prostetic groups. Purine metabolism
W10	Pc18g06000	gi211587160	strong similarity to pyruvate kinase <i>pkA</i> - <i>Aspergillus niger</i>	59	6.6	59	7.6	20	20	340	41	2.46	9.97E-03	Carbohydrate metabolism. Glycolysis
W11	Pc12g09430	gi211582389	strong similarity to alanine transaminase ALA2 - <i>Hordeum vulgare</i>	60	6.4	55	5.9	16	33	319	40	2.58	6.32E-04	Amino Acid metabolism
W12	Pc12g04370	gi211581925	strong similarity to phosphoserine transaminase Ser1 - <i>Saccharomyces cerevisiae</i>	48	5.5	49	6.1	21	17	631	49	2.31	2.61E-04	Amino Acid metabolism
W13	Pc13g05010	gi211583556	strong similarity to cytoplasmic serine hydroxymethyltransferase Shm2 - <i>Saccharomyces cerevisiae</i>	58	8.7	50	8.1	16	15	284	32	4.62	1.87E-04	Amino Acid Metabolism
W14	Pc12g05400	gi211582004	strong similarity to citrate synthase <i>citA</i> - <i>Aspergillus niger</i>	52	8.8	44	8.7	20	43	574	44	3.77	2.19E-03	Carbohydrate metabolism. TCA cycle

Table 3.3 continued

W15	Pc16g05740	gi 2111585536	strong similarity to FMN(H2)-dependent aliphatic sulfonate monooxygenase ssuD - <i>Escherichia coli</i>	40	5.6	37	6.2	17	47	395	63	N/A	N/A	Sulfur metabolism
W16	Pc20g06210	gi 2111587885	strong similarity to Fe(II)-dependent sulfonate/alpha-ketoglutarate dioxygenase YLL057c - <i>Saccharomyces cerevisiae</i>	43	6.0	37	7.1	12	18	430	36	4.96	4.69E-04	Sulfur metabolism
W17	Pc12g04750	gi 2111581963	strong similarity to malate dehydrogenase precursor MDH - <i>Mus musculus</i>	34	5.4	33	5.8	20	21	606	79	3.14	5.45E-04	Precursor of MDH, Carbohydrate metabolism, TCA cycle
W18	Pc16g05740	gi 2111585536	strong similarity to FMN(H2)-dependent aliphatic sulfonate monooxygenase ssuD - <i>Escherichia coli</i>	40	5.6	28	6.6	7	8	198	21	2.91	7.18E-03	Sulfur metabolism
W19	Pc22g10040	gi 2111592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	28	7.9	17	27	588	68	1.50	1.44E-03	Carbohydrate metabolism
W20	Pc14g01430	gi 2111584758	strong similarity to phosphonamidotriase Sec53 - <i>Saccharomyces cerevisiae</i>	30	5.1	28	5.0	10	15	261	35	2.04	1.75E-03	Carbohydrate metabolism
W21	Pc21g16950	gi 2111590406	strong similarity to transaldolase TalI - <i>Saccharomyces cerevisiae</i>	36	5.5	33	5.4	16	49	141	48	N/A	N/A	Carbohydrate metabolism, Non-oxidative pentose phosphate pathway
W22	Pc18g00110	gi 2111586591	weak similarity to isoflavone reductase IFR - <i>Medicago sativa</i>	36	5.8	35	6.5	12	53	153	50	N/A	N/A	Secondary metabolism, (Medicarpin biosynthesis)
RESPONSE TO STIMULI														
W23	Pc22g01260	gi 2111591187	strong similarity to small G-protein Gsp1 - <i>Candida albicans</i>	24	6.5	25	7.5	17	45	324	78	1.85	2.44E-03	Cellular communication /Signal transduction mechanism
W24	Pc18g00980	gi 2111586677	strong similarity to hypothetical trunk lateral cell specific gene HTLC1 - <i>Halocynthia roretzi</i>	57	5.6	55	6.4	24	36	548	50	2.76	4.35E-04	Cell rescue, defense and virulence. (It contains a Pyridine nucleotide-disulphide oxidoreductase conserved domain)
W25	Pc18g00980	gi 2111586677	strong similarity to hypothetical trunk lateral cell specific gene HTLC1 - <i>Halocynthia roretzi</i>	57	5.6	56	6.2	25	36	722	50	2.46	3.20E-03	Cell rescue, defense and virulence. (It contains a Pyridine nucleotide-disulphide oxidoreductase conserved domain)
W26	Pc18g00980	gi 2111586677	strong similarity to hypothetical trunk lateral cell specific gene HTLC1 - <i>Halocynthia roretzi</i>	57	5.6	58	4.7	20	37	362	39	16.72	1.65E-04	Cell rescue, defense and virulence. (It contains a Pyridine nucleotide-disulphide oxidoreductase conserved domain)
W27	Pc15g00720	gi 2111584920	strong similarity to 1,4-butanediol diacrylate esterase BDA1 - <i>Brevibacterium linens</i>	43	5.1	40	5.0	18	39	554	78	4.64	3.09E-03	Cell rescue, defense and virulence. (It contains Beta-lactamase class C and other penicillin binding proteins conserved domain)
INFORMATION PATHWAYS														
W28	Pc18g05190	gi 2111587079	strong similarity to cyclophilin-like peptidyl prolyl cis-trans isomerase cypA - <i>Aspergillus niger</i>	19	8.6	18	9.0	12	52	324	60	2.58	4.77E-03	Protein folding, modification and destination
W29	Pc16g13060	gi 2111586228	strong similarity to cyclophilin cypB - <i>Aspergillus nidulans</i>	18	6.9	16	8.0	10	52	291	64	2.34	9.83E-05	Protein folding, modification and destination
W30	Pc12g06650	gi 2111582124	strong similarity to translation elongation factor 3 Yef3 - <i>Saccharomyces cerevisiae</i>	118	6.0	111	7.2	9	54	154	12	6.35	2.23E-03	Protein synthesis, Translation.

Table 3.3 continued

W31	Pc22g02800	gi 211591341	strong similarity to calcium-binding protein precursor <i>cox1p</i> - <i>Schizosaccharomyces pombe</i>	62	4.8	72	4.4	19	46	289	37	2.01	4.35E-03	Protein folding, modification and destination
W32	Pc16g11070	gi 211586036	strong similarity to mitochondrial heat shock protein Hsp60 - <i>Saccharomyces cerevisiae</i>	62	5.6	60	5.2	8	5	250	12	3.65	1.17E-03	Protein folding, modification and destination
W33	Pc12g10670	gi 211582507	strong similarity to <i>dnaK</i> -type molecular chaperone <i>bipA</i> - <i>Aspergillus niger</i>	74	4.8	30	4.1	8	12	324	14	3.40	3.69E-04	Protein folding, modification and destination
DEVELOPMENTAL PROCESSES														
W34	Pc13g04170	gi 211583473	similarity to cell polarity protein <i>tea1p</i> - <i>Schizosaccharomyces pombe</i>	36	5.2	37	5.2	21	14	518	78	2.54	2.00E-03	Cell type differentiation. Microtubule end-associated factor. Cell polarity
MISCELLANEOUS														
W35	Pc22g08080	gi 211591841	strong similarity to actin interacting protein like protein <i>Ano2g14620</i> - <i>Aspergillus niger</i>	65	6.3	73	7.7	34	17	797	69	2.66	1.67E-03	Subcellular localization
W36	Pc15g00640	gi 211584913	strong similarity to GDP dissociation inhibitor in the secretory pathway <i>Gdi1</i> - <i>Saccharomyces cerevisiae</i>	52	5.3	52	5.5	21	39	449	51	2.47	1.97E-03	Cellular transport, transport facilitation and transport routes
W37	Pc22g24640	gi 211593369	strong similarity to peroxisomal membrane protein <i>PMP20</i> - <i>Candida boidinii</i>	18	5.4	16	5.2	11	16	403	68	1.87	1.37E-03	Subcellular localization
UNKNOWN														
W38	Pc22g17870	gi 211592713	strong similarity to hypothetical ECM33 homolog <i>SPCC1223.12c</i> - <i>Schizosaccharomyces pombe</i>	41	4.6	65	4.3	7	11	112	19	3.38	1.09E-02	unknown
W39	Pc18g02780	gi 211586852	strong similarity to hypothetical protein <i>contig1488_2.tfa_1170cg</i> - <i>Aspergillus fumigatus</i>	25	5.2	24	5.1	13	7	428	58	4.81	3.48E-05	unknown
W40	Pc13g14920	gi 211584514	strong similarity to hypothetical protein <i>Ano9g01070</i> - <i>Aspergillus niger</i>	54	5.5	52	5.9	22	29	350	53	2.55	4.79E-03	unknown
W41	Pc21g02210	gi 211588997	strong similarity to hypothetical protein <i>contig31_part_ii.tfa_2190wg</i> - <i>Aspergillus fumigatus</i>	35	5.6	34	6.2	10	25	272	45	5.61	7.73E-03	unknown
W42	Pc22g05690	gi 211591618	strong similarity to hypothetical protein <i>contig12.tfa_1730cg</i> - <i>Aspergillus fumigatus</i>	60	4.9	30	4.6	13	41	393	20	8.71	2.11E-03	unknown
W43	Pc20g02780	gi 211587567	strong similarity to hypothetical protein <i>PA3829</i> - <i>Pseudomonas aeruginosa</i>	34	5.4	33	5.4	12	48	624	60	N/A	N/A	unknown

Table 3.4. Proteins overrepresented in the AS-P-78 strain compared to the Wisconsin 54-1255 strain. Fold change and p-value are indicated for those proteins present in both strains. Otherwise, non-available (N/A) is shown.

Spot	ORF name	Accession No.	Title	Theoretical		Estimated		peptides identified	Un-matched peptides	Score	Percent coverage (%)	Fold change	p-value	Function
				Mass (kDa)	pI	Mass (kDa)	pI							
A1	Pc12g00830	gi 211581603	strong similarity to sorbitol utilization protein <i>sou2</i> - <i>Candida albicans</i>	29	5.3	27	5.7	17	40	474	80	8.45	8.37E-04	Carbohydrate metabolism. (Related to short-chain alcohol dehydrogenases)
A2	Pc22g02090	gi 211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39	5.5	42	5.7	15	44	566	57	1.94	4.66E-02	Carbohydrate Metabolism
A3	Pc12g12060	gi 211582641	strong similarity to branched-chain amino acid aminotransferase <i>Bat2</i> - <i>Saccharomyces cerevisiae</i>	43	6.4	39	7.7	20	40	684	50	4.07	1.58E-02	Amino Acid Metabolism
A4	Pc20g05830	gi 211587847	strong similarity to enoyl reductase of the lovastatin biosynthesis <i>lovC</i> - <i>Aspergillus terreus</i>	37	6.2	36	7.5	17	48	625	73	2.70	1.56E-04	Secondary metabolism. It is necessary for the correct processing of the growing polypeptide chain and production of dihydromonacolin L, a lovastatin precursor
A5	Pc18g02900	gi 211586864	lysophospholipase phospholipase B <i>plb1</i> - <i>Penicillium chrysogenum</i>	69	4.4	111	4.4	11	53	248	20	4.35	2.09E-06	Lipid Metabolism
A6	Pc13g05320	gi 211583586	strong similarity to hypothetical cystathione beta-synthase <i>cysB</i> - <i>Dictyostelium discoideum</i>	56	5.9	57	7.0	26	35	498	63	1.85	1.37E-03	Amino Acid Metabolism
A7	Pc13g03600	gi 211583417	strong similarity to thiamin-phosphate pyrophosphorylase/hydroxyethylthiazole kinase <i>Thi6</i> - <i>Saccharomyces cerevisiae</i>	54	6.0	54	7.0	16	18	408	39	N/A	N/A	Metabolism of vitamins, cofactors, and prosthetic groups
A8	Pc12g00830	gi 211581603	strong similarity to sorbitol utilization protein <i>sou2</i> - <i>Candida albicans</i>	29	5.3	24	7.1	12	26	257	57	3.21	1.29E-03	Carbohydrate metabolism. (Related to short-chain alcohol dehydrogenases)
A9	Pc22g04840	gi 211591538	strong similarity to hypothetical phosphatidyl synthase <i>SPAC22A12.08c</i> - <i>Schizosaccharomyces pombe</i>	53	5.4	44	6.6	20	26	314	43	N/A	N/A	Lipid metabolism
A10	Pc12g05380	gi 211582002	strong similarity to coproporphyrinogen oxidase III <i>Hem13</i> - <i>Saccharomyces cerevisiae</i>	50	7.2	40	6.8	13	18	315	26	3.20	3.65E-05	Metabolism of vitamins, cofactors, and prosthetic groups. Secondary metabolism (metabolism of porphyrins)
A11	Pc18g02920	gi 211586866	strong similarity to 2-dehydro-3-deoxyphosphoheptanate aldolase - <i>Arabidopsis thaliana</i>	50	6.0	36	6.7	15	43	428	39	2.10	1.37E-02	Amino Acid Metabolism
A12	Pc21g14560	gi 211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase <i>gndA</i> - <i>Aspergillus niger</i>	36	6.2	35	7.0	12	52	281	42	4.96	2.31E-03	Carbohydrate Metabolism. Glycolysis

Table 3.4 continued

A13	Pc22g21440	gi 211593057	strong similarity to ribose-5-phosphate isomerase <i>rpIB</i> - <i>Escherichia coli</i>	17	6.7	33	6.5	10	36	370	71	N/A	N/A	Carbohydrate Metabolism. Non-oxidative pentose phosphate pathway
A14	Pc22g21440	gi 211593057	strong similarity to ribose-5-phosphate isomerase <i>rpIB</i> - <i>Escherichia coli</i>	17	6.7	32	6.5	9	33	287	71	N/A	N/A	Carbohydrate Metabolism. Non-oxidative pentose phosphate pathway
A15	Pc12g06820	gi 211582045	strong similarity to esterase D ESD - <i>Homo sapiens</i>	31	5.9	32	7.0	16	35	450	83	2.12	3.11E-03	General Metabolism
A16	Pc22g21440	gi 211593057	strong similarity to ribose-5-phosphate isomerase <i>rpIB</i> - <i>Escherichia coli</i>	17	6.7	30	5.6	7	58	176	38	N/A	N/A	Carbohydrate Metabolism. Non-oxidative pentose phosphate pathway
A17	Pc12g00830	gi 211581603	strong similarity to sorbitol utilization protein <i>sou2</i> - <i>Candida albicans</i>	29	5.3	25	6.5	6	59	86	21	N/A	N/A	Carbohydrate metabolism. (Related to short-chain alcohol dehydrogenases)
A18	Pc21g15310	gi 211590252	weak similarity to hypothetical intracellular protease/amidase related enzyme of the ThiJ family CAC2826 - <i>Clostridium acetobutylicum</i>	25	5.6	24	5.8	10	19	192	53	N/A	N/A	Metabolism of vitamins, cofactors, and prosthetic groups
A19	Pc21g15310	gi 211590252	weak similarity to hypothetical intracellular protease/amidase related enzyme of the ThiJ family CAC2826 - <i>Clostridium acetobutylicum</i>	25	5.6	24	6.2	12	38	262	71	N/A	N/A	Metabolism of vitamins, cofactors, and prosthetic groups
RESPONSE TO STIMULI														
A20	Pc18g00790	gi 211586658	strong similarity to glutathione S-transferase like protein An02g06560 - <i>Aspergillus niger</i>	29	6.6	25	8.1	12	50	384	51	3.93	1.32E-04	Cell rescue, defense and virulence
A21	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	91	5.7	27	34	568	43	2.15	6.97E-03	Cell rescue, defense and virulence
A22	Pc22g25220	gi 211593426	strong similarity to 1,4-benzoquinone reductase <i>qr</i> - <i>Phanerochaete chrysosporium</i>	21	5.7	21	6.8	11	53	260	64	1.64	1.88E-03	Cell rescue, defense and virulence
A23	Pc06g01800	gi 211581398	strong similarity to quinone oxidoreductase P1 - <i>Arabidopsis thaliana</i>	37	6.5	37	8.1	19	46	813	63	6.07	1.29E-04	Cell rescue, defense and virulence
A24	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	85	5.9	23	40	484	36	N/A	N/A	Cell rescue, defense and virulence
A25	Pc16g13280	gi 211586250	strong similarity to glutathione reductase Glr1 - <i>Saccharomyces cerevisiae</i>	51	6.2	53	8.0	21	37	825	57	1.65	6.29E-03	Cell rescue, defense and virulence
A26	Pc16g13280	gi 211586250	strong similarity to glutathione reductase Glr1 - <i>Saccharomyces cerevisiae</i>	51	6.2	53	7.8	16	44	522	50	3.11	2.42E-03	Cell rescue, defense and virulence
A27	Pc12g07100	gi 211582165	strong similarity to alcohol dehydrogenase <i>adhIII</i> - <i>Aspergillus nidulans</i>	37	6.7	36	8.2	14	51	478	55	N/A	N/A	Cell rescue, defense and virulence
A28	Pc20g05270	gi 211587798	strong similarity to aflatoxin B1 aldehyde reductase AFAR - <i>Raffus norvegicus</i>	39	5.5	36	5.5	18	47	492	58	N/A	N/A	Cell rescue, defense and virulence
A29	Pc12g11220	gi 211582559	weak similarity to 2-hydroxyisoflavone reductase IRL - <i>Zea mays</i>	31	5.3	33	5.4	19	34	674	78	N/A	N/A	Cell rescue, defense and virulence
A30	Pc12g14390	gi 211582863	strong similarity to hypothetical glutathione S-transferase CAA19067.1 - <i>Schizosaccharomyces pombe</i>	27	5.6	26	6.4	10	53	330	56	N/A	N/A	Cell rescue, defense and virulence

Table 3.4 continued

A31	Pc18g00790	gj 211586658	strong similarity to glutathione S-transferase like protein An02g06560 - <i>Aspergillus niger</i>	29	6.6	25	7.2	8	27	202	32	N/A	N/A	Cell rescue, defense and virulence
A32	Pc18g00790	gj 211586658	strong similarity to glutathione S-transferase like protein An02g06560 - <i>Aspergillus niger</i>	29	6.6	24	7.3	17	25	579	54	N/A	N/A	Cell rescue, defense and virulence
A33	Pc18g00790	gj 211586658	strong similarity to glutathione S-transferase like protein An02g06560 - <i>Aspergillus niger</i>	29	6.6	25	7.7	17	21	557	56	10.79	9.45E-04	Cell rescue, defense and virulence
A34	Pc18g00790	gj 211586658	strong similarity to glutathione S-transferase like protein An02g06560 - <i>Aspergillus niger</i>	29	6.6	23	7.7	19	44	589	59	N/A	N/A	Cell rescue, defense and virulence
A35	Pc18g00790	gj 211586658	strong similarity to glutathione S-transferase like protein An02g06560 - <i>Aspergillus niger</i>	29	6.6	25	7.7	18	46	545	59	N/A	N/A	Cell rescue, defense and virulence
A36	Pc18g00790	gj 211586658	strong similarity to glutathione S-transferase like protein An02g06560 - <i>Aspergillus niger</i>	29	6.6	24	7.7	17	42	604	57	N/A	N/A	Cell rescue, defense and virulence
A37	Pc18g00790	gj 211586658	strong similarity to glutathione S-transferase like protein An02g06560 - <i>Aspergillus niger</i>	29	6.6	24	7.7	8	57	287	40	N/A	N/A	Cell rescue, defense and virulence
INFORMATION PATHWAYS														
A38	Pc22g09990	gj 211592025	strong similarity to carboxypeptidase S1 - <i>Penicillium janthinellum</i>	62	4.7	90	4.6	9	36	262	23	10.08	3.03E-02	Protein folding, modification and destination
A39	Pc21g23710	gj 211591049	strong similarity to gamma subunit of translation elongation factor eEF-1 - <i>Homo sapiens</i>	25	5.1	25	5.4	11	54	262	53	1.76	6.61E-03	Protein synthesis
DEVELOPMENTAL PROCESSES														
A40	Pc15g01030	gj 211584950	strong similarity to 1,3-beta-glucanase transferase gel1 - <i>Aspergillus fumigatus</i>	50	5.2	44	4.7	12	53	337	34	2.76	4.84E-05	Biogenesis of cellular components. Required for cell wall assembly and morphogenesis
A41	Pc22g09380	gj 211591967	Strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanase transferase gel3 - <i>Aspergillus fumigatus</i>	58	4.9	75	4.8	12	52	175	24	3.35	1.92E-03	Biogenesis of cellular components. Required for cell wall assembly and morphogenesis
UNKNOWN														
A42	Pc21g12310	gj 211589973	hypothetical protein [Penicillium chrysogenum]	30	5.6	29	6.3	16	30	419	72	7.24	1.35E-05	unknown
A43	Pc21g06950	gj 211589448	strong similarity to hypothetical protein YML079w - <i>Saccharomyces cerevisiae</i>	24	5.1	27	5.1	11	48	417	62	6.44	2.02E-04	unknown
A44	Pc22g13590	gj 211592302	strong similarity to hypothetical protein - <i>Gloeobacter violaceus</i>	70	5.9	73	7.2	16	4	491	35	3.82	9.19E-04	unknown
A45	Pc12g04310	gj 211581920	strong similarity to acetate-inducible gene acIA - <i>Aspergillus nidulans</i>	50	6.3	42	7.7	22	31	446	56	2.60	1.69E-03	unknown
A46	Pc13g10260	gj 211584068	strong similarity to hypothetical protein contig_1_84_scaffold_5.tfa_850cg - <i>Aspergillus nidulans</i>	38	5.0	36	5.0	6	43	100	20	N/A	N/A	unknown

Table 3.4 continued

A47	Pc22g01530	gi 211591214	strong similarity to hypothetical protein YAL049c - <i>Saccharomyces cerevisiae</i>	27	5.6	29	5.5	4	31	164	23	18.68	1.12E-03	unknown
A48	Pc22g24530	gi 211593358	similarity to hypothetical protein MDB19 - <i>Arabidopsis thaliana</i>	28	6.5	27	7.1	11	54	95	57	N/A	N/A	unknown
A49	Pc22g24530	gi 211593358	similarity to hypothetical protein MDB19 - <i>Arabidopsis thaliana</i>	28	6.5	27	6.5	9	53	124	43	N/A	N/A	unknown
A50	Pc12g10000	gi 211582444	similarity to hypothetical protein CC3654 - <i>Caulobacter crescentus</i>	19	5.0	19	4.9	12	38	392	87	7.82	9.27E-06	unknown
A51	Pc13g06880	gi 211583741	strong similarity to hypothetical protein contig49_tfa_1800cg - <i>Aspergillus fumigatus</i>	18	5.9	18	6.0	7	15	353	50	11.64	1.64E-05	unknown
A52	Pc21g06950	gi 211589448	strong similarity to hypothetical protein YML079w - <i>Saccharomyces cerevisiae</i>	24	5.1	16	5.2	5	45	171	28	N/A	N/A	unknown

Spots W1 (probable mitochondrial aconitate hydratase), W3 (probable alpha subunit E1 of the pyruvate dehydrogenase complex), W10 (probable pyruvate kinase), W14 (probable citrate synthase) and W17 (probable malate dehydrogenase precursor) are underrepresented in the AS-P-78 strain. These proteins belong to the glycolysis or tricarboxylic acids cycle. In addition, spot W4, which contains a probable sorbitol dehydrogenase catalyzing the oxidation of sorbitol to fructose, is also underrepresented in the AS-P-78 strain (6.26 times). This large decrease in protein W4 in the AS-P-78 strain may be compensated in part by three isoforms of a different probable sorbitol utilization protein Sou2 (spots A1, A8 and A17). Spot A45 is 2.6-fold overrepresented in the AS-P-78 strain. It contains a protein encoded by an acetate-inducible gene (*aciA*), which shows strong similarity to a NAD-dependent formate dehydrogenase AciA/Fdh from *Neosartorya fischeri* (90% homology, 84% identity). It catalyses the oxidation of formate to bicarbonate generating NADH (required for penicillin biosynthesis). Formate may serve as an auxiliary energy substrate for yeasts and fungi; it has been reported that mixed-substrate feeding (glucose/formate) can be used to increase the yield of β -lactams (Harris *et al.*, 2007); therefore, this enzyme might help to increase the penicillin levels in this high producer strain. Spot W21 contains a probable transaldolase only present in the Wis 54-1255 strain. This protein is involved in the non-oxidative steps of the pentose phosphate pathway, connecting it to glycolysis. The lack of this protein in the AS-P-78 strain supports an important implication of the pentose-phosphate pathway in penicillin production. An increased flux through the pentose pathway and a decreased glycolysis correlate with strain improvement.

3.1.5.2. Penicillin precursors

Spot A6 (1.85-fold overrepresented in the high-producer strain) includes a hypothetical cystathione beta synthase that is involved in the biosynthesis of cystathionine, a precursor of the cysteine in the ACV tripeptide (Kosalková *et al.*, 2001). In this strain, a probable branched-chain amino acid aminotransferase Bat2p (spot A3) is 4.07-fold overrepresented. This protein participates in the metabolism of valine, leucine and isoleucine and has been reported to be essential in *S. cerevisiae* for fusel alcohol production; this aminotransferase may provide additional valine for penicillin biosynthesis (Schoondermark-Stolk *et al.*, 2005). A probable alcohol

dehydrogenase AlkJ (Pc22g10020), which converts aliphatic medium-chain-length alcohols into aldehydes, is 4.53-fold underrepresented in AS-P-78 (spot W6).

Two proteins, a probable thiamine-phosphate pyrophosphorylase/hydroxyethylthiazole kinase (spot A7) and a protein with weak similarity to a hypothetical intracellular protease/amidase related enzyme of the ThiJ family CAC2826 (spots A18 and A19), are only detected in the AS-P-78 strain. These proteins are involved in the *de novo* synthesis of thiamine pyrophosphate (Nosaka *et al.*, 1994; Mizote *et al.*, 1999) that works as a cofactor of aminotransferases. Several enzymes involved in metabolism of unusual sulfur compounds and in valine catabolism have less representation in the AS-P-78 strain suggesting that they are unfavourable for penicillin biosynthesis. Spot W7 includes a probable dihydroxy-acid dehydratase Ilv3 (Pc 22g22710), which is involved in the biosynthesis of branched-chain amino acids (valine, leucine and isoleucine), is 3.54-fold overrepresented in the Wis 54-1255 strain.

In the AS-P-78 strain spot W42 is 8.71-fold underrepresented as compared to Wis 54-1255. It contains a protein with strong similarity to the phytanoyl-CoA dioxygenase family protein from *A. fumigatus* (84% homology, 74% identity). This is a peroxisomal enzyme catalysing the first step of phytanic acid alpha-oxidation involved in lipid and branched chain fatty acids degradation. Its decreased level in the AS-P-78 may save fatty acid precursors for penicillin biosynthesis.

The overrepresentation of spots A47, A48 and A49 in the AS-P-78 strain is also interesting. The A47 protein has strong similarity to the dienelactone hydrolase family protein from *A. fumigatus* (84% homology, 73% identity), which is involved in chlorocatechol degradation via the modified ortho cleavage pathway (Moiseeva *et al.*, 2002). Spots A48 and A49 include the same protein (Pc22g24530), which shares a high similarity to the dienelactone hydrolase family protein of *A. fumigatus* (84% homology, 75% identity). The high representation of putative dienelactone hydrolases proves the ability of the high-producer strain to degrade catechol and related compounds as an additional carbon source.

Surprisingly, the AS-P-78 strain does not show a significant overrepresentation of the proteins directly involved in the penicillin pathway when compared with the Wis 54-1255, despite the fact that AS-P-78 contains 5 or 6 copies of the amplified region that includes the penicillin coding genes (Fierro *et al.*, 1995; 2006). In order to test

whether the expression of the *pcbC* and *penDE* genes (coding IPN synthase and IAT, respectively) was different in the Wis 54-1255 and AS-P-78 strains, RT-PCR experiments were conducted using RNA extracted from cultures of these strains at 40 h and 60 h. As it can be observed in Figure 3.8, the AS-P-78 strain showed up to 1.7-fold and 1.3-fold increase in the steady-state levels of the *pcbC* and *penDE* transcripts, respectively. This light transcriptional increase was not translated in a proportional increase of either the IPN synthase or the IAT in the AS-P-78 strain (see Discussion).

Figure 3.8. Transcriptional analysis of the *pcbC* and *penDE* genes in the Wis 54-1255 and AS-P-78 strains. A. Electrophoresis of the RT-PCR products using RNA extracted at 40 h and 60 h from cultures of the Wis 54-1255 strain (W) and the AS-P-78 strain (A) grown under the same conditions. The transcript of the β -actin was used as control. The absence of contaminant DNA in the samples was tested by PCR (data not shown). **B.** The bands obtained after the electrophoresis were quantified by densitometry and their intensity was normalized to that provided by the β -actin band [Relative Integral Optical Density (IOD)]. The intensity values of the *pcbC* and *penDE* transcripts obtained from the Wis 54-1255 strain at 40 h, were set to 100%.

3.1.5.3. Other secondary metabolites

In the AS-P-78 strain, spot A4 (2.70-fold overrepresented) corresponds to a probable enoylreductase of the lovastatin pathway (Pc20g05830), which is involved in the biosynthesis of the lovastatin precursor dihydromonacolin N (see Discussion).

Spot W22 is present in the Wis 54-1255 but absent in the AS-P-78 strain. It is a protein with weak similarity to an isoflavone reductase, a key enzyme of the medicarpin biosynthetic pathway (Dixon, 2001). Probably, strain AS-P-78 has lost the ability to synthesize a flavonoid molecule of this type.

It is also interesting to note that only the AS-P-78 strain shows spot A28, which includes a probable aflatoxin B aldehyde reductase (Pc20g05270). This is the major enzyme providing protection against aflatoxin B1 (see Discussion).

3.1.5.4. Virulence and defense mechanisms

The Wis 54-1255 strain shows overrepresentation as compared to AS-P-78 of three isoforms (spots W24, W25 and W26) of a protein similar to a UDP-galactopyranose mutase from *A. fumigatus* (96% homology, 90% identity), which is in charge of galactofuranose biosynthesis. This compound plays a crucial role in cell surface formation and infectious cycle (Bakker *et al.*, 2005; Beverley *et al.*, 2005).

A probable glucose oxidase (spot W5), involved in oxidation of D-glucose into D-glucono-1,5-lactone (further hydrolyzed to gluconic acid), is also overrepresented in the Wis 54-1255 strain as compared to AS-P-78. Gluconic acid and glucose oxidase have been associated with the pathogenicity of *P. expansum* in apples (Hadas *et al.*, 2007).

Spot W27 is 4.64-fold overrepresented in the Wis 54-1255 strain and contains a 1,4-butanediol diacrylate esterase BDA1 (Pc15g00720) that might act as a β -lactamase (see Discussion). In general, these enzymes involved in fungal attack to host plants decrease during the penicillin strain improvement program.

3.1.5.5. Oxidative stress

The most striking finding in the AS-P-78 strain consists of the overexpression in several proteins involved in oxidative stress response. These proteins are: two probable glutathione S-transferase like proteins (eight isoforms of Pc18g00790 and one isoform

of Pc12g14390; spots A30 to A37, A20), two isoforms of a probable glutathione reductase (spots A25 and A26), two probable catalases R (spots A21 and A24), a probable quinone oxidoreductase (spot A23), a probable 1,4-benzoquinone reductase (spot A22), a protein with weak similarity to 2-hydroxyisoflavone reductase (spot A29) and a probable alcohol dehydrogenase AdhIII, which is an oxidoreductase induced by periods of anaerobic stress (Kelly *et al.*, 1990).

3.1.5.6. Protein targeting and processing

In the Wis 54-1255 strain two probable cyclophilins CypA (spot W28) and CypB (spot W29) are overrepresented, as well as a probable Hsp60 (spot W32) and chaperone BipA (spot W33). In contrast, the AS-P-78 strain contains a highly represented probable carboxypeptidase S1 (spot A38).

3.1.5.7. Cell wall biosynthesis and/or integrity

In the AS-P-78 strain, several proteins involved in cell wall biosynthesis and/or integrity are overrepresented (spots A40 and A41). In addition, spot A46 is only present in the high-producer strain. This spot contains a protein with strong similarity to a hypothetical protein (contig_1_84_scaffold_5.tfa_850cg) from *A. nidulans* that shares similarity to a dolichol-phosphate mannosyltransferase from *Aspergillus terreus* (72% homology, 57% identity), which is involved in N-glycan biosynthesis.

In the high-producer strain, spot A5 is 4.45-fold overrepresented. It includes a lysophospholipase (phospholipase B), which hydrolyzes both the acyl ester bonds of diacylphospholipids (diacyl-hydrolase) and the acyl ester bond of monoacylphospholipids or lysophospholipids. The estimated mass of this protein (111 kDa) is bigger than the theoretical mass (68.69 kDa), indicating post-translational modifications events. It has been reported that the purified phospholipase B from *P. notatum* is a glycoprotein with a molecular weight determined by gel filtration of about 116,000 (Kawasaki *et al.*, 1975). This enzyme showed a carbohydrate content of approximately 30%, explaining the difference between the expected and predicted masses.

3.1.6. Functional analysis of two genes with different expression in the strains obtained during the strain improvement program

Comparison of the intracellular proteome of the *P. chrysogenum* strains showed some proteins involved in pathways or functions that have been modified during the strain improvement program.

LovC (enoylreductase, Pc20g05830) is a 345 amino acids protein encoded by *lovC* gene with 1038 bp of length. This protein, which is similar to a protein involved in the biosynthesis of the lovastatin precursor dihydromonacolin N, is overrepresented in the Wis 54-1255 strain compared to the wild-type strain and also in the AS-P-78 strain compared to the Wis 54-1255 strain, i.e. is a clear example of a protein associated with industrial strain improvement.

Another interesting finding of intracellular proteome comparison of the *P. chrysogenum* strains is an esterase protein (Pc15g00720: strong similarity to 1,4-butanediol diacrylate esterase BDA1) that may be related to penicillin modification or degradation. In the present study this protein will be called “EST” hereafter. The reduction of this protein in the AS-P-78 as compared to the wild-type and Wis 54-1255 strains might be one of the mechanisms that contributed to increase the penicillin titers in this industrial strain.

EST has 399 amino acids and its corresponding gene has the length of 1200 bp and converts 1,4-butanediol diacrylate to 4-hydroxybutyl acrylate. In addition, it contains a domain conserved in class C β -lactamases and other penicillin binding proteins.

In this section we analyze the effects of gene silencing of *lovC* and *est* genes on penicillin production in *P. chrysogenum* Wis 54-1255.

3.1.6.1. Construction of silencing vectors and transformation

Plasmids pJL43RNAi-*lovC* or pJL43RNAi-*est* were designed to generate knock-down mutants based on the small interfering RNAs machinery, which triggered the gene attenuation. Primers LOV-*NcoI*-F and LOV-*NcoI*-R were used for PCR

isolation and amplification of the first 452 bp of the *lovC* gene from the *P. chrysogenum* Wis 54-1255 strain.

For the amplification of the first 431 bp of the *est* gene, primers EST-*NcoI*-F and EST-*NcoI*-R, were used. The *NcoI* restriction site was added to the primer sequence for further enzymatic digestions.

The amplified bands were digested using *NcoI* and ligated into the *NcoI*-digested pJL43RNAi silencing plasmid to give rise to the final constructions pJL43RNAi-*lovC* (6459 bp) and pJL43RNAi-*est* (6438 bp).

A total of 2×10^7 protoplasts of *P. chrysogenum* Wis 54-1255 were transformed with 10 µg of the pJL43RNAi-*lovC* or pJL43RNAi-*est* silencing vectors. Finally, 20 phleomycin resistant transformants were selected for each transformation and cultured in PMMY media for 48 h. The genomic DNA was extracted from all the transformants and checked by PCR using the primers FP-pcbC and RP-gpd2 designed from the vector sequence.

The positive transformants were then analyzed by Southern blotting and the confirmed transformants were studied in details by RT-PCR (for gene transcription analysis) and bioassay (for penicillin production analysis) as it is described below.

3.1.6.2. Functional analysis of *lovC* gene

■ Analysis of the *lovC* knock-down transformants

For the analysis of the *lovC* knock-down transformants, Southern blotting was performed with genomic DNA from *P. chrysogenum* transformants as well as the parental *P. chrysogenum* Wis 54-1255 strain (as the negative control). The hybridization probe was created using the 482-bp attenuation fragment of the *lovC* gene. The enzymes *SphI* and *StuI* were used for double-digestion of DNA.

As it is shown in Figure 3.9 the expected bands from this digestion are a 2118-bp attenuation fragment from the integrative cassette and a 16822-bp fragment with the endogenous *lovC* gene.

Figure 3.9. Silencing of the *lovC* gene in *P. chrysogenum* Wis 54-1255. Plasmid pJL43RNAi-*lovC* was transformed into the *P. chrysogenum* Wis 54-1255 strain. Genomic DNA was digested with *SphI* and *StuI*. **A and B.** Digestions release the 16822 bp fragment with the endogenous *lovC* gene (A), and a 2118 bp attenuation fragment from the integrative cassette (B). **C.** The parental strain (W) and three *lovC*-attenuated transformants (L1, L2 and L3) were examined by Southern blotting. The transformation in L1 and L2 is confirmed since they have both of expected bands. The λ -HindIII molecular weight marker (M) indicates 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bp bands.

■ Transcriptional analysis of the *lovC* gene

Silencing of the *lovC* gene expression was confirmed by the semi-quantitative RT-PCR analysis using the total RNA extracted from the *P. chrysogenum* Wis 54-1255 parental strain (control) and transformants L1 and L2 grown for 24 h, 48 h and 72 h in MCFP medium.

RNA was treated with RNase-free DNaseI and 3 μ g of total RNA was loaded on the agarose gel. The quality of RNA samples were also checked by loading 1 μ l of each sample on the wells of the Agilent RNA 6000 Nano chip and analysed by the bio-analyzer system. In addition, the absence of contaminant DNA in the samples was tested by PCR (data not shown).

Figure 3.10. Differential transcription of the *lovC* gene in *P. chrysogenum* Wis 54-1255 and *lovC* knock-down transformants. A. Relative transcription level in different strains, Transformant L1 shows lower levels of transcription at 24 h and 72 h (but not at 48 h) compared to the parental strain. The level of the *lovC* transcript is drastically decreased in the transformant L2. **B.** β -actin gene was used as a control of the amount of RNA in the RT-PCR reaction. The levels of the *lovC* and β -actin transcripts were determined by densitometry. The absence of contaminant DNA in the samples was tested by PCR.

From the total RNA samples, 200 ng were used for each RT-PCR reaction. Oligonucleotides β -actin-F and β -actin-R were used for the control RT-PCR reaction. The signals provided by the RT-PCR were quantified by densitometry using the “Gel-Pro Analyzer” software (Media Cybernetics). The transcription of *lovC* gene was analysed in transformants L1 and L2 comparing them to *P. chrysogenum* Wis 54-1255 (W). The bands obtained after the optimization of RT-PCR and electrophoresis were quantified by densitometry and their intensity was normalized to that provided by the β -actin band [Relative Integral Optical Density (IOD)]. The intensity values of the *lovC* transcripts obtained from the Wis 54-1255 strain at 24 h, 48 h and 72 h, were set to

100% and the intensity of the signal in transformant L1 and L2 were evaluated as the corresponding percentage regarding the parental strain Wis 54-1255 (W).

As it can be observed in Figure 3.10 the most intense PCR band belongs to the parental strain (W). Transformant L1 shows lower levels of *lovC* mRNA at 24 h and 72 h (but not at 48 h) comparing to the *lovC* mRNA levels in the control strain Wis 54-1255 (W). Interestingly, the level of the *lovC* transcript is drastically decreased (69% at 24 h, 55% at 48h and 84% at 72 h) in the transformant L2, which indicates the efficient silencing of the *lovC* gene in this transformant.

■ Analysis of penicillin production

Penicillin production was estimated by bioassay from three independent experiments performed in triplicates. The standard control samples with different concentrations of penicillin G were used to create a control line according to the inhibition halo. Penicillin concentration in the samples was inferred according to the next formula: $Y=13.181X + 35.902$; $R^2=0.9849$.

For easy visualization of the results the specific penicillin production (per milligram of dry weight) of Wis 54-1255 at 24 h was set to 100 and the production in all the other cases was considered as the “Relative Specific Production”. Figure 3.11 shows the relative specific penicillin production in the Wis 54-1255 strain and the *lovC*-attenuated transformants L1 and L2 at 24 h, 48 h and 72 h.

The penicillin production was increased in all of the strains during the time course. However, for every specific time point the production of transformants L1 and L2 was less than in the parental strain (W), especially for the transformant L2, which showed a dramatic decrease in the penicillin titers (61% at 24 h, 91% at 48 h and 93% at 72 h).

These results are in concordance with the previous findings about the *lovC* expression pattern in transformants L1, L2 and in the parental Wis 54-1255 strain.

Figure 3.11. Relative specific penicillin production of the Wis 54-1255 strain and the *lovC* knock-down transformants L1 and L2 grown in MCFP production medium for 24 h, 48 h and 72 h. The specific penicillin production (per milligram of dry weight) of Wis 54-1255 (W) at 24 h (1.017 $\mu\text{g}/\text{mg}$) was set to 100 and the production in all the other cases was considered as the “Relative Specific Production”. Error bars indicate the standard deviation of three analyses made from three independent cultures.

■ Effects of *lovC* gene silencing on pigmentation

Attenuation of *lovC* gene also affects the phenotypic characteristics of *P. chrysogenum* Wis 54-1255. As shown in figure 3.12 the efficient silencing of the *lovC* gene resulted in a decrease in the pigmentation (typical green color) of the conidia, especially in transformant L2.

These results are also in concordance with the previous findings about the *lovC* expression pattern in transformants L1, L2 and in the parental Wis 54-1255 strain, and indicate that this gene may be involved in the control of different secondary metabolism pathways (see Discussion).

Figure 3.12. Phenotype of the Wis 54-1255 strain and the *lovC* knock-down transformants L1 and L2. After 5 days of cultivation on solid Power the transformant L1 shows lighter typical green color during the sporulation. The more efficient attenuation of *lovC* gene in transformant L2 has resulted in the absence of the typical green color of the conidia.

3.1.6.3. Functional analysis of *est* gene

■ Analysis of the *est* knock-down transformants

For the analysis of the *est* knock-down transformants, Southern blotting was performed with genomic DNA from *P. chrysogenum* transformants as well as the parental *P. chrysogenum* Wis 54-1255 strain (as the negative control). The hybridization probe was created using the 461-bp already utilized as silencing fragment of the *est* gene. The enzymes *Sph*I and *Hind*III were used for double-digestion of DNA.

As it is shown in Figure 3.13 the expected bands from this digestion are a 1813-bp silencing fragment from the integrative cassette and a 11172-bp fragment with the endogenous *lovC* gene.

Figure 3.13. Silencing of the *est* gene in *P. chrysogenum* Wis 54-1255. Plasmid pJL43RNAi-*est* was transformed into the *P. chrysogenum* Wis 54-1255 strain. Genomic DNA was digested with *Sph*I and *Hind*III. **A and B.** Digestions release the 11172-bp fragment with the endogenous *est* gene (A), and a 1813-bp attenuation fragment from the integrative cassette (B). **C.** The parental strain (W) and three *est*-attenuated transformants (E1 and E2) were examined by Southern blotting. The transformation is confirmed since they have both of expected bands. The λ -*Hind*III molecular weight marker (M) indicates 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bp bands.

■ Transcriptional analysis of the *est* gene

Silencing of the *est* gene expression was confirmed by the semi-quantitative RT-PCR analysis using the total RNA extracted from the *P. chrysogenum* Wis 54-1255 parental strain (control) and transformants E1 and E2 grown for 24 h, 48 h and 72 h in MCFP medium. RNA was treated with RNase-free DNaseI and 3 μ g of total RNA was checked on the agarose gel. The quality of RNA samples were also checked by loading 1 μ l of each sample on the wells of the Agilent RNA 6000 Nano chip and analysed by the bio-analyzer system. In addition, the absence of contaminant DNA in the samples was tested by PCR (data not shown). From the total RNA, 200 ng was used for each RT-PCR reaction. The intensity of bands was normalized to that provided by the β -actin band [Relative Integral Optical Density (IOD)] as described before (3.1.6.2).

The intensity values of the *est* transcripts obtained from the Wis 54-1255 strain at 24 h, 48 h and 72 h, were set to 100% and the intensity of the signal in transformant E1 and E2 were evaluated as the corresponding percentage regarding the parental strain Wis 54-1255 (W).

Figure 3.14 shows the results of transcriptional analysis of the *est* gene in transformant E1, E2 and in the parental control strain Wis 54-1255 (W). Compared to the control strain (W) *est* mRNA levels drastically decreased in transformant E1 at all time points (63% at 24 h, 71% at 48 h and 76% at 72 h). The transformant E2, however, showed a much less efficient silencing effect.

Figure 3.14. Differential transcription of the *est* gene in *P. chrysogenum* Wis 54-1255 and *est* knock-down transformants. A. The *est* mRNA levels drastically decreased in transformant E1 at all the time points, but the attenuation in transformant E2 is less efficient than in transformant E1. **B.** The bands obtained after the electrophoresis were quantified by densitometry and their intensity was normalized to that provided by the β -actin band [Relative Integral Optical Density (IOD)]. The transcript of the β -actin was used as control. The absence of contaminant DNA in the samples was tested by PCR.

■ Analysis of penicillin production

Penicillin production was estimated from three independent experiments performed in triplicates by bioassays. The standard control samples with different

concentrations of penicillin G were used to create a control line according to the inhibition halo. Penicillin concentration in the samples was inferred according to the next formula: $Y=13.181X + 35.902$; $R^2=0.9849$.

For easy visualization of the results the specific penicillin production (per milligram of dry weight) of Wis 54-1255 at 24 h was set to 100 and the production in all the other cases was considered as the “Relative Specific Production”. Figure 3.15 shows the relative specific penicillin production in the Wis 54-1255 strain and the *est*-attenuated transformants E1 and E2 at 24 h, 48 h and 72 h.

The penicillin production by the *est*-attenuated transformant E1 highly increased at all time points (120% at 24 h, 66% at 48h and 79% at 72 h) regarding the parental strain (W), whereas the transformant E2 showed a very slight increase in penicillin production at 24 h and 48 h but a significant decrease (21%) at 72 h, i.e. transformant E2 dose not seem to have a significant alteration of penicillin biosynthesis. These findings are in concordant with the results of *est* transcriptional study and demonstrate an inverse relationship between the EST protein and the penicillin production.

Figure 3.15. Relative specific penicillin production of the Wis 54-1255 strain and the *est* knock-down transformants E1 and E2 grown in MCFP production medium for 24 h, 48 h and 72 h.

The specific penicillin production (per milligram of dry weight) of Wis 54-1255 (W) at 24 h (1.024 µg/mg) was set to 100 and the production in all the other cases was considered as the “Relative Specific Production”. Error bars indicate the standard deviation of three analyses made from three independent cultures.

3.1.7. Discussion

One of the major efforts to modify microorganisms of biotechnological interest has been provided by the antibiotics industry. *P. chrysogenum* has been extensively mutated during the last decades to increase the penicillin titers, but despite the importance of this microorganism, the biochemical bases that have been modified during the strain improvement process remain poorly understood.

In this study, we have performed for the first time an extensive 2-DE proteome reference map of the penicillin producer *P. chrysogenum* with a high resolution and reproducibility, together with a detailed study by PMF and tandem mass spectrometry analyses of the protein changes produced during the industrial strain improvement program. We have been able to identify a large number of proteins (950 representing 549 different proteins), which have been classified according to their function with a similar distribution to that reported for the ascomycete *S. pombe* (Hwang *et al.*, 2006). Since *P. chrysogenum* is important for the production of secondary metabolites, especially penicillins, we focused on the proteins related to the biosynthesis of these compounds. As indicated in the Results section, we identified the last two proteins involved in the penicillin pathway (IPN synthase and IAT), but not the ACV synthetase due to its big molecular weight (425 kDa). Only the β -subunit of the IAT was detected, suggesting that the α subunit is further processed or degraded. The IAT is a peroxisomal enzyme synthesized as an inactive 40-kDa preprotein that undergoes self-processing between residues Gly102-Cys103, giving rise to an active heterodimer with subunits α (11 kDa, corresponding to the N-terminal fragment) and β (29 kDa, corresponding to the C-terminal region) (Whiteman *et al.*, 1990; Tobin *et al.*, 1993; García-Estrada *et al.*, 2008b). Although the 11-kDa α -subunit has not been identified in the reference map, Kiel and coworkers (2009) found both subunits (the α subunit in small amounts) during the analysis of *P. chrysogenum* peroxisomal proteins by nano-LC-MS/MS. It is likely that the 11-kDa α subunit is further processed hampering its identification by PMF. The optimization of the 2-DE analysis for *P. chrysogenum* allowed us to perform a detailed comparative analysis of the wild-type strain NRRL 1951, the low-producer Wis 54-1255 and the high-producer AS-P-78. In the wild-type strain, those proteins involved in virulence and plant cell wall degradation for the obtention of nutrients are predominant (the wild-type strain isolated from a cantaloupe

is able to infect and grow on fruits) (Raper *et al.*, 1944). One of the reasons for the very low amount of penicillin produced by the wild-type strain might be the use of precursors and energy for the biosynthesis of other secondary metabolites, such as terpenoids (3-hydroxy-3-methylglutaryl-CoA synthase), porphyrins (coproporphyrinogen oxidase) or pigments like violaxanthin (zeaxanthin epoxidase) and melanin (scytalone dehydratase), which could divert the metabolic fluxes away from the penicillin biosynthetic pathway. It is known that during the mutagenic treatments (from the Q-176 strain to the BL3-D10 strain) strains without the chrysogenin yellow pigment were selected (García-Rico *et al.*, 2008).

Proteins involved in biosynthesis or degradation of precursor amino acids are distinctly represented in the three strains. In the wild-type NRRL 1951 strain, the increased levels of the methylmalonate semialdehyde dehydrogenase might be one reason for the low penicillin biosynthesis in this strain. This enzyme is involved in the 'distal pathway' of valine catabolism (Goodwin *et al.*, 1989; Zhang *et al.*, 1996), leading to valine pool depletion. Cysteine biosynthesis seems to be one of the major improvements occurred during the selection process. Penicillin production flux is greatly influenced by cysteine availability for ACV formation (Nasution *et al.*, 2008). Cysteine synthase (forming cysteine from serine) is overrepresented in the Wis 54-1255 compared to the wild-type strain. This improvement is conserved in the AS-P-78 strain, which in addition, showed increased content of the cystathionine beta synthase, an enzyme involved in the biosynthesis of the cysteine from methionine by transsulfuration. This indicates that the AS-P-78 strain has improved the two cysteine biosynthetic pathways. In the Wis 54-1255 improved producer strain those proteins in charge of energy obtention from carbohydrates are predominant. These proteins mainly belong to the tricarboxylic acid cycle, although there are also enzymes from glycolysis and oxidative phosphorylation pathways. It is likely that during the strain improvement process, those mechanisms for energy obtention were of benefit to meet the increasing demand for penicillin biosynthesis.

An interesting finding is that the Wis 54-1255 strain overproduces, as compared to the AS-P-78 strain, one protein that may be related to penicillin modification or degradation. This enzyme is similar to a 1,4-butanediol diacrylate esterase BDA1 (converting 1,4-butanediol diacrylate to 4-hydroxybutyl acrylate), which contains a

domain conserved in class C β -lactamase and other penicillin binding proteins. It shows similarity to a putative transesterase (LovD), from *Aspergillus clavatus* (85% homology, 72% identity) and to a β -lactamase from *Paracoccidioides brasiliensis* (48% homology, 32% identity). Transesterase LovD adds 2-methylbutyrate to monacolin J to form lovastatin. Therefore, this LovD-like protein may bind the nucleus of penicillin contributing to its modification or degradation. The reduction of this protein in the AS-P-78 as compared to the wild-type and Wis 54-1255 strains might be one of the mechanisms that contributed to increase the penicillin titers in this industrial strain. Our findings from the functional analysis of this gene demonstrate an inverse correlation between the *est* gene expression level and the penicillin production, which suggests that the EST protein may act as a β -lactamase.

It is known that during the generation of the penicillin high-producer strain AS-P-78, amplification of the penicillin biosynthetic gene cluster (*pcbAB-pcbC-penDE*) occurred (Fierro *et al.*, 1995). However, this amplification has not led to correlative protein synthesis even though there was a light increase in transcription. This is in agreement with previous results obtained for the *pcbC* gene (Kosalková *et al.*, 2007). In fact, it has been reported that there is no linear relationship between the increasing number of penicillin gene clusters and the penicillin titer (Newbert *et al.*, 1997). These results indicate that, although gene amplification has an impact on penicillin production, it is not the main mechanism that transformed the Wis 54-1255 strain in a penicillin over-producer.

Another modification that might have provided the basis for the increased penicillin titers of the AS-P-78 strain is the overproduction of enzymes of the pentose phosphate pathway. Enzymes from the non-oxidative phase of this pathway, like ribose-5-phosphate isomerase or transketolase, are predominant in the high-producer strain. In addition, some enzymes involved in *de novo* synthesis of thiamine pyrophosphate, were overrepresented. Thiamine pyrophosphate is a prosthetic group present in many enzymes. One of these enzymes is the transketolase (Shreve *et al.*, 1983), which connects the pentose phosphate pathway to glycolysis, feeding excess sugar phosphates into the main carbohydrate metabolic pathways. It is likely that high concentration of ribulose-5-phosphate (together with reducing power in the form of NADPH) is formed in the high-producer strain through the oxidative phase of the

pentose phosphate pathway; therefore, the excess of this sugar phosphate would be converted by the ribose-5-phosphate isomerase in ribulose-5-phosphate (precursor of nucleotides and nucleic acids) and by the transketolase in precursors for glycolysis. An increase in the NADPH levels has been strongly correlated to β -lactam production (Jørgensen *et al.*, 1995; Henriksen *et al.*, 1996; van Gulik *et al.*, 2000). [(biosynthesis of 1 mol of penicillin requires 8–10 mol of NADPH)] It is accepted that penicillin production in the high-producer strains constitutes a major burden on the supply of NADPH (Kleijn *et al.*, 2007). In addition, it has been reported that penicillin production is greatly influenced by the ATP level and the cysteine concentration. Since NADPH is required for the biosynthesis of this amino acid, there is a positive relationship between cysteine and NADPH levels and penicillin production (Nasution *et al.*, 2008). NADPH is also required to reduce the oxidized glutathione during oxidative stress that results from intense aeration rates (Díez *et al.*, 1998). Our results have established that several proteins involved in the response to oxidative stress are overrepresented in the AS-P-78 strain. Obviously during the selection procedure in shake flasks with strong agitation those mutants with increased resistant to oxidative stress were selected.

Some differences in protein expression observed in the AS-P-78 strain show good correlation with the transcriptional responses described in a *P. chrysogenum* strain engineered to produce adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (ad7-ACCCA); this may be related to the fact that the host *P. chrysogenum* strain used in that study is a penicillin overproducer strain. These responses involved transcriptional upregulation of a putative aflatoxin B1 aldehyde reductase, two glutathione S-transferases, a cytochrome p450 monooxygenase and a NADPH quinone oxidoreductase (Harris *et al.*, 2009). These results point to oxidative stress responses as adaptation mechanisms to penicillin overproduction.

A global view of the proteins differentially expressed in these three strains revealed that the representativeness of some pathways and functions have been magnified during the improvement process whereas others have decreased (Figure 3.16). Glucose oxidase is a protein with low content in the AS-P-78 strain as compared to the wild-type. The underrepresentation of this enzyme associated with plant pathogenicity is not surprising, since submerged liquid cultures have replaced the natural growth on fruits.

Figure 3.16. Schematic representation of some pathways and networks modified during the strain improvement program, according to the results obtained after the proteome comparison of NRRL 1951, Wis 54-1255 and AS-P-78 strains. Font-size is in concordance with the protein amount observed by CC staining strength in the comparative proteome analyses. **αAA**: α-amino adipic acid; **BDA1**: 1,4-butanediol diacrylate esterase (putative β-lactamase); **CBS**: cystathione beta synthase; **CS**: cysteine synthase; **Cys**: cysteine; **FDH**: Formate dehydrogenase; **GR**: Glutathione Reductase; **GT**: Glutathione S-transferase; **M**: Mitochondrion; **MSD**: methylmalonate semialdehyde dehydrogenase; **N**: Nucleus; **NADH**: Reduced form of Nicotinamide adenine dinucleotide; **NADPH**: Reduced form of nicotinamide adenine dinucleotide phosphate; **P**: Peroxisome; **PenG**: Benzylpenicillin; **PPP**: Pentose Phosphate Pathway; **QO**: quinone oxidoreductase; **TCAC**: Tricarboxylic Acid cycle; **TPP**: thiamine pyrophosphate; **Val**: Valine.

Probably the most striking loss of some pathways occurred during the selection of these strains is the reduction of many secondary metabolism routes to potentiate the biosynthesis of β -lactams. Yellow pigments constitute one of the best examples of secondary metabolites lost during the manipulation of the wild-type strain to obtain the Wis 54-1255 strain. During the selection of the high-producer strain AS-P-78, other pathways for secondary metabolism seemed to be blocked, as it is suggested by the absence in AS-P-78 of a putative isoflavone reductase (involved in the latter part of the medicarpin biosynthetic pathway).

On the other hand, there are proteins involved in pathways or functions that have increased during the strain improvement program. It is intriguing that one of the proteins (enoylreductase LovC, Pc20g05830) similar to a protein involved in the biosynthesis of the lovastatin precursor dihydromonacolin N, is overrepresented in the Wis 54-1255 strain compared to the wild-type strain and also in the AS-P-78 strain compared to the Wis 54-1255 strain. Upregulation of the gene encoding the enoylreductase, has also been reported as one of the transcriptional responses in the ad7-ACCCA production in *P. chrysogenum* (Harris *et al.*, 2009). Although other lovastatin-related genes occur in the genome of *P. chrysogenum* they are not clustered together, making it unlikely to serve for lovastatin biosynthesis. The results of functional analysis of the *P. chrysogenum lovC* gene reflect a direct relation between the *lovC* transcription level and the penicillin production.

P. chrysogenum LovC has an alcohol dehydrogenase GroEs-like domain (from residues 25 to 89) and a Zinc-binding dehydrogenase domain (residues 161 to 242). The amino acid sequence of the *P. chrysogenum* LovC shares 84% similarity (74% identity) to a putative zinc-binding oxidoreductase ToxD from *A. clavatus* NRRL 1 and 54% similarity (38% identity) to the LovC enoylreductase of *A. terreus*. In the *Aspergillus* sp. LovC is involved in the biosynthesis of the polyketide lovastatin. The lovastatin biosynthesis cluster of *Aspergillus* sp. contains two type I polyketide synthase genes. Synthesis of the main nonaketide-derived skeleton was found to require the previously known iterative lovastatin nonaketide synthase (LNKS), plus at least one additional protein (LovC) that interacts with LNKS and is necessary for the correct processing of the growing polyketide chain and production of dihydromonacolin L. The noniterative lovastatin diketide synthase (LDKS) enzyme specifies formation of

2-methylbutyrate and interacts closely with an additional transesterase (LovD) responsible for assembling lovastatin from this polyketide and monacolin J (Kennedy *et al.*, 1999). A similar mechanism of action has been postulated for ToxD, which is involved in cyclic peptide biosynthesis in the plant pathogenic fungus *Cochliobolus carbonum* that produce the host-selective compound HC-toxin (Pedley *et al.*, 2001). Taking into account the similarities between ToxD, LovC and the *P. chrysogenum* LovC and due to the positive correlationship between this protein and penicillin production, a role of LovC in the biosynthesis and/or processing of the ACV tripeptide is likely. In addition, the fact that *lovC* knock-down mutants also show a decrease in the green color of conidia indicates that the LovC protein may be involved in different secondary metabolism pathways. The nature and function of the green pigment is unknown, but it may be a polyketide whose synthesis and processing is also modified by LovC. The polyketide nature of this green pigment is supported by the fact that defective mutants in either the phosphopantetheinyl transferase (necessary for the activation of polyketide synthases and non-ribosomal peptide synthetases), or the secondary metabolism global regulator LaeA, have lost this green pigment (García-Estrada *et al.*, 2008a; Kosalková *et al.*, 2009).

3.2 THE EXTRACELLULAR PROTEOME

In order to fully characterize *P. chrysogenum* and how the modifications acquired during the industrial strain improvement programs affected the wild-type plant pathogenicity, analysis of the secreted proteins present in the culture broths was carried out. Using 2-DE gels coupled to peptide mass fingerprint (PMF) and tandem MS we describe here the extracellular proteome of *P. chrysogenum* and the differences found in secreted protein among the wild type and two improved strains of this microorganism which represent different stages of the industrial improvement programs.

3.2.1. Growth phases

The presence of extracellular proteins in the culture medium is correlated to the growth phase (Peberdy, 1994). Therefore, first of all we determined the optimal conditions for sample preparations. The growth of *P. chrysogenum* in submerged cultures was analyzed at different time points. Mycelia were collected at 24 h, 40 h, 68 h, 74 h and 86 h to quantify the dry weight.

Figure 3.17. Relationship between growth phase and extracellular protein concentration. **A.** Dry weight obtained from samples taken from *P. chrysogenum* Wis 54-1255 liquid cultures at different time points. Data correspond to three biological replicates performed in duplicate. Results for the NRRL 1951 and AS-P-78 strains were similar to those obtained for the Wis 54-1255 strain (data not shown). **B.** Extracellular protein concentration and plotted versus the culture time. Data correspond to three biological replicates performed in duplicate.

As shown in Figure 3.17.A, *P. chrysogenum* hyphal growth reached the end of the linear phase at 68 h, where it entered the stationary phase until 74 h. A small decrease in the dry weight was observed from this time point, indicating that hyphal lysis and fragmentation coupled with a progressive loss of biomass was taking place. Hence, in order to avoid contamination with intracellular proteins resulting from the cell lysis, we determined the extracellular protein concentration at 24 h, 40 h and 68 h by the Bradford method. The amount of proteins present in the culture medium (Figure 3.17.B) was very low at 24 h (0.86 µg/ml), reaching the concentration of 8.5 µg/ml at 40 h. This concentration rapidly increased from this time point until 68 h reaching 31.7 µg/ml. Protein concentration rised till the end of the culture reaching a maximum concentration of 43.8 µg/ml at 86 h. However, since dry weight remains constant after 68 h and falls down after 72 h, the increase in the protein concentration after 68 h likely corresponds to cell lysis events. According to these results, we selected 68 h for the characterization of the *P. chrysogenum* extracellular proteome reference map.

3.2.2. The secretome reference map analysis

The extracellular proteome (secretome) reference map was made using 68 hour cultures of *P. chrysogenum* Wis 54-1255, which was the reference strain for the genome sequencing project (van den Berg *et al.*, 2008) and the cytoplasmatic proteome reference map (Jami *et al.*, 2010). Initially, wide range IPG strips (pH 3-10, NL) were used, but the majority of spots appeared distributed in the acidic side of the gel (Figure 3.18). Therefore, to get a better resolution of the spots, IPG strips with a pH range from 4 to 7 were used. Visualization of 317 spots by “Blue Silver” staining was obtained under this condition and allowed the correct identification by PMF and tandem MS of the proteins present in 279 spots (Figure 3.19 and Supplementary Table 3 in the appendix section). The 279 spots represented 328 correctly identified proteins (with an average error of 7 ppm), since some spots contained more than one protein. The extracellular proteome reference map with a direct link to the identified proteins can be accessed through the URL: http://isa.uniovi.es/P_chrysogenum_secretome/.

Figure 3.18. Proteins separation by 2-DE using 18-cm wide-range IPG strips (pH 3-10 NL). The majority of spots appeared overlapped on each other in the acidic side of the gel.

All the significant MASCOT searches provided an exact match with proteins inferred from the *P. chrysogenum* Wis 54-1255 genome (except spots 185 and 257, see below), which indicates high accuracy of the genome annotation process. From the 328 proteins identified in the reference map, 62 proteins showed a total of 259 isoforms and 69 proteins had no isoforms (Supplementary Table 4 in the appendix section).

Figure 3.19. Reference map of the *P. chrysogenum* extracellular proteome. A total of 317 spots were visualized in a 4-7 pH range and 279 spots (numbered in the Figure) were identified by PMF and tandem mass spectrometry (see Supplementary Table 3 in the appendix section). Molecular weights are shown on the left. Assigned numbers over each spot correlate with those shown in Supplementary Table 3.

Some examples of the proteins showing a high number of isoforms are a probable catalase R (Pc16g11860, 18 isoforms), two probable 6-hydroxy-D-nicotine oxidases 6-HDNO (Pc21g12590, 12 isoforms; Pc18g05530, 8 isoforms), a probable pectate lyase PlyA (Pc22g24890, 11 isoforms), a probable acid phosphatase AphA (Pc22g09580, 11 isoforms) and a probable cell wall protein Crh1 (Pc21g23210, 11 isoforms).

The 10 spots showing more relative volume represented 31.31% of the total spot volume and are spots 279 (5.22%) and 249 (3.19%), both including a hypothetical cell wall protein BinB (Pc22g00190); spot 76 (3.54%), which includes an extracellular acid phosphatase PhoA (Pc12g14860); spot 19 (3.15%), which contains a probable catalase R (Pc16g11860); spot 129 (2.92%), which includes a probable acid phosphatase AphA (Pc22g09580) and a probable 6-hydroxy-D-nicotine oxidase 6-HDNO (Pc18g05530); spot 155 (2.88%), which includes a probable polygalacturonase PgaI (Pc22g20290); spot 189 (2.86%), which contains a probable pectate lyase PlyA (Pc22g24890); spots 121 (2.58%) and 123 (2.49%), both including a sulphhydryl oxidase Sox (Pc22g22810) (patent EP565172-A1) and spot 242 (2.49%), which includes a hypothetical necrosis and ethylene inducing protein BH0395 (Pc12g13600) and one IgE-binding protein (Pc22g22290).

The final number of different proteins that were found in the extracellular proteome of *P. chrysogenum* was 131 (62 different proteins having isoforms + 69 individual proteins without isoforms).

Seventy three proteins out of 131 were predicted by the SignalP software (Bendtsen *et al.*, 2004a) to have classical signal peptide sequences (Supplementary Table 4 in the appendix section).

Fifty-eight of the different proteins identified lacked a predicted N-terminal signal peptide sequence, indicating that these proteins might be secreted through a non-classical secretory mechanism, like that reported for yeasts (Nombela *et al.*, 2006).

In order to assess whether the 58 proteins lacking the classical N-terminal signal peptide sequence include non-classical secretion signals, the SecretomeP prediction software (Bendtsen *et al.*, 2004b) was used. This tool predicted 29 proteins out of 58 having a non-classical secretory targeting signal (Supplementary Table 4 in the appendix section), pointing to the existence of non-classical secretory pathways in *P. chrysogenum* (see Discussion).

3.2.3. Functional classification of extracellular proteins

The 328 proteins identified (131 different proteins and isoforms) were functionally classified according to their biological roles. As shown in Figure 3.20 and Supplementary Table 3 (see appendix section), 84 proteins were implicated in general

metabolism (25.61%); 70 proteins were involved in utilization of nutrients (21.34%); and 7 additional proteins had protease activity that are relevant for nutrient utilization (2.13%); 51 proteins were in charge of cell wall morphogenesis (15.55%); 43 proteins were involved in cell rescue, defense and virulence (13.11%); 38 proteins were in charge of plant cell wall degradation (11.59%); 21 proteins had an unknown function (6.40%); 13 proteins were involved in the control of cellular functions (3.96%); and one protein belonged to the cytoskeleton (0.31%). The main findings for each functional category are summarized below.

Figure 3.20. Functional classification of the proteins identified in the extracellular reference map. The 328 correctly identified proteins (131 different proteins and isoforms) were classified according to their biological function.

3.2.3.1. General metabolism

Some proteins identified in the culture medium of *P. chrysogenum* are involved in metabolic reactions of special interest for food industry (see Discussion). Examples are provided by a probable isoamyl alcohol oxidase (Pc22g16510, spots 49, 58 and 59) and a sulphhydryl oxidase (Pc22g22810, spots 97, 120, 121 and 123), which looks specially important due to the high representativity of its isoforms in the *P. chrysogenum* secretome (5.48%). Enzymes related to the metabolism of branched-chain amino acids

are represented by a probable dihydroxy-acid dehydratase Ilv3 (Pc22g22710, spots 64 and 65), a probable ketol-acid reductoisomerase Ilv2 (Pc13g15810, spots 173 and 174) and a probable branched-chain-amino-acid aminotransferase Bat2p (Pc22g16780, spots 176 and 178). Branched-chain amino acids and their by-products are very important for the flavour of alcoholic beverages (see Discussion).

The finding of a probable glyceraldehyde-3-phosphate dehydrogenase (Pc21g14560, spot 202), which is predicted to be a non-classical secreted protein (Supplementary Table 4 in the appendix section), in the extracellular proteome is highly interesting, since it appears to be located on several subcellular locations in eukaryotes (see Discussion). Other examples of enzymes predicted to be secreted that might play a dual role according to their location are a fumarylacetoacetase (Pc12g09030, spot 147), an orotidine 5-phosphate decarboxylase (Pc13g04420, spot 227), a pyruvate dehydrogenase beta chain precursor (Pc14g00690, spot 200), an aspartate transaminase like protein An08g01000 (Pc16g02790, spot 167), or an adenosylhomocysteinase (Pc16g05080, spot 143), a precursor of dihydrolipoamide dehydrogenase (Pc20g04720, spots 109, 112, 114 and 115), among others (see Supplementary Table 4 in the appendix section).

3.2.3.2. Utilization of nutrients

Several proteins identified by mass spectrometry were grouped according to their ability to utilize phosphate, carbon or nitrogen sources from the culture medium. Phosphatases (specially acid phosphatases) were highly represented in the extracellular proteome both in a number of isoforms and relative volumes, such as the extracellular acid phosphatase PhoA (Pc12g14860; spots 75, 76, 79, 86 and 133) or other probable acid phosphatases like aphA (Pc22g09580; spots 60, 127, 129, 138, 141, 142, 145, 183, 251, 253 and 254) or AFPhoA (Pc18g02580; spots 6, 10, 11, 34, 38, 43 and 277). The latter (AFPhoA) from *Aspergillus ficuum* has 64% homology to a phosphate-repressible acid phosphatase from *P. chrysogenum* and has been reported to be secreted (Ehrlich *et al.*, 1994). Probable alkaline phosphatases were also identified in spots 2-4 (Pc12g15470; 85% homology and 73% identity to an alkaline phosphatase from *A. clavatus*) and 5, 7, 8 and 9 (Pc22g06490). In addition, spot 74 contained a protein (Pc22g12670) that shared 72% homology (60% identity) with a phytase from

Ajellomyces dermatitidis. This protein can break down undigestible phytates, which are the principal storage form of phosphorus in cereals and leguminous plants (Reddy *et al.*, 1982), thus releasing digestible phosphorus, calcium and other nutrients.

Enzymes for the utilization of several carbon sources were also identified. Spots 119 and 136 include the glucan 1,4- α -glucosidase or glucoamylase (Pc22g01850), which catalyses the release of glucose from the non-reducing ends of starch and related polysaccharides. This activity is of industrial importance in the production of sugar from starch (Kumar and Satyanarayana, 2009). Spots 164-166 include a protein (Pc12g04310, encoded by a probable acetate-inducible gene *aciA*) with strong similarity to a NAD-dependent formate dehydrogenase AciA/Fdh from *Neosartorya fischeri* (90% homology, 84% identity). This enzyme catalyses the oxidation of formate, which serves as an auxiliary energy substrate for yeasts and fungi (Harris *et al.*, 2007). Protein Pc16g04220 (probable trehalose metabolism factor Pmu1) was identified in spots 84 and 274. This protein is required in addition to the MAL4 gene-encoded protein, for both constitutive maltase synthesis and maltose utilization in *S. cerevisiae* (Khan, 1982). Probable sorbitol (a sugar alcohol found in stone fruits) utilization proteins Sou2 (Pc12g00830; spots 230, 233, 234 and 238) and sorbitol dehydrogenases (Pc16g08460; spots 173 and 175), also known as glucitol-, polyol-, or L-iditol-dehydrogenases, were present in the extracellular fraction. Although these enzymes were not predicted to be secreted by the classical system (Supplemental Table 4), extracellular sorbitol dehydrogenase activity (oxidation of sorbitol to fructose) has been detected extracellularly in fungal species of the genus *Fusarium* (Bilař *et al.*, 1986), thus supporting our results.

Another interesting finding is the presence of several isoforms of two probable 6-hydroxy-D-nicotine oxidases (Pc18g05530 in spots 107, 127, 129, 138, 141, 142, 145 and 197; Pc21g12590 in spots 89, 95, 96, 100, 111, 113, 118, 148, 266-269). All these isoforms are also highly represented and implies that nicotine degradation is an important source of carbon and nitrogen in this fungus. This is well known for some soil bacteria, which use nicotine as a suitable carbon and nitrogen source and degrade it through a single pathway involving this enzyme (Hochstein and Rittenberg, 1959). A probable fructosyl amine oxygen oxidoreductase (Pc21g09780) is present in spot 102. This enzyme is an amadoriase enzyme that catalyzes the oxidative deglycation of

Amadori products (glycated low molecular weight amino acids) to yield amino acids, glucosone and H₂O₂. The role of this protein is not clear, but might contribute to increase the pool of free extracellular amino acids as nitrogen source.

3.2.3.3. Cell wall morphogenesis

Enzymes relevant for fungal cell wall biosynthesis and morphogenesis are β -1,3-glucanosyltransferases. Probable 1,3- β -glucanosyltransferases Bgt1 and Gel1 (Pc13g08730 with 6 isoforms, Pc15g01030 with 6 isoforms and Pc21g22000 with one isoform) are identified in the extracellular proteome of *P. chrysogenum* (spots 61, 95, 96, 148, 150, 151, 153, 203, 212, 215, 216, 218 and 220). In addition, a probable glycosylphosphatidylinositol-anchored β (1-3) glucanosyltransferase gel3 (Pc22g09380) is present in spots 32, 33, 35, 37, 48, 50, 54 and 57. Chitin and its partially or fully deacetylated derivative chitosan are major components of most fungal cell walls. Probable chitinases are identified in spots 138 (Pc13g09520) and 106 (Pc20g02250). Related to this protein is the identification of a probable chitosanase (Pc12g07820, spot 194), which has been purified from the culture medium of the koji mold *A. oryzae* (Zhang *et al.*, 2001), and 11 isoforms of a cell wall protein Crh1 (Pc21g23210, spots 22, 40, 74, 99, 101, 103, 112, 115, 116, 232 and 235), which transfers chitin to 1,6- β glucan and has been located at the cell surface, particularly in chitin-rich areas at the incipient bud site, around the septum area in later stages of budding, and in ascospore envelopes, suggesting that it might exert a common role in cell wall organization of *S. cerevisiae* (Rodríguez-Peña *et al.*, 2000).

Spots 51, 52, 55 and 56 include a β -N-acetylhexosaminidase (Pc20g10360), which is widely distributed in nature including fungi (Horsch *et al.*, 1997; Diez *et al.*, 2005). Several roles have been given to fungal β -N-acetylhexosaminidases, β -N-acetylglucosaminidases and chitobiases, such as the invasion of host organisms, the digestion of chitinous nutrients, the apical growth and branching of hyphae, the autolysis of mycelia and fruiting bodies in chitinous fungi and in the recycling and mobilisation of intracellular polysaccharides and glycoproteins especially during starvation (Horsch *et al.* 1997), although it has been reported that chitin is not utilised by *P. chrysogenum* even in the state of carbon starvation (Pusztahelyi *et al.*, 1997).

3.2.3.4. Cell rescue, defense and virulence

One of the proteins showing a high representativity in the extracellular proteome both in number of isoforms (18) and intensity of these isoforms (6.03%) is a probable catalase R (Pc16g11860, spots 12-24, 26, 27, 29, 31 and 275). Correlated with this finding is the presence of a probable glucose oxidase (Pc20g09560) in spot 41. This enzyme, initially known as notatin, has been isolated from the culture filtrate of a selected strain *Penicillium notatum* Westling and possesses antibacterial properties (Coulthard *et al.*, 1945). This protein catalyzes the oxidation of β -D-glucose into D-glucono-1,5-lactone, which then is hydrolyzed to gluconic acid, which is involved in virulence (see Discussion).

Other proteins important for virulence are phospholipases (see Discussion). Proteins with a putative phospholipase activity are found in spot 1 (Pc18g02900; lysophospholipase phospholipase B), spots 69, 77, 78 and 80 (Pc14g00170; 81% homology and 68% identity to a phosphatidylglycerol specific phospholipase from *A. clavatus*), spots 81-83 and 100 (Pc15g01880; probable non-hemolytic phospholipase C). Another virulence protein is found in spots 181, 270 and 272 (Pc16g06700). This protein shares 82% homology and 75% identity with a muraminidase (lysozyme) from *A. flavus*, which functions as an antibacterial agent. This protein has been reported to be secreted also in *Agaricus bisporus* (Lincoln *et al.*, 1997). Spots 85, 87 and 90 include a protein (Pc18g00980) with 96% similarity (90% identity) to an UDP-galactopyranose mutase from *A. fumigatus*. This enzyme synthesizes UDP-galactofuranose, which is the precursor of the D-galactofuranose residues found in bacterial and parasitic cell walls, including those of many pathogens, such as *Mycobacterium tuberculosis* and *Trypanosoma cruzi*. The mutase enzyme is essential for the viability of mycobacteria (Pan *et al.*, 2001). Galactofuranose containing molecules have been described at the cell surface of several eukaryotes and shown to contribute to the virulence of *A. fumigatus* (Schmalhorst *et al.*, 2008).

Spot 242 includes a protein (Pc12g13600) with strong similarity to hypothetical necrosis and ethylene inducing protein BH0395. A similar protein, which induces ethylene and necrosis in leaves of *Erythroxylum coca*, has been purified from culture filtrates of *Fusarium oxysporum* (Bailey, 1997). These proteins are elicitors of defense responses in tobacco and tomato and induce ethylene biosynthesis, production of

phytoalexins, and pathogenesis-related proteins and cause cell death resembling the hypersensitive response reaction (Bailey *et al.*, 1992; Yano *et al.*, 1998).

Other proteins identified in the extracellular fraction were involved in cell rescue or defense. Spots 211 and 228 contain a probable ribonuclease T2 precursor (Pc06g00430). RNase T2 was first reported by Sato and Egami (1957) as a ribonuclease in Takadiastase produced from a culture extract of *A. oryzae*. This enzyme might be a defense mechanism against viral pathogens (Irie, 1999). Oxidative stress response proteins were also found. Spot 250 contains a type 2 peroxiredoxin (Pc22g23760), a potent thiol-specific antioxidant protein with alkyl hydroperoxidase activity involved in osmotic stress resistance and detoxification of the cell (Jeong *et al.*, 1999). Spot 105 contains a probable glutathione reductase (Pc16g13280), although it was not predicted to be secreted (Supplemental Table 4). Spot 47 includes a probable L-galactonolactone oxidase Alo1p (Pc16g04640), which participates in the biosynthesis of vitamin C (l-ascorbic acid) in plants. Some fungi, such as *S. cerevisiae* do not normally synthesize ascorbate, but Alo1p, which is embedded in the mitochondrial membrane, can convert a number of related substrates to either dehydro-D-arabino-1,4-lactone or ascorbate, two antioxidants (Hancock *et al.*, 2000).

The finding of a probable cephalosporin esterase (Pc12g13400) in spot 53 is also remarkable. This enzyme, like other cephalosporin acetylhydrolases, is present in many organisms and may be in charge of cephalosporin deacetylation, thus generating deacetylcephalosporin. The presence of cephalosporin acetylhydrolases in the culture broths of *A. chrysogenum* has been described since 1975 (Fujisawa *et al.* 1975) and a extracellular cephalosporin C acetylhydrolase has been purified from this microorganism (Velasco *et al.*, 2001). Although *P. chrysogenum* does not produce cephalosporin C, cephalosporin esterases use a variety of acetylated substrates and deacetylation of these compounds might constitute a defense mechanism from other fungi.

Several extracellular fungal enzymes, such as polygalacturonase, pectate lyase, xylanase and proteases, have been shown or postulated to be required for virulence in at least one host/pathogen interaction and certain polygalacturonases and xylanases are elicitors of plant defense responses (Monod and Borg-von, 2002; Paper *et al.*, 2007). These proteins have been included in subsequent categories (see below).

3.2.3.5. Plant cell wall degradation

Cellulolytic enzymes are represented by endoglucanases (Pc22g19230, spots 22, 23, 40 and 42; Pc20g07340, spot 271; Pc13g13110, spot 273) and β -glucosidases (Pc15g00210, spots 270 and 272; Pc18g01940, spot 276). For hemicellulose degradation, *P. chrysogenum* secretes an endo-1,4- β -xylanase, which was found to be the major protein in the culture filtrate of this fungus when grown on 1% xylan (Haas *et al.*, 1992). It is well-known that *P. chrysogenum* is able to grow on xylan as a sole carbon source (Haas *et al.*, 1993). The precursor of this endo-1,4- β -xylanase (Pc20g07020) is present in spot 193.

Pectins form another group of heteropolysaccharides from the plant cell wall. It is noteworthy that 11 isoforms (3.56% of the total intensity) of a probable pectate lyase (Pc22g24890) have been identified in spots 184, 187-194, 206 and 265. In addition, a putative pectate lyase is also found in spot 222 (Pc22g05880, 80% homology and 65% identity to a pectate lyase D from *Pyrenophora tritici-repentis*). Other pectinolytic enzymes found in the extracellular fraction are rhamnogalacturonase B (Pc14g00370, spot 88), which has a positive effect in the apple hot-mash liquefaction process (Suykerbuyk *et al.*, 1995) and polygalacturonases (Pc20g13360, spot 149; Pc22g20290, spots 154-157). The finding of a probable ferulic acid esterase (Pc20g07010) in spots 206 and 208 is also remarkable (see Discussion).

Other accessory enzymes involved in the degradation of plant cell wall polysaccharides have been described in the genus *Aspergillus* (de Vries and Visser, 2001). Putative endoarabinases, like those found in spots 219 (Pc12g01330, 79% homology and 69% identity with a putative endoarabinase from *A. clavatus*), 186 (Pc12g10800), 217 (Pc20g15730), 221 and 224 (Pc13g15920), arabinoxylan arabinofuranohydrolase, like that found in spots 223 and 225 (Pc20g07030) or acetyl xylan esterase, like that found in spot 198 (Pc20g02780, 63% homology and 48% identity with an acetyl xylan esterase from *Rhodococcus jostii*) are also found in the *P. chrysogenum* secretome.

3.2.3.6. Proteases

Proteolytic enzymes secreted by fungi are important enzymes for fungal pathogenicity and some of them are important in the food industry. Examples of

probable carboxypeptidases are found in spots 94 (Pc12g14680), 56 (Pc20g10240) and 22 (Pc21g06670), the latter two being probable serine carboxypeptidases. Extracellular serine carboxypeptidases are found in the vacuoles of fungi and higher plants and in the lysosomes of animal cells and additionally, many fungi secrete serine carboxypeptidases (Breddam, 1986). Other probable secreted serine protease (Pc20g15140) with putative antigenic properties (see below) is found in spot 264. The group of aspartyl proteases is represented by homologues of candidapepsin (Pc13g09680, spot 181) and aspergillopepsin (Pc21g02370, spot 271), the latter being one of the major proteins secreted by *A. fumigatus* (Schwienbacher *et al.*, 2005) and *A. awamori* (Moralejo *et al.*, 2000). Some of these proteins may contribute to pathogenicity as has been reported for other fungi (Monod and Borg-von, 2002).

It is also important to indicate that spot 45 contains an aminopeptidase-like protein (Pc22g15910) that lacks signals to be secreted. This protein shares 92% homology and 84% identity with a putative aminopeptidase P from *A. flavus*. This enzyme, also known as proline aminopeptidase, and aminoacylproline aminopeptidase catalyses the release of any N-terminal amino acid, including proline itself, that is linked with proline. Prolyl aminopeptidase activity is important during the ripening of the Camembert cheese by decreasing the amount of bitter tasting peptides (Fuke *et al.*, 1988).

3.2.3.7. Allergenic proteins

In *Penicillium* spp., many allergens show higher IgE-binding activity in culture filtrate extracts than in cellular extracts (Chiu *et al.*, 2008). Orthologs of human IgE-reactive proteins from *Penicillium citrinum* are identified in the filtrate extracts of *P. chrysogenum*, such as pectate lyase (Pc22g24890) with 11 isoforms (spots 184, 187-194, 206 and 265), catalase R (Pc16g11860) with 18 isoforms (spots 12-24, 26, 27, 29, 31 and 275) or 1,3- β -glucanosyltransferase (Pc15g01030) with 6 isoforms (spots 95, 96, 148, 150, 151 and 153). In addition, two isoforms of a probable IgE binding protein (Pc22g22290) are present in spots 236 and 242. Spot 257 includes a 16-kDa allergen specific of *P. chrysogenum* and spots 51, 52, 55 and 56 contain the 68-kDa allergen N-acetylhexosaminidase (Pc20g10360) specific for *P. chrysogenum* (Shen *et al.*, 1995). This protein is one of the highly represented proteins in *P. chrysogenum*, increasing

along the fermentation process (Diez *et al.*, 2005). Orthologs of proteins that have been shown to possess antigenic properties in the filamentous fungus *P. citrinum* are also present in the *P. chrysogenum* secretome. Spot 264 contains a protein (Pc20g15140) with strong similarity to a secreted serine protease (19 kDa CS antigen) from *Coccidioides immitis*. In addition, an enolase (Pc14g01740, spots 53-59) and a putative elongation factor 1-beta (Pc13g08810, spot 255) are present in the culture medium of *P. chrysogenum*, although these proteins are not predicted to be secreted as shown in Supplementary Table 4 (see Discussion). These findings remark the importance of *P. chrysogenum* as an indoor allergen.

3.2.3.8. Proteins of unknown function

Those proteins present in the extracellular proteome of *P. chrysogenum* that lacked conserved domains are grouped under this category. Some members from this group are described as hypothetical cell wall proteins (Pc22g00190, spots 249 and 279) or hypothetical membrane proteins (Pc22g14360, spot 181). With the only exception of Pc13g10260 (spot 184) the rest of these proteins are predicted to be secreted (Supplemental Table 4), although the function of these proteins remains unclear.

3.2.4. Differences in the extracellular proteome of the wild type *P. chrysogenum* NRRL 1951 and Wis 54-1255

In order to find the extracellular protein variations introduced during the early strain improvement program, the wild-type strain NRRL 1951 was compared to the improved, but still low producer Wis 54-1255. Analysis of the 2-DE gels (Figure 3.21) showed 22 spots that were overrepresented in the NRRL 1951 strain (totalling 31 proteins with isoforms), whereas in the Wis 54-1255, 46 spots (51 proteins and isoforms) had higher representation compared to the wild-type strain. Tables 5 and 6 display the findings summarized below.

Figure 3.21. Comparison of the extracellular proteomes of *P. chrysogenum* NRRL 1951 and Wis 54-1255. The word “Nw” is used for those spots overrepresented or present only in the wild-type strain, whereas “Wn” is used for those spots overrepresented or present only in the Wis 54-1255 strain.

Table 3.5. Spots overrepresented in the extracellular proteome of the NRRL-1951 strain compared to the Wisconsin 54-1255 strain. Fold change and p-value are indicated. For those spots only detected in one of the strains (N/A) is shown.

Spot	ORF name	Accession No.	Title	Theoretical		Estimated		Matched	Non-Matched	Score	Protein coverage (%)	Fold change	p-value	Signal peptide probability	Signal peptide	Non-classically secreted protein
				Mass (kDa)	pI	Mass (kDa)	pI									
Cell rescue, defense and virulence																
Nw1	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	49.0	4.9	14	45	689	35	N/A	N/A	0.9990	YES	
Nw2	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	59.6	5.2	22	39	784	48	12.19	1.74E-05	0.9990	YES	
Nw3	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	61.0	5.2	13	40	217	37	9.36	8.14E-06	0.9990	YES	
Nw5	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	57.2	5.4	12	53	143	28	N/A	N/A	0.9990	YES	
Nw6	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	57.3	5.5	16	48	298	44	N/A	N/A	0.9990	YES	
Nw8	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	58.0	5.7	14	50	234	36	N/A	N/A	0.9990	YES	
Nw9	Pc20g09560	gil211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakense</i>	66.5	5.2	76.1	5.4	24	38	700	56	N/A	N/A	0.9930	YES	
Nw10	Pc20g09560	gil211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakense</i>	66.5	5.2	74.5	5.5	22	41	758	49	N/A	N/A	0.9930	YES	
Nw11	Pc06g00430	gil211581271	strong similarity to ribonuclease T2 precursor <i>rntB</i> - <i>Aspergillus oryzae</i>	28.75	4.28	30.5	4.4	10	55	894	39	2.48	1.33E-04	0.9950	YES	
Nw12	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	33.1	4.2	11	54	157	26	N/A	N/A	0.9990	YES	
Nw15	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	32.0	5.0	23	42	588	55	N/A	N/A	0.9990	YES	
Nw16	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	34.9	4.8	7	54	115	21	N/A	N/A	0.9990	YES	
Nw17	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	32.8	4.8	12	53	159	26	N/A	N/A	0.9990	YES	
Nw18	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	33.7	4.9	11	29	390	35	N/A	N/A	0.9990	YES	
Nw19	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	34.0	5.0	15	50	279	44	N/A	N/A	0.9990	YES	
Nw20	Pc15g01880	gil255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	42.7	5.0	15	50	418	34	N/A	N/A	0.9990	YES	

Table 3.5 continued

Nw21	Pc15g01880	gj 255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	47.3	5.1	11	35	496	32	N/A	N/A	0.9990	YES
Nw22	Pc15g01880	gj 255939267	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	27.6	6.0	11	54	448	24	N/A	N/A	0.9990	YES
Utilization of nutrients															
Nw3	Pc12g14860	gj 211582909	extracellular acid phosphatase PhoA - <i>Penicillium chrysogenum</i>	45.9	5.2	61.0	5.2	14	47	553	41	9.36	8.14E-06	0.9990	YES
Nw4	Pc12g14860	gj 211582909	extracellular acid phosphatase PhoA - <i>Penicillium chrysogenum</i>	45.9	5.2	59.9	5.3	15	44	715	48	4.05	9.50E-06	0.9990	YES
Nw5	Pc12g14860	gj 211582909	extracellular acid phosphatase PhoA - <i>Penicillium chrysogenum</i>	45.9	5.2	57.2	5.4	14	46	588	41	N/A	N/A	0.9990	YES
Nw6	Pc12g14860	gj 211582909	extracellular acid phosphatase PhoA - <i>Penicillium chrysogenum</i>	45.9	5.2	57.3	5.5	14	48	415	41	N/A	N/A	0.9990	YES
Nw7	Pc12g14860	gj 211582909	extracellular acid phosphatase PhoA - <i>Penicillium chrysogenum</i>	45.9	5.2	56.3	5.6	8	23	405	24	N/A	N/A	0.9990	YES
Nw8	Pc12g14860	gj 211582909	extracellular acid phosphatase PhoA - <i>Penicillium chrysogenum</i>	45.9	5.2	58.0	5.7	14	48	463	36	N/A	N/A	0.9990	YES
Nw19	Pc22g09580	gj 211591986	strong similarity to acid phosphatase <i>aphA</i> - <i>Aspergillus ficuum</i>	55.07	4.84	34.0	5.0	8	57	361	26	N/A	N/A	0.7250	YES
Nw20	Pc12g14860	gj 211582909	extracellular acid phosphatase PhoA - <i>Penicillium chrysogenum</i>	45.9	5.2	42.7	5.0	7	58	146	22	N/A	N/A	0.9990	YES
Cell wall morphogenesis															
Nw19	Pc13g08730	gj 211583918	strong similarity to 1,3-beta-glucanosyltransferase <i>bgt1</i> - <i>Aspergillus fumigatus</i>	34.67	5.00	34.0	5.0	9	56	107	54	N/A	N/A	1.0000	YES
Plant cell wall degradation															
Nw17	Pc22g20290	gj 211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39.01	5.47	32.8	4.8	7	58	695	48	N/A	N/A	1.0000	YES
Other proteins															
Nw12	Pc21g16870	gj 255955869	strong similarity to hypothetical protein <i>smik_17056</i> - <i>Saccharomyces mikatae</i>	46.0	4.6	33.1	4.2	12	53	313	33	N/A	N/A	0.0000	NO
Nw13	Pc21g16870	gj 255955869	strong similarity to hypothetical protein <i>smik_17056</i> - <i>Saccharomyces mikatae</i>	46.0	4.6	35.0	4.3	3	11	149	9	N/A	N/A	0.0000	NO
Nw14	Pc13g13310	gj 255937437	hypothetical protein [<i>Penicillium chrysogenum</i>]	19.6	5.03	20.6	4.9	6	57	225	23	N/A	N/A	0.9990	YES

Table 3.6. Spots overrepresented in the extracellular proteome of the Wisconsin 54-1255 strain compared to the NRRL 1951 strain. Fold change and p-value are indicated. For those spots only detected in one of the strains (N/A) is shown.

Spot	ORF name	Accession No.	Title	Theoretical		Estimated		Matched	Non-Matched	Score	Protein coverage (%)	Fold change	p-value	Signal peptide probability	Signal peptide	Non-classically secreted protein
				Mass (kDa)	pI	Mass (kDa)	pI									
General metabolism																
Wn5	Pc21g09860	gil211589733	strong similarity to hypothetical amidase - <i>Schizosaccharomyces pombe</i>	62.9	4.7	77.1	4.8	20	32	369	39	N/A	N/A	0.9990	YES	
Wn8	Pc22g16510	gil211592582	strong similarity to isoamyl alcohol oxidase <i>mreA</i> - <i>Aspergillus oryzae</i>	61.9	4.8	65.6	4.8	17	47	558	43	N/A	N/A	1.0000	YES	
Wn9	Pc22g16510	gil211592582	strong similarity to isoamyl alcohol oxidase <i>mreA</i> - <i>Aspergillus oryzae</i>	61.9	4.8	65.3	4.7	11	54	117	24	N/A	N/A	1.0000	YES	
Wn15	Pc22g09680	gil211591995	similarity to hypothetical protein PA4204 - <i>Pseudomonas aeruginosa</i>	43.6	5.2	47.6	5.2	17	44	873	66	N/A	N/A	0.9930	YES	
Wn23	Pc20g04720	gil211587749	strong similarity to precursor of dihydrolipoamide dehydrogenase Lpd1 - <i>Saccharomyces cerevisiae</i>	54.6	7.7	50.0	6.6	33	28	992	68	N/A	N/A	0.0690	NO	YES
Wn25	Pc18g01390	gil211586717	strong similarity to phosphoglucomutase <i>pgmB</i> - <i>Aspergillus nidulans</i>	60.5	5.7	61.6	5.9	24	41	349	52	N/A	N/A	0.0000	NO	YES
Wn26	Pc22g23810	gil211593289	strong similarity to fumarate hydratase <i>fumR</i> - <i>Rhizopus oryzae</i>	58.1	7.7	48.7	6.1	21	41	617	55	N/A	N/A	0.6190	YES	
Wn27	Pc20g03610	gil211587647	strong similarity to precursor of mitochondrial isocitrate dehydrogenase <i>icdA</i> - <i>Aspergillus niger</i>	54.4	8.4	48.3	5.9	24	40	694	52	N/A	N/A	0.2800	NO	YES
Wn34	Pc22g04850	gil211591539	strong similarity to D-arabinose dehydrogenase Ara1 - <i>Saccharomyces cerevisiae</i>	36.63	5.54	35.39	5.72	20	43	690	61	N/A	N/A	0.1470	NO	NO
Wn36	Pc21g16950	gil211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	35.59	5.45	34.29	5.49	17	30	539	57	N/A	N/A	0.0010	NO	NO
Wn40	Pc21g16950	gil211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	35.59	5.45	34.14	5.37	13	52	398	48	N/A	N/A	0.0010	NO	NO
Wn41	Pc21g16950	gil211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	35.59	5.45	34.64	5.34	15	31	272	54	N/A	N/A	0.0010	NO	NO
Wn42	Pc14g01740	gil211584789	<i>enolase</i> BAC82549 - <i>Penicillium chrysogenum</i>	47.3	5.3	47.0	5.6	23	39	871	65	2.10	7.47E-05	0.0000	NO	NO
Wn46	Pc22g02000	gil211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	83.9	6.1	33	24	814	52	N/A	N/A	0.2920	NO	NO
Utilization of nutrients																
Wn1	Pc12g15470	gil211582966	strong similarity to hypothetical protein contig1492_0.tfa_1880cg - <i>Aspergillus fumigatus</i>	70.7	5.2	95.3	5.0	28	31	698	54	N/A	N/A	0.9980	YES	

Table 3.6 continued

Wn2	Pc22g06490	gj211591694	strong similarity to alkaline phosphatase - <i>Neurospora crassa</i>	73.8	4.8	87.8	4.9	27	35	858	51	N/A	N/A	0.9990	YES
Wn3	Pc22g06490	gj211591694	strong similarity to alkaline phosphatase - <i>Neurospora crassa</i>	73.8	4.8	87.8	4.8	27	34	863	48	N/A	N/A	0.9990	YES
Wn4	Pc22g06490	gj211591694	strong similarity to alkaline phosphatase - <i>Neurospora crassa</i>	73.8	4.8	89.4	4.8	23	39	473	41	N/A	N/A	0.9990	YES
Wn12	Pc22g01850	gj211591246	glucan 1,4-alpha-glucosidase BAC8255- <i>Penicillium chrysogenum</i>	52.4	5.0	47.6	5.1	27	38	811	69	N/A	N/A	0.9970	YES
Wn13	Pc21g12590	gj211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrotrabacter oxidans</i>	54.7	5.2	46.8	5.1	18	47	467	43	N/A	N/A	0.9990	YES
Wn22	Pc21g12590	gj211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrotrabacter oxidans</i>	54.72	5.18	18.15	5.44	13	52	302	27	20.35	6.97E-06	0.9990	YES
Wn24	Pc12g00830	gj211581603	strong similarity to sorbitol utilization protein sou2 - <i>Candida albicans</i>	28.71	5.33	27.29	5.52	18	41	606	80	2.71	1.29E-05	0.0210	NO
Wn25	Pc22g09390	gj211591968	strong similarity to mamitol dehydrogenase mtlD - <i>Pseudomonas fluorescens</i>	59.5	5.6	61.6	5.9	24	41	436	48	N/A	N/A	0.0000	NO
Wn28	Pc21g12590	gj211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrotrabacter oxidans</i>	54.7	5.2	49.5	5.0	18	45	705	43	2.15	4.13E-06	0.9990	YES
Wn33	Pc12g04310	gj211581920	strong similarity to acetate-inducible gene aciA - <i>Aspergillus nidulans</i>	50.35	6.25	42.02	6.30	26	37	591	59	N/A	N/A	0.0020	NO
Wn38	Pc22g09580	gj211591986	strong similarity to acid phosphatase apha - <i>Aspergillus ficum</i>	55.07	4.84	37.00	5.37	7	58	146	23	N/A	N/A	0.7250	YES
Cell wall morphogenesis															
Wn6	Pc22g09380	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanotransferase gel3 - <i>Aspergillus fumigatus</i>	57.6	4.9	72.4	4.9	15	49	510	30	N/A	N/A	1.0000	YES
Wn7	Pc22g09380	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanotransferase gel3 - <i>Aspergillus fumigatus</i>	57.6	4.9	69.4	4.9	16	49	451	29	N/A	N/A	1.0000	YES
Wn10	Pc22g17870	gj211592713	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>	41.2	4.6	59.3	4.6	14	48	516	38	14.21	5.32E-06	0.9920	YES
Wn11	Pc22g17870	gj211592713	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>	41.2	4.6	59.7	4.7	15	45	580	38	7.28	3.84E-04	0.9920	YES
Wn13	Pc15g01030	gj211584950	strong similarity to 1,3-beta-glucanotransferase gel1 - <i>Aspergillus fumigatus</i>	49.5	5.2	46.8	5.1	11	54	315	36	N/A	N/A	0.9710	YES
Wn14	Pc20g10360	gj211588263	beta-N-acetylhexosaminidase hex - <i>Penicillium chrysogenum</i> (68 kDa allergen)	66.8	5.3	68.6	5.2	22	38	661	45	N/A	N/A	0.9970	YES
Wn18	Pc13g08730	gj211583918	strong similarity to 1,3-beta-glucanotransferase bgf1 - <i>Aspergillus fumigatus</i>	34.67	5.00	31.23	5.10	22	38	685	82	6.77	1.56E-05	1.0000	YES
Wn30	Pc22g09380	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanotransferase gel3 - <i>Aspergillus fumigatus</i>	57.6	4.9	78.5	5.0	15	50	370	28	4.14	2.27E-06	1.0000	YES

Table 3.6 continued

Plant cell wall degradation															
Wn17	Pc22g24890	gj 211593394	strong similarity to pectate lyase <i>plyA</i> - <i>Aspergillus niger</i>	34.41	5.28	35.34	5.19	16	47	707	63	N/A	N/A	1.0000	YES
Wn19	Pc22g05880	gj 211591637	strong similarity to hypothetical protein 1117_scaffold_1.tfa_300cg - <i>Fusarium graminearum</i>	25.56	4.47	30.79	4.48	8	57	543	49	N/A	N/A	0.9990	YES
Wn29	Pc15g00210	gj 211584871	similarity to glucan 1,3-beta-glucosidase Bgl2 - <i>Saccharomyces cerevisiae</i>	48.50	4.47	50.00	5.08	9	56	353	27	2.06	2.68E-04	0.9990	YES
Wn31	Pc13g15920	gj 211584606	strong similarity to endo 1,5-alpha arabinanase <i>abrA</i> - <i>Aspergillus niger</i>	34.33	5.53	31.55	5.38	5	49	239	23	2.07	7.11E-04	0.8760	YES
Wn32	Pc20g15730	gj 211588780	strong similarity to endo 1,5-alpha-arabinanase <i>abrA</i> - <i>Aspergillus niger</i>	34.78	5.35	32.01	5.39	3	62	194	20	N/A	N/A	0.9950	YES
Wn37	Pc22g24890	gj 211593394	strong similarity to pectate lyase <i>plyA</i> - <i>Aspergillus niger</i>	34.41	5.28	36.26	5.33	13	33	487	54	N/A	N/A	1.0000	YES
Wn39	Pc20g02780	gj 211587567	strong similarity to hypothetical protein PA3829 - <i>Pseudomonas aeruginosa</i>	33.82	5.39	35.04	5.47	15	47	454	58	N/A	N/A	0.0000	NO
Cell rescue, defense and virulence															
Wn16	Pc16g11860	gj 211586109	strong similarity to catalase R <i>catR</i> - <i>Aspergillus niger</i>	79.9	5.4	81.2	5.5	27	34	699	44	24.45	6.84E-05	0.0000	NO
Wn29	Pc16g06700	gj 211585628	strong similarity to hypothetical protein contig_1_7_scaffold_1.tfa_1160wg - <i>Aspergillus nidulans</i>	26.00	4.69	50.00	5.08	5	60	138	22	2.06	2.66E-04	0.9910	YES
Wn35	Pc12g09820	gj 211582426	strong similarity to glyoxalase I Glo1 - <i>Saccharomyces cerevisiae</i>	35.17	5.45	37.94	5.67	18	35	477	64	N/A	N/A	0.0010	NO
Control of cellular functions															
Wn9	Pc22g02800	gj 211591341	strong similarity to calcium-binding protein precursor <i>crx1p</i> - <i>Schizosaccharomyces pombe</i>	62.0	4.8	65.3	4.7	10	55	176	20	N/A	N/A	0.9980	YES
Wn22	Pc21g02110	gj 211588987	strong similarity to hypothetical protein mg01387.1 - <i>Magnaporthe grisea</i>	48.82	4.89	18.15	5.44	5	60	148	29	20.35	6.97E-06	1.0000	YES
Specific allergens															
Wn20	Pc22g22290	gj 211593141	strong similarity to IgE-binding protein - <i>Aspergillus fumigatus</i>	20.16	4.79	25.66	4.76	2	51	200	16	8.83	2.25E-04	0.9990	YES
Wn45		gj 144952798	16 kDa allergen - <i>Penicillium chrysogenum</i>	16.45	5.20	18.21	4.67	4	61	278	21	4.37	1.35E-03	0.9990	YES
Other proteins															
Wn21	Pc22g00190	gj 211591082	strong similarity to hypothetical cell wall protein <i>binB</i> - <i>Aspergillus nidulans</i>	19.11	5.61	18.56	5.44	2	22	172	18	3.38	1.40E-03	0.9960	YES
Wn43	Pc21g14220	gj 211590148	strong similarity to hypothetical protein contig1495_2.tfa_630cg - <i>Aspergillus fumigatus</i>	22.21	4.51	21.00	4.52	8	57	289	40	2.20	1.10E-02	0.9990	YES
Wn44	Pc12g10010	gj 211582445	strong similarity to protein copper regulated - <i>Aspergillus nidulans</i>	16.75	4.59	18.45	4.59	5	25	139	60	N/A	N/A	0.9990	YES

3.2.4.1. Cell rescue, defense and virulence

It is striking that 18 out of the 22 spots overrepresented or only present in the wild-type strain NRRL 1951 include proteins related to fungal virulence. Isoforms of a probable non-hemolytic phospholipase C are highly abundant (15 isoforms, spots Nw1-Nw3, Nw5, Nw6, Nw8, Nw12, Nw15-Nw22). In addition, two isoforms of a glucose oxidase GOX are only detected in this strain (spots Nw9 and Nw10). As discussed below, these proteins are related to pathogenesis, tissue invasion and infectivity of fruits, results that correlate well with the fact that the wild-type strain was isolated from an infected cantaloupe.

All these proteins showed lower concentrations in the secretome of the improved penicillin producer Wis 54-1255 strain.

However, in the Wis 54-1255 strain, proteins involved in stress defense mechanisms are predominant, such as a probable catalase R (spot Wn16), a probable glyoxalase I (spot Wn35), which detoxifies methylglyoxal -a typical 2-oxoaldehyde in living cells that inhibits the growth of various kinds of cells from microorganisms to mammals -by condensation with glutathione, and the hypothetical protein present in spot Wn29. The latter shares 82% similarity and 75% identity with a muramidase (lysozyme) from *Aspergillus flavus* NRRL3357. This antibacterial enzyme catalyzes the hydrolysis of 1,4- β -linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrin.

3.2.4.2. Utilization of nutrients

Acid phosphatases are predominant in the wild-type strain. Seven isoforms of PhoA (spots Nw3-Nw8, Nw20) and one isoform of AphA (spot Nw19) are overrepresented in the NRRL 1951 strain, whereas alkaline phosphatases are overrepresented in the Wis 54-1255 strain (spots Wn1-Wn4). Glucan 1,4- α -glucosidase (glucoamylase) is only detected in the Wis 54-1255 strain (spot Wn12). Since it catalyses the release of glucose from the non-reducing ends of starch and related polysaccharides, the overproduction of this enzyme may have represented one important milestone during the industrial improvement program, allowing *P. chrysogenum* to utilize carbon sources more efficiently.

3.2.4.3. General metabolism

One of the proteins that are overrepresented in the Wis 54-1255 strain is a probable isoamyl alcohol oxidase, which is included in spots Wn8 and Wn9. This protein is of relevant interest for the production of alcoholic beverages (see Discussion).

Strikingly some proteins overrepresented or only detected in the Wis 54-1255 strain lack predicted secretion signal sequences. These proteins might play different roles according to their location (see Discussion).

Examples are provided by a probable D-arabinose dehydrogenase Ara1 (spot Wn34), an enolase (spot Wn42), a probable mitochondrial aconitate hydratase Aco1 (spot Wn46) and a probable transaldolase Tal1 (spots Wn36, Wn40 and Wn41).

3.2.4.4. Plant cell wall degradation

One interesting finding is the overrepresentation of several enzymes involved in degradation of complex nutrients in the Wis 54-1255 strain probably as the result of selection of mutants showing good growth in complex plant materials. These enzymes are a probable pectate lyase PlyA (spots Wn19 and Wn37), glucan 1,3- β -glucosidase Bgl2 (spot Wn29) and endo 1,5- α arabinanase AbnA (spots Wn31 and Wn32).

3.2.4.5. Specific allergens

The Wis 54-1255 strain also overreproduces some specific antigens, such as one ortholog of the IgE-binding protein from *A. fumigatus* (spot Wn20) and a 16 kDa allergen (spot Wn45).

3.2.4.6. Other proteins

There are two proteins that were not identified in the extracellular proteome reference map of Wis 54-1255 and therefore, seem to be specific of the wild-type strain NRRL 1951 under the conditions used. These proteins are a hypothetical protein of unknown function (spots Nw14) and a protein with strong similarity to a hypothetical protein smik_17056 (spots Nw12 and Nw13). The latter shows a 58% similarity and a 51% identity with the Ddr48p protein of *S. cerevisiae*. This corresponds to a DNA damage-responsive protein, whose expression is increased in response to heat-shock stress or treatments that produce DNA lesions (Sheng and Schuster, 1993).

3.2.5. Extracellular proteome differences between the Wis 54-1255 and the high producer strain AS-P-78

To assess some of the protein variations that occurred during the development of the high producer AS-P-78 from Wis 54-1255, extracellular proteins from these two strains were compared to each other as indicated above. Analysis of the 2-DE gels (Figure 3.22) identified 65 spots (68 proteins and isoforms) that were overrepresented in the Wis 54-1255 strain, whereas 44 spots (49 proteins and isoforms) had higher representation in the AS-P-78 strain. The findings summarized below are detailed in Tables 7 and 8.

3.2.5.1. General metabolism

One of the proteins that are overrepresented in the Wis 54-1255 strain when compared with the high producer AS-P-78 is a probable sulphhydryl oxidase Sox (spots Wa7 and Wa8), an important enzyme for food industry (see Discussion). Another enzyme overrepresented in the Wis 54-1255 that is related to food industrial processes is a dihydroxy-acid dehydratase Ilv3 (Wa62 and Wa63), which is involved in the biosynthesis of branched-chain amino acids (see Discussion). As it was observed during the comparison of the the wild-type strain versus the Wis 54-1255 strain, some proteins overrepresented or only detected in the latter strain lack predicted secretion signal sequences. Examples are provided by an enolase (spots Wa13-Wa15), a probable mitochondrial (homo) aconitate hydratase Aco1 (spots Wa20-Wa22), a probable glucose-6-phosphate 1-dehydrogenase (spot Wa27), a probable ribose 5-phosphate isomerase RPI (spot Wa40), a probable transaldolase Tal1 (spots Wa48) and the glutamate dehydrogenase GDHA (spots Wa26, Wa30), the latter reported to be involved in regulation of β -lactam production (Thykaer *et al.*, 2008). Several of these enzymes play roles related to the mitochondrial formation of the penicillin precursor α -aminoadipic acid and may be released by some type of vesicles. As it was indicated before, these proteins might play different roles according to their location (see Discussion). In the AS-P-78 strain, a probable isoamyl alcohol oxidase is overrepresented (spot Aw11). In addition, a hypothetical amidase is overrepresented in this strain (spots Aw4-Aw6).

Figure 3.22. Comparison of the extracellular proteomes of *P. chrysogenum* Wis 54-1255 and AS-P-78. Proteins were separated by 2-DE. The word “Wa” is used for those spots overrepresented or present only in the Wis 54-1255 strain, whereas “Aw” is used for those spots overrepresented or present only in the AS-P-78 strain. The spots differentially represented at each time point are numbered and correspond to those proteins listed in Table 7 (for Wa) and Table 8 (for Aw).

Table 3.7. Spots overrepresented in the extracellular proteome of the Wisconsin 54-1255 strain compared to the AS-P-78 strain. Fold change and p-value are indicated. For those spots only detected in one of the strains (N/A) is shown.

Spot	ORF name	Accession No.	Title	Theoretical		Estimated		Matched	Non-Matched	Score	Protein coverage (%)	Fold change	p-value	Signal peptide probability	Signal peptide	Non-classically secreted protein
				Mass (kDa)	pI	Mass (kDa)	pI									
General metabolism																
Wa7	Pc22g22810	gil211593192	strong similarity to sulphhydryl oxidase Sox from patent EP565172-A1 - <i>Aspergillus niger</i>	42.3	4.9	47.4	4.9	18	43	570	47	52.71	1.24E-05	1.0000	YES	
Wa8	Pc22g22810	gil211593192	strong similarity to sulphhydryl oxidase Sox from patent EP565172-A1 - <i>Aspergillus niger</i>	42.3	4.9	47.1	5.0	18	42	647	49	55.42	1.18E-06	1.0000	YES	
Wa9	Pc22g09680	gil211591995	similarity to hypothetical protein P44204 - <i>Pseudomonas aeruginosa</i>	43.6	5.2	47.6	5.2	17	44	873	66	N/A	N/A	0.9930	YES	
Wa13	Pc14g01740	gil211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47.3	5.3	47.9	5.5	24	37	881	63	N/A	N/A	0.0000	NO	NO
Wa14	Pc14g01740	gil211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47.3	5.3	47.0	5.6	23	39	871	65	N/A	N/A	0.0000	NO	NO
Wa15	Pc14g01740	gil211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47.3	5.3	48.0	5.7	25	35	897	63	N/A	N/A	0.0000	NO	NO
Wa18	Pc18g01390	gil211586717	strong similarity to phosphoglucomutase pgmB - <i>Aspergillus nidulans</i>	60.5	5.7	61.6	5.9	24	41	349	52	N/A	N/A	0.0000	NO	YES
Wa20	Pc22g02000	gil211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	83.9	6.1	33	24	814	52	N/A	N/A	0.2920	NO	NO
Wa21	Pc22g02000	gil211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	86.0	6.0	33	28	911	50	N/A	N/A	0.2920	NO	NO
Wa22	Pc22g02000	gil211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	86.5	6.2	27	31	648	42	N/A	N/A	0.2920	NO	NO
Wa23	Pc16g05080	gil211585478	strong similarity to adenosylhomocysteinase - <i>Homo sapiens</i>	49.5	5.6	47.6	5.9	24	39	614	42	N/A	N/A	0.0040	NO	YES
Wa24	Pc20g03610	gil211587647	strong similarity to precursor of mitochondrial isocitrate dehydrogenase icdA - <i>Aspergillus niger</i>	54.4	8.4	48.3	5.9	24	40	694	52	N/A	N/A	0.2800	NO	YES
Wa25	Pc22g23810	gil211593289	strong similarity to fumarate hydratase fumR - <i>Rhizopus oryzae</i>	58.1	7.7	48.7	6.1	21	41	617	55	N/A	N/A	0.6190	YES	
Wa26	Pc22g17560	gil211592684	glutamate dehydrogenase gdhA - <i>Penicillium chrysogenum</i>	50.0	6.0	51.3	6.2	31	30	791	73	N/A	N/A	0.0000	NO	NO
Wa27	Pc20g03330	gil211587620	strong similarity to glucose-6-phosphate 1-dehydrogenase gsdA - <i>Aspergillus niger</i>	58.6	6.0	56.0	6.3	21	44	301	52	N/A	N/A	0.0010	NO	NO
Wa30	Pc22g17560	gil211592684	glutamate dehydrogenase gdhA - <i>Penicillium chrysogenum</i>	39.8	6.1	57.0	6.4	12	52	493	30	N/A	N/A	0.9990	YES	

Table 3.7 continued

Wa31	Pc20g04720	gil211587749	strong similarity to precursor of dihydroilpoamide dehydrogenase Lpd1 - <i>Saccharomyces cerevisiae</i>	54.6	7.7	50.0	6.6	33	28	992	68	N/A	N/A	0.0690	NO	YES
Wa34	Pc16g02790	gil211585272	strong similarity to aspartate transaminase like protein An08g01000 - <i>Aspergillus niger</i>	41.33	6.13	41.87	6.48	16	49	365	49	N/A	N/A	0.0020	NO	YES
Wa36	Pc20g15580	gil211588765	strong similarity to NADPH-dependent aldehyde reductase - <i>Sporobolomyces salmonicolor</i>	37.33	6.14	38.01	6.50	9	56	261	38	N/A	N/A	0.0030	NO	NO
Wa37	Pc22g04850	gil211591539	strong similarity to D-arabinose dehydrogenase Ara1 - <i>Saccharomyces cerevisiae</i>	36.63	5.54	35.39	5.72	20	43	690	61	N/A	N/A	0.1470	NO	NO
Wa39	Pc12g05820	gil211582045	strong similarity to esterase D ESD - <i>Homo sapiens</i>	31.36	5.89	32.47	6.20	19	43	720	87	N/A	N/A	0.1650	NO	YES
Wa40	Pc21g20440	gil211590742	strong similarity to ribose 5-phosphate isomerase RPI - <i>Mus musculus</i>	29.00	5.76	30.66	5.92	8	37	206	26	N/A	N/A	0.0000	NO	NO
Wa41	Pc22g25470	gil211593449	strong similarity to Sol1 - <i>Saccharomyces cerevisiae</i>	29.06	5.51	28.74	5.79	14	51	285	70	N/A	N/A	0.0010	NO	YES
Wa48	Pc21g16950	gil211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	35.59	5.45	34.29	5.49	17	30	539	57	N/A	N/A	0.0010	NO	NO
Wa56	Pc14g01740	gil211584789	enolase BAC62549 - <i>Penicillium chrysogenum</i>	47.3	5.3	18.43	4.86	23	39	871	65	9.56	1.33E-06	0.0000	NO	NO
Wa61	Pc22g02000	gil211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	64.5	5.5	14	51	209	20	N/A	N/A	0.2920	NO	NO
Wa62	Pc22g22710	gil211593182	strong similarity to dihydroxy-acid dehydratase Ilv3 - <i>Saccharomyces cerevisiae</i>	65.3	6.2	63.6	5.6	9	56	202	13	N/A	N/A	0.0440	NO	YES
Wa63	Pc22g22710	gil211593182	strong similarity to dihydroxy-acid dehydratase Ilv3 - <i>Saccharomyces cerevisiae</i>	65.3	6.2	64.0	5.7	20	42	484	40	N/A	N/A	0.0440	NO	YES
Wa64	Pc18g01390	gil211586717	strong similarity to phosphoglucosyltransferase <i>pgrMB</i> - <i>Aspergillus nidulans</i>	60.5	5.7	62.7	5.8	29	34	557	57	N/A	N/A	0.0000	NO	YES
Wa65	Pc18g01390	gil211586717	strong similarity to phosphoglucosyltransferase <i>pgrMB</i> - <i>Aspergillus nidulans</i>	60.5	5.7	63.6	5.8	21	44	216	42	N/A	N/A	0.0000	NO	YES
Utilization of nutrients																
Wa1	Pc22g06490	gil211591694	strong similarity to alkaline phosphatase - <i>Neurospora crassa</i>	73.8	4.8	89.4	4.8	23	39	473	41	N/A	N/A	0.9990	YES	
Wa2	Pc22g06490	gil211591694	strong similarity to alkaline phosphatase - <i>Neurospora crassa</i>	73.8	4.8	87.8	4.8	27	34	863	48	N/A	N/A	0.9990	YES	
Wa3	Pc22g06490	gil211591694	strong similarity to alkaline phosphatase - <i>Neurospora crassa</i>	73.8	4.8	87.8	4.9	27	35	858	51	N/A	N/A	0.9990	YES	
Wa4	Pc12g15470	gil211582966	strong similarity to hypothetical protein contig1492_0_fta_1880cg - <i>Aspergillus fumigatus</i>	70.7	5.2	95.3	5.0	28	31	698	54	N/A	N/A	0.9980	YES	
Wa18	Pc22g09390	gil211591988	strong similarity to mannitol dehydrogenase <i>mtd</i> - <i>Pseudomonas fluorescens</i>	59.5	5.6	61.6	5.9	24	41	436	48	N/A	N/A	0.0000	NO	NO

Table 3.7 continued

Wa28	Pc16g04220	gil211585400	strong similarity to trehalose metabolism factor Pmu1 - <i>Saccharomyces cerevisiae</i>	39.8	6.1	57.0	6.4	12	52	493	30	N/A	N/A	0.9990	YES
Wa29	Pc16g04220	gil211585400	strong similarity to trehalose metabolism factor Pmu1 - <i>Saccharomyces cerevisiae</i>	39.75	6.05	52.07	6.36	11	37	314	27	N/A	N/A	0.9990	YES
Wa32	Pc12g04310	gil211581920	strong similarity to acetate-inducible gene <i>aciA</i> - <i>Aspergillus nidulans</i>	50.35	6.25	42.02	6.30	26	37	591	59	N/A	N/A	0.0020	NO
Wa33	Pc12g04310	gil211581920	strong similarity to acetate-inducible gene <i>aciA</i> - <i>Aspergillus nidulans</i>	50.35	6.25	42.25	6.42	23	42	368	53	N/A	N/A	0.0020	NO
Wa35	Pc12g04310	gil211581920	strong similarity to acetate-inducible gene <i>aciA</i> - <i>Aspergillus nidulans</i>	50.35	6.25	42.02	6.55	26	37	570	60	N/A	N/A	0.0020	NO
Wa38	Pc12g08930	gil211582340	strong similarity to hypothetical protein contig1492_0.tfa_1890cg - <i>Aspergillus fumigatus</i>	34.14	6.11	33.85	6.40	15	27	406	74	N/A	N/A	0.0020	NO
Wa42	Pc12g00830	gil211581603	strong similarity to sorbitol utilization protein <i>sou2</i> - <i>Candida albicans</i>	28.71	5.33	25.00	5.96	12	52	312	49	N/A	N/A	0.0210	NO
Wa43	Pc12g00830	gil211581603	strong similarity to sorbitol utilization protein <i>sou2</i> - <i>Candida albicans</i>	28.71	5.33	27.29	5.52	18	41	606	80	N/A	N/A	0.0210	NO
Wa44	Pc12g00830	gil211581603	strong similarity to sorbitol utilization protein <i>sou2</i> - <i>Candida albicans</i>	28.71	5.33	28.21	5.52	17	39	393	74	N/A	N/A	0.0210	NO
Cell rescue, defense and virulence															
Wa6	Pc14g00170	gil211584633	strong similarity to hypothetical protein contig1490_3.tfa_630cg - <i>Aspergillus fumigatus</i>	47.1	4.8	57.6	4.8	15	50	641	52	N/A	N/A	1.0000	YES
Wa16	Pc16g11860	gil211586109	strong similarity to catalase <i>R catR</i> - <i>Aspergillus niger</i>	79.9	5.4	84.4	5.5	30	30	687	43	2.43	3.62E-03	0.0000	NO
Wa17	Pc16g11860	gil211586109	strong similarity to catalase <i>R catR</i> - <i>Aspergillus niger</i>	79.9	5.4	81.2	5.5	27	34	699	44	5.18	2.37E-04	0.0000	NO
Wa19	Pc18g00980	gil211586677	strong similarity to hypothetical trunk lateral cell specific gene <i>HrTLC1</i> - <i>Halocynthia roretzi</i>	57.2	5.6	57.5	6.0	26	38	573	50	N/A	N/A	0.0460	NO
Wa53	Pc12g13600	gil211582786	strong similarity to hypothetical necrosis and ethylene inducing protein <i>BH0395</i> - <i>Bacillus halodurans</i>	26.26	5.22	23.51	4.76	7	58	315	46	58.02	1.56E-03	0.9970	YES
Cell wall morphogenesis															
Wa5	Pc20g10360	gil211588263	beta-N-acetylhexosaminidase <i>hex</i> - <i>Penicillium chrysogenum</i> (68 kDa allergen)	66.8	5.3	68.6	5.2	22	38	661	45	N/A	N/A	0.9970	YES
Wa10	Pc22g17870	gil211592713	strong similarity to hypothetical ECM33 homolog <i>SPCC1223.12c</i> - <i>Schizosaccharomyces pombe</i>	41.2	4.6	52.1	4.7	15	46	591	37	4.13	3.18E-04	0.9920	YES
Wa11	Pc22g17870	gil211592713	strong similarity to hypothetical ECM33 homolog <i>SPCC1223.12c</i> - <i>Schizosaccharomyces pombe</i>	41.2	4.6	52.8	4.7	15	50	275	38	2.85	9.84E-03	0.9920	YES
Wa12	Pc15g01030	gil211584950	strong similarity to 1,3-beta-glucanase/yltransferase <i>gel1</i> - <i>Aspergillus fumigatus</i>	49.54	5.16	45.88	5.12	19	42	703	53	N/A	N/A	0.9710	YES

Table 3.7 continued

Wa52	Pc13g08730	gil211583918	strong similarity to 1,3-beta-glucanase <i>bgt1</i> - <i>Aspergillus fumigatus</i>	34.67	5.00	31.23	5.10	22	38	685	82	7.66	3.19E-05	1.0000	YES
Plant cell wall degradation															
Wa45	Pc13g15920	gil211584606	strong similarity to endo 1,5-alpha arabinanase <i>abra</i> - <i>Aspergillus niger</i>	34.33	5.53	31.55	5.38	5	49	239	23	N/A	N/A	0.8760	YES
Wa51	Pc22g05880	gil211591637	strong similarity to hypothetical protein 1117_scaffold_1.tfa.300cg - <i>Fusarium graminearum</i>	25.56	4.47	30.79	4.48	8	57	543	49	N/A	N/A	0.9990	YES
Wa54	Pc22g24890	gil211593394	strong similarity to pectate lyase <i>plyA</i> - <i>Aspergillus niger</i>	34.41	5.28	35.34	5.19	16	47	707	63	N/A	N/A	1.0000	YES
Wa60	Pc22g20290	gil211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39.01	5.47	42.32	5.38	19	46	966	67	N/A	N/A	1.0000	YES
Proteases															
Wa11	Pc12g14680	gil211582891	strong similarity to hypothetical lysosomal pepstatin insensitive protease CLN2 - <i>Canis lupus</i>	65.7	4.8	52.8	4.7	14	51	456	38	2.85	9.84E-03	1.0000	YES
Wa58	Pc20g15140	gil211588723	strong similarity to secreted serine protease 19 kDa CS antigen CS-Ag - <i>Coccidioides immitis</i>	15.68	4.70	17.08	4.53	4	52	117	30	N/A	N/A	1.0000	YES
Control of cellular functions															
Wa46	Pc20g04810	gil211587757	strong similarity to estrogen receptor-binding cyclophilin <i>cypD</i> - <i>Bos primigenius taurus</i>	41.31	5.91	24.28	5.22	7	15	138	28	N/A	N/A	0.0000	NO
Wa47	Pc20g04810	gil211587757	strong similarity to estrogen receptor-binding cyclophilin <i>cypD</i> - <i>Bos primigenius taurus</i>	41.31	5.91	23.39	5.23	12	33	391	40	N/A	N/A	0.0000	NO
Specific allergens															
Wa49	Pc22g22290	gil211593141	strong similarity to IgE-binding protein - <i>Aspergillus fumigatus</i>	20.16	4.79	25.66	4.76	2	51	200	16	N/A	N/A	0.9990	YES
Wa53	Pc22g22290	gil211593141	strong similarity to IgE-binding protein - <i>Aspergillus fumigatus</i>	20.16	4.79	23.51	4.76	2	63	154	16	58.02	1.56E-03	0.9990	YES
Other proteins															
Wa50	Pc21g14220	gil211590148	strong similarity to hypothetical protein contig1495_2.tfa.630cg - <i>Aspergillus fumigatus</i>	22.21	4.51	21.00	4.52	8	57	289	40	N/A	N/A	0.9990	YES
Wa55	Pc22g00190	gil211591082	strong similarity to hypothetical cell wall protein <i>binB</i> - <i>Aspergillus nidulans</i>	19.11	5.61	18.56	5.44	2	22	172	18	N/A	N/A	0.9960	YES
Wa57	Pc16g00900	gil211585131	strong similarity to hypothetical protein contig10.tfa.360cg - <i>Aspergillus fumigatus</i>	16.12	4.58	17.30	4.53	8	44	461	81	N/A	N/A	1.0000	YES
Wa59	Pc13g06930	gil211583746	strong similarity to hypothetical protein contig_1_7_scaffold_1.tfa_1010wg - <i>Aspergillus nidulans</i>	15.47	4.03	17.00	4.20	4	59	121	31	N/A	N/A	1.0000	YES

Table 3.8. Spots overrepresented in the extracellular proteome of the AS-P-78 compared to the Wisconsin 54-1255 strain. Fold change and p-value are indicated. For those spots only detected in one of the strains (N/A) is shown.

Spot	ORF name	Accession No.	Title	Theoretical		Estimated				Non-Matched	Score	Protein coverage (%)	Fold change	p-value	Signal peptide probability	Signal peptide	Non-classically secreted protein
				Mass (kDa)	pI	Mass (kDa)	pI	Matched									
Utilization of nutrients																	
Aw23	Pc12g14860	gil211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	55.16	5.23	9	56	193	31	N/A	N/A	0.9999	YES		
Aw24	Pc12g14860	gil211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	55.26	5.30	10	55	388	34	N/A	N/A	0.9999	YES		
Aw25	Pc12g14860	gil211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	59.85	5.36	8	24	379	29	N/A	N/A	0.9999	YES		
Aw26	Pc12g14860	gil211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	58.75	5.50	9	21	333	32	N/A	N/A	0.9999	YES		
Aw27	Pc12g14860	gil211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	53.95	5.37	12	53	384	35	N/A	N/A	0.9999	YES		
Aw28	Pc12g14860	gil211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	53.35	5.47	11	54	362	41	N/A	N/A	0.9999	YES		
Aw29	Pc12g14860	gil211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	57.67	5.56	14	51	422	49	N/A	N/A	0.9999	YES		
Aw30	Pc12g14860	gil211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	55.26	5.60	13	48	620	48	N/A	N/A	0.9999	YES		
Aw31	Pc12g14860	gil211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	55.78	5.77	11	54	452	35	N/A	N/A	0.9999	YES		
Aw32	Pc12g14860	gil211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	55.99	5.91	14	50	850	51	N/A	N/A	0.9999	YES		
Aw41	Pc18g02580	gil211586833	strong similarity to acid phosphatase AFPhoA - <i>Aspergillus ficum</i>	50.2	5.0	72.29	4.99	8	57	498	14	N/A	N/A	1.0000	YES		
Aw42	Pc18g02580	gil211586833	strong similarity to acid phosphatase AFPhoA - <i>Aspergillus ficum</i>	50.2	5.0	71.63	5.12	9	56	440	17	N/A	N/A	1.0000	YES		
Aw43	Pc18g02580	gil211586833	strong similarity to acid phosphatase AFPhoA - <i>Aspergillus ficum</i>	50.2	5.0	72.83	5.26	8	57	283	14	N/A	N/A	1.0000	YES		

Cell rescue, defense and virulence

Aw1	Pc20g09560	gij211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakense</i>	66.48	5.2	68.89	5.49	23	37	793	55	N/A	N/A	0.9930	YES
Aw2	Pc20g09560	gij211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakense</i>	66.48	5.2	67.88	5.57	25	37	800	55	N/A	N/A	0.9930	YES
Aw3	Pc20g09560	gij211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakense</i>	66.48	5.2	68.77	5.65	20	42	661	46	N/A	N/A	0.9930	YES
Aw23	Pc15g01880	gij211585025	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	55.16	5.23	18	47	508	44	N/A	N/A	0.9999	YES
Aw24	Pc15g01880	gij211585025	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	55.26	5.30	16	49	238	45	N/A	N/A	0.9999	YES
Aw33	Pc20g09560	gij211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakense</i>	66.48	5.2	70.05	5.32	19	44	539	45	N/A	N/A	0.9930	YES
Aw34	Pc20g09560	gij211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakense</i>	66.48	5.2	69.79	5.36	21	40	699	49	N/A	N/A	0.9930	YES
Aw35	Pc16g11860	gij211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	79.9	5.4	70.31	5.80	18	47	516	30	N/A	N/A	0.0000	NO
Aw36	Pc20g09560	gij211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakense</i>	66.48	5.2	70.31	5.80	18	47	311	41	N/A	N/A	0.9930	YES
Aw36	Pc16g11860	gij211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	79.9	5.4	70.57	5.88	12	48	308	19	N/A	N/A	0.0000	NO
Aw36	Pc20g09560	gij211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakense</i>	66.48	5.2	70.57	5.88	16	43	292	32	N/A	N/A	0.9930	YES
Aw37	Pc06g00430	gij211581271	strong similarity to ribonuclease T2 precursor rntB - <i>Aspergillus oryzae</i>	28.75	4.28	30.75	4.66	8	57	706	33	2.22	1.85E-03	0.9950	YES
roteases															
Aw7	Pc21g03510	gij255953325	strong similarity to asperillopepsin apnS - <i>Aspergillus phoenicis</i>	41.40	4.9	40.94	4.71	14	34	906	59	N/A	N/A	0.9980	YES
Aw8	Pc21g03510	gij255953325	strong similarity to asperillopepsin apnS - <i>Aspergillus phoenicis</i>	41.40	4.9	40.26	4.74	18	47	806	63	N/A	N/A	0.9980	YES
Aw9	Pc21g03510	gij255953325	strong similarity to asperillopepsin apnS - <i>Aspergillus phoenicis</i>	41.40	4.9	40.64	4.77	14	34	965	54	N/A	N/A	0.9980	YES
Aw11	Pc20g10240	gij211588252	strong similarity to serine-type carboxypeptidase precursor cpdS - <i>Aspergillus phoenicis</i>	57.9	5.2	62.80	4.92	8	57	341	17	N/A	N/A	1.0000	YES
Aw12	Pc20g10240	gij211588252	strong similarity to serine-type carboxypeptidase precursor cpdS - <i>Aspergillus phoenicis</i>	57.9	5.2	62.57	4.98	6	23	334	12	N/A	N/A	1.0000	YES
Aw13	Pc20g10240	gij211588252	strong similarity to serine-type carboxypeptidase precursor cpdS - <i>Aspergillus phoenicis</i>	57.9	5.2	63.50	5.05	6	59	337	14	N/A	N/A	1.0000	YES

Table 3.8 continued

Aw38	Pc21g06670	gl 211589422	strong similarity to carboxypeptidase S1 - <i>Penicillium janthinellum</i>	63.2	4.6	73.51	4.73	10	55	296	21	N/A	N/A	0.9770	YES
Aw39	Pc21g06670	gl 211589422	strong similarity to carboxypeptidase S1 - <i>Penicillium janthinellum</i>	63.2	4.6	73.51	4.75	11	51	395	27	N/A	N/A	0.9770	YES
Aw40	Pc22g09990	gl 255948482	strong similarity to carboxypeptidase S1 - <i>Penicillium janthinellum</i>	62.19	4.7	71.63	4.84	11	50	663	35	N/A	N/A	0.9730	YES
Plant cell wall degradation															
Aw14	Pc22g20290	gl 211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39.01	5.5	44.17	5.11	16	49	807	68	N/A	N/A	1.0000	YES
Aw15	Pc22g20290	gl 211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39.01	5.5	43.20	5.21	15	31	680	49	N/A	N/A	1.0000	YES
Aw16	Pc22g20290	gl 211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39.01	5.5	43.60	5.27	14	40	459	50	N/A	N/A	1.0000	YES
Aw17	Pc22g20290	gl 211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39.01	5.5	42.80	5.35	12	26	702	53	N/A	N/A	1.0000	YES
Aw18	Pc22g20290	gl 211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39.01	5.5	43.20	5.42	16	49	901	65	N/A	N/A	1.0000	YES
Aw19	Pc22g20290	gl 211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39.01	5.5	41.94	5.48	19	46	1050	68	N/A	N/A	1.0000	YES
Aw20	Pc22g20290	gl 211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39.01	5.5	42.17	5.67	19	36	1060	68	N/A	N/A	1.0000	YES
Aw21	Pc22g20290	gl 211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39.01	5.5	41.70	5.81	16	49	719	68	N/A	N/A	1.0000	YES
Aw22	Pc22g20290	gl 211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	39.01	5.5	42.48	5.93	18	47	719	68	N/A	N/A	1.0000	YES
General metabolism															
Aw4	Pc22g01750	gl 211591236	strong similarity to hypothetical amidase - <i>Schizosaccharomyces pombe</i>	54.03	5.9	68.13	5.75	17	46	854	40	N/A	N/A	0.0000	NO
Aw5	Pc22g01750	gl 211591236	strong similarity to hypothetical amidase - <i>Schizosaccharomyces pombe</i>	54.03	5.9	68.38	5.83	17	46	907	40	N/A	N/A	0.0000	NO
Aw6	Pc22g01750	gl 211591236	strong similarity to hypothetical amidase - <i>Schizosaccharomyces pombe</i>	54.03	5.9	68.77	5.97	15	50	634	32	N/A	N/A	0.0000	NO
Aw11	Pc22g16510	gl 211592582	strong similarity to isoamyl alcohol oxidase <i>meA</i> - <i>Aspergillus oryzae</i>	61.9	4.8	62.80	4.92	10	55	131	25	N/A	N/A	1.0000	YES
Other proteins															
Aw10	Pc20g15500	gl 255946509	strong similarity to hypothetical protein contig1492_0.fta_3030cg - <i>Aspergillus fumigatus</i>	23.96	5.4	24.28	5.50	3	62	420	24	N/A	N/A	0.9970	YES

3.2.5.2. Utilization of nutrients

Several proteins involved in the utilization of alternative carbon sources as auxiliary energy substrates (see before) are overrepresented in the Wis 54-1255 strain, such as three isoforms of a protein encoded by an acetate-inducible gene *aciA* (spots Wa32, Wa33 and Wa35), two isoforms of a probable trehalose metabolism factor Pmu1 (spots Wa28 and Wa29) and two isoforms of a probable sorbitol utilization protein Sou2 (spots Wa42-Wa44). One of the most remarkable differences found between AS-P-78 strain and the Wis 54-1255 strain is represented by phosphatases. Ten isoforms of the acid phosphatase PhoA (spots Aw23-Aw32) and three isoforms of another probable acid phosphatase AFPhoA (spots Aw41-Aw43) are overexpressed in the high producer strain. On the contrary, the Wis 54-1255 strain overexpresses three isoforms of a probable alkaline phosphatase (spots Wa1-Wa3).

3.2.5.3. Cell rescue, virulence and plant cell wall degradation

Another interesting feature shown by the AS-P-78 strain is the overrepresentation of 7 isoforms of a probable glucose oxidase GOX (spots Aw1-Aw3, Aw33-Aw36) and 2 isoforms of a probable non-hemolytic phospholipase C (spots Aw23 and Aw24). Although both strains secrete plant cell wall-degrading enzymes, the overrepresentation of 9 isoforms of a probable polygalacturonase in the AS-P-78 strain is remarkable (spots Aw14-Aw22). The high content of a polygalacturonase is likely due to the selection of mutant colonies showing better growth on plant polymeric materials during the selection procedure to compensate for the slow growth rate of the high penicillin producing strains (Lein, 1986). This pectinolytic enzyme is of relevant importance for beverage industries (see Discussion).

3.2.5.4. Proteases

The high producer strain AS-P-78 showed several proteases overrepresented, such as a probable aspergillopepsin (spots Aw7-Aw9) or carboxypeptidases (spots Aw11-Aw13, Aw38-Aw40). Strikingly, the carboxypeptidase protein included in spot Aw40 is not present in the reference map (strain Wis 54-1255), which indicates that it is specific of the AS-P-78 strain under the conditions used.

3.2.5.5. Specific allergens and other proteins

The Wis 54-1255 strain contained two overrepresented isoforms of the IgE-binding protein ortholog from *A. fumigatus* (spots Wa49 and Wa53). As it was indicated before, some of the proteins overrepresented in the Wis 54-1255 strain lacked predicted signal sequences. This is the case of a probable cyclophilin CypD, which is found in spots Wa46 and Wa47.

3.2.6 Discussion

In this work, we present for the first time, the extracellular proteome reference map of the filamentous fungus *P. chrysogenum*. Of the total number of different proteins in the extracellular proteome of *P. chrysogenum*, 102 out of 131 were predicted to contain either classical or non-classical signal sequences using the SignalP and SecretomeP programs. As it was indicated before, prediction of secretory proteins has been improved by the implementation of the Fungal Secretome Database (Choi *et al.*, 2010), which is based on nine prediction programs (including SignalP and SecretomeP) and has been used to identify putative secretory proteins in 158 fungal/oomycete genomes. The presence of proteins with non-predicted signal sequences in the secreted fraction has also been described in the filamentous fungus *Botrytis cinerea* (Shah *et al.*, 2009). These authors suggested that these proteins could be truly secreted multifunctional proteins with different activities according to their intracellular or extracellular location, a fact that has been confirmed in other organisms (McGoldrick *et al.*, 2005). Therefore, a dual role for those 29 proteins identified in *P. chrysogenum* cannot be excluded, especially when major cell lysis events were not observed at 68h (Figure 3.17). Interestingly, Kiel and coworkers (2009) also identified some of these extracellular proteins in the microbody (peroxisomes) matrix of *P. chrysogenum*, even those presumed to be located on the cytosol, mitochondria or vacuoles (Supplementary Table 5 in the appendix section). An alternative explanation might be given by the selective autophagic degradation of peroxisomes (pexophagy). It is known that in late stage cultures, peroxisomes are integrated into vacuoles by pexophagy (Sakai *et al.*, 2006). Integration of peroxisomes into vacuoles and further degradation may lead to secretion of the proteins located in the peroxisomal matrix by exocytosis, a mechanism that has been discussed as an alternative route for the release of penicillin from

peroxisomes to the culture medium (Martín *et al.*, 2010). In addition, the presence of 29 identified proteins lacking classical or non-classical predicted signal sequences could also be explained by cell lysis. This is very unlikely because proteins that have been reported to be the most abundant in the intracellular proteome of *P. chrysogenum*, such as a flavohemoglobin, a hypothetical ECM33 homolog SPCC1223.12c, a manganese superoxide dismutase or a methyltransferase (Jami *et al.*, 2010) are not detected in the extracellular proteome. Only traces of a probable glyceraldehyde-3-phosphate dehydrogenase (Pc21g14560), which is also relatively abundant in the intracellular proteome of *P. chrysogenum* (Jami *et al.*, 2010), are found in the secretome, suggesting that contribution of proteins from cell lysis to the extracellular proteome is minimal. The case of the latter protein is especially relevant, since it appears to be a multifunctional protein found in several subcellular locations in eukaryotes, displaying functions unrelated to glycolysis, such as membrane transport and fusion, as well as nuclear RNA transport (Singh and Green, 1993; Sirover, 1999). In some pathogenic fungi, this enzyme has been found on the cell wall, where it may have various roles in host–pathogen interactions (Gil-Navarro *et al.*, 1997; Gozalbo *et al.*, 1998; Barbosa *et al.*, 2006). A similar extracellular role for this enzyme is likely in *P. chrysogenum* (Supplementary Table 5 in the appendix section). As an additional control of cell lysis events that might contaminate the secretome at 68 h, we analysed the extracellular proteome at 40 h (Figure 3-23) and searched for those proteins detected at 68 h that lacked a classical secretion signal peptide (58 different proteins included in 118 spots). Results (Table 3.9) indicated that 32 out of the 118 spots were not present at 40 h. This may indicate that 32 out of the 279 spots analyzed in this work (11.47%) may be “contaminating” the extracellular reference map at 68 h. Although some spots including one specific protein are not detected at 40 h, other spots including isoforms of the same protein are detected at this time-point (e.g. Pc16g11860), which indicates that the presence of such protein at 68 h may not be due to cell lysis. In addition, 15 out of the 32 spots not detected at 40 h, included isoforms of 10 different proteins predicted to be secreted through a non-classical pathway. Therefore, it seems that only 17 out of the 279 spots analysed (6.09%) might correspond to contamination from intracellular proteins.

Figure 3.23. Comparison of the extracellular proteomes of *P. chrysogenum* Wis 54-1255 at 40 h and 68 h. Proteins were separated by 2-DE. Those spots present at each time-point were numbered according to the numbers given in Figure 3.19 and Supplementary Table 3. The differences between these time-points are listed in Table 3.9.

Table 3.9. Comparison between the spots detected at 68 h lacking a classical signal peptide and those detected at 40 h.

Protein (ORF name)	Accession Number	Description	Spot	Isoforms	Function	Signal peptide probability	Signal peptide	Non-classically secreted protein	Present in the 40h extracellular proteome?
Pc12g00830	gi211581603	strong similarity to sorbitol utilization protein <i>sou2</i> - <i>Candida albicans</i>	230						NO
			233	4	Utilization of nutrients	0.0210	NO	NO	YES
			234						YES
			238						YES
			164						NO
Pc12g04310	gi211581920	strong similarity to acetate-inducible gene <i>aciA</i> - <i>Aspergillus nidulans</i>	165	3	Utilization of nutrients	0.0020	NO	YES	YES
			166						YES
Pc12g04370	gi211581925	strong similarity to phosphoserine transaminase <i>Ser1</i> - <i>Saccharomyces cerevisiae</i>	117	1	General metabolism	0.0140	NO	NO	NO
Pc12g05380	gi211582002	strong similarity to coproporphyrinogen oxidase III <i>Hem13</i> - <i>Saccharomyces cerevisiae</i>	168	1	General metabolism	0.0000	NO	YES	NO
Pc12g05820	gi211582045	strong similarity to esterase D <i>ESD</i> - <i>Homo sapiens</i>	211	2	General metabolism	0.1650	NO	YES	YES
			213						YES
Pc12g08930	gi211582340	strong similarity to hypothetical protein contig1492_0.tfa_1890cg - <i>Aspergillus fumigatus</i>	205	1	Utilization of nutrients	0.0020	NO	YES	NO
Pc12g09030	gi211582350	strong similarity to fumarylacetoacetase - <i>Homo sapiens</i>	147	1	General metabolism	0.1500	NO	YES	YES
Pc12g09430	gi211582389	strong similarity to alanine transaminase <i>ALA2</i> - <i>Hordeum vulgare</i>	98	1	General metabolism	0.1210	NO	NO	NO
Pc12g09820	gi211582426	strong similarity to glyoxalase I <i>Glo1</i> - <i>Saccharomyces cerevisiae</i>	177	2	Cell rescue, defense and virulence	0.0010	NO	NO	NO
			179						YES
Pc12g10940	gi211582533	strong similarity to phosphogluconate dehydrogenase <i>Gnd1</i> - <i>Saccharomyces cerevisiae</i>	144	1	General metabolism	0.0570	NO	NO	YES
Pc13g04420	gi211583497	orotidine 5-phosphate decarboxylase <i>pyrG</i> - <i>Penicillium chrysogenum</i>	227	1	General metabolism	0.0010	NO	YES	YES

Table 3.9 continued

Pc13g08810	gil211583926	strong similarity to elongation factor 1 beta EF-1 - <i>Oryctolagus cuniculus</i>	255	1	General metabolism	0.0150	NO	NO	YES
Pc13g09520	gil211583995	strong similarity to chitinase 1 precursor <i>chs1</i> - <i>Coccidioides immitis</i>	138	1	Cell wall morphogenesis	0.0030	NO	YES	YES
Pc13g09540	gil211583997	strong similarity to proteasome 20S subunit <i>Scl1</i> - <i>Saccharomyces cerevisiae</i>	231	1	General metabolism	0.0000	NO	NO	YES
Pc13g10260	gil211584068	strong similarity to hypothetical protein contig_1_84_scaffold_5.tfa_850cg - <i>Aspergillus nidulans</i>	184	1	Unknown	0.0000	NO	NO	YES
Pc14g00690	gil211584684	strong similarity to pyruvate dehydrogenase beta chain precursor <i>Pdb1</i> - <i>Saccharomyces cerevisiae</i>	200	1	General metabolism	0.7480	NO	YES	YES
Pc14g01140	gil211584729	strong similarity to proteasome 20S subunit <i>Pre10</i> - <i>Saccharomyces cerevisiae</i>	214	1	General metabolism	0.0000	NO	NO	YES
Pc14g01230	gil211584738	strong similarity to proteasome 20S subunit <i>Pre7</i> - <i>Saccharomyces cerevisiae</i>	237	1	General metabolism	0.0050	NO	YES	YES
			133						YES
			134						YES
			135						YES
Pc14g01740	gil211584789	enolase BAC82549- <i>Penicillium chrysogenum</i>	137	7	General metabolism	0.0000	NO	NO	YES
			139						YES
			140						NO
			278						YES
Pc16g02790	gil211585272	strong similarity to aspartate transaminase like protein Ar08g01000 - <i>Aspergillus niger</i>	167	1	General metabolism	0.0020	NO	YES	YES
Pc16g05080	gil211585478	strong similarity to adenosylhomocysteinase - <i>Homo sapiens</i>	143	1	General metabolism	0.0040	NO	YES	YES
Pc16g08460	gil211585789	strong similarity to sorbitol dehydrogenase <i>gudB</i> - <i>Bacillus subtilis</i>	173	2	Utilization of nutrients	0.0000	NO	NO	YES
			175						NO
Pc16g09710	gil211585905	strong similarity to proliferating cell nuclear antigen <i>pcn1p</i> - <i>Schizosaccharomyces pombe</i>	207	2	Control of cellular functions	0.0000	NO	YES	NO
			209						YES

Table 3.9 continued

12	YES
13	YES
14	NO
15	YES
16	NO
17	NO
18	NO
19	YES
20	YES
21	YES
22	YES
23	NO
24	YES
26	YES
27	YES
29	YES
31	YES
275	YES
105	NO
162	YES

Table 3.9 continued

Pc18g00980	gil211586677	strong similarity to hypothetical trunk lateral cell specific gene HrTLC1 - <i>Halocynthia roretzi</i>	85					YES
			87	3	Cell rescue, defense and virulence	0.0460	NO	YES
			90					YES
			66					YES
Pc18g01390	gil211586717	strong similarity to phosphoglucosyltransferase <i>pgrMB</i> - <i>Aspergillus nidulans</i>	67	4	General metabolism	0.0000	NO	YES
			7					YES
			71					YES
Pc18g02740	gil211586848	strong similarity to regucalcin also known as senescence marker protein-30 like protein An04g03420 - <i>Aspergillus niger</i>	215	2	Control of cellular functions	0.0000	NO	NO
			218					YES
Pc18g05720	gil211587132	strong similarity to hypothetical protein contig. 1_89_scaffold_5.tfa_270cg - <i>Aspergillus nidulans</i>	157	1	General metabolism	0.0000	NO	YES
Pc20g02780	gil211587567	strong similarity to hypothetical protein PA3829 - <i>Pseudomonas aeruginosa</i>	198	1	Plant cell wall degradation	0.0000	NO	NO
Pc20g03330	gil211587620	strong similarity to glucose-6-phosphate 1-dehydrogenase <i>gsdA</i> - <i>Aspergillus niger</i>	92	1	General metabolism	0.0010	NO	YES
Pc20g03610	gil211587647	strong similarity to precursor of mitochondrial isocitrate dehydrogenase <i>icdA</i> - <i>Aspergillus niger</i>	131	2	General metabolism	0.2800	NO	NO
			132					NO
			109					YES
Pc20g04720	gil211587749	strong similarity to precursor of dihydrolipoamide dehydrogenase Lpd1 - <i>Saccharomyces cerevisiae</i>	112	4	General metabolism	0.0690	NO	YES
			114					YES
			115					YES
			239					NO
Pc20g04810	gil211587757	strong similarity to estrogen receptor-binding cyclophilin <i>cypD</i> - <i>Bos primigenius taurus</i>	240	4	Control of cellular functions	0.0000	NO	YES
			241					NO
			243					NO

Table 3.9 continued

Pc20g05320	gil211587803	strong similarity to phosphomutase homolog Pmu1 - <i>Saccharomyces cerevisiae</i>	182	1	Utilization of nutrients	0.0160	NO	NO	YES
Pc20g07230	gil211587983	strong similarity to inorganic pyrophosphatase <i>lpp1</i> - <i>Saccharomyces cerevisiae</i>	210	1	General metabolism	0.0000	NO	NO	YES
Pc20g08410	gil211588099	strong similarity to UDP-glucose 4-epimerase Gal10 - <i>Saccharomyces cerevisiae</i>	170	1	General metabolism	0.1540	NO	NO	YES
Pc20g11630	gil211588384	gamma-actin <i>act-Penicillium chrysogenum</i>	36	1	Cytoskeleton	0.0000	NO	YES	YES
Pc20g15580	gil211588765	strong similarity to NADPH-dependent aldehyde reductase - <i>Sporobolomyces salmonicolor</i>	180	1	General metabolism	0.0030	NO	NO	NO
Pc21g14560	gil211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase <i>gpdA</i> - <i>Aspergillus niger</i>	202	1	General metabolism	0.0000	NO	YES	YES
Pc21g14970	gil211590220	strong similarity to protein D-lactate dehydrogenase YEL071w - <i>Saccharomyces cerevisiae</i>	93	1	General metabolism	0.4280	NO	YES	NO
			199						NO
Pc21g16950	gil211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	201	3	General metabolism	0.0010	NO	NO	YES
			204						YES
Pc21g20440	gil211590742	strong similarity to ribose 5-phosphate isomerase RPI - <i>Mus musculus</i>	226	1	General metabolism	0.0000	NO	NO	YES
Pc21g23240	gil211591002	strong similarity to alpha-isopropylmalate synthase Leu4 - <i>Saccharomyces cerevisiae</i>	146	1	General metabolism	0.0000	NO	NO	NO
Pc22g01750	gil211591236	strong similarity to hypothetical amidase - <i>Schizosaccharomyces pombe</i>	44	2	General metabolism	0.0000	NO	YES	YES
			46						YES
			13						YES
			16						NO
			17						NO
Pc22g02000	gil211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	25	7	General metabolism	0.2920	NO	NO	YES
			28						YES
			30						YES
			62						YES

Table 3.9 continued

Pc22g03140	gil211591374	strong similarity to Gbeta like protein cpcB - <i>Aspergillus nidulans</i>	196	1	Control of cellular functions	0.0050	NO	NO	YES
Pc22g04850	gil211591539	strong similarity to D-arabinose dehydrogenase Ara1 - <i>Saccharomyces cerevisiae</i>	177	2	General metabolism	0.1470	NO	NO	NO
			195						YES
Pc22g09390	gil211591968	strong similarity to mannitol dehydrogenase mtlD - <i>Pseudomonas fluorescens</i>	68	2	Utilization of nutrients	0.0000	NO	NO	YES
			70						YES
			169						YES
Pc22g13130	gil211592257	strong similarity to mitochondrial aspartate aminotransferase mAspAT - <i>Mus musculus</i>	171	3	General metabolism	0.0190	NO	YES	NO
			172						YES
Pc22g15910	gil211592524	strong similarity to cytoplasmic form of aminopeptidase like protein An03g04230 - <i>Aspergillus niger</i>	45	1	Proteases	0.0000	NO	NO	YES
Pc22g17560	gil211592684	glutamate dehydrogenase gdhA - <i>Penicillium chrysogenum</i>	108	2	General metabolism	0.0000	NO	NO	YES
			110						YES
Pc22g19730	gil211592892	strong similarity to glucose-6-phosphate isomerase Pg1 - <i>Saccharomyces cerevisiae</i>	91	1	Utilization of nutrients	0.0000	NO	NO	YES
Pc22g22710	gil211593182	strong similarity to dihydroxy-acid dehydratase Ilv3 - <i>Saccharomyces cerevisiae</i>	64	2	General metabolism	0.0440	NO	YES	NO
			65						YES
Pc22g23760	gil211593284	strong similarity to type 2 peroxiredoxin like protein An12g08570 - <i>Aspergillus niger</i>	250	1	Cell rescue, defense and virulence	0.0000	NO	YES	YES
Pc22g25470	gil211593449	strong similarity to Sol1 - <i>Saccharomyces cerevisiae</i>	229	1	General metabolism	0.0010	NO	YES	YES
	gil119482686	ATP phosphoribosyltransferase - <i>Neosartorya fischeri</i> NRRL 181	185	1	General metabolism	0.0000	NO	YES	YES

■ *Many of the observed extracellular enzymes are clearly related to the saprophytic growth of the fungus on plant material*

The major structural component of the fungal wall is β -1,3-glucan, which serves as an anchor for other polysaccharides, such as chitin and galactomannan (Fontaine *et al.*, 1997). After the biosynthesis of β -1,3-glucan, this compound is subjected to downstream processing leading to extensive branching and crosslinking to other cell wall components (Klis *et al.*, 2002).

The β -1,3-glucanosyltransferases are key enzymes involved in this processing and play active roles in fungal cell wall biosynthesis and morphogenesis. These enzymes have also being related to virulence, since one of the key factors underlying fungal attack on plants is the capacity to grow as hyphae (Madhani and Fink, 1998), a process that requires the polarized biogenesis of the cell wall at the apical tip (Harris and Momany, 2004).

The β -1,3-glucanosyltransferases are present in the extracellular proteome of *P. chrysogenum* together with chitinases and chitosanases. *Coccidioides immitis* secretes in both the saprophytic and parasitic phases a 48-kDa antigen acting as a chitinase (Johnson and Pappagianis, 1992). It was suggested that the active chitinase associates with the autolysis of the parasitic cell wall during early asexual reproductive cycle endosporulation (Cole *et al.*, 1993).

Glucose oxidases are enzymes involved in virulence, since gluconic acid and glucose oxidase have been associated with the pathogenicity of *Penicillium expansum* in apples (Hadas *et al.*, 2007). An extracellular localization of glucose oxidase activity implies an extracellular production of hydrogen peroxide. The sequestration of this toxic compound will prevent damage to the cell and extracellular catalases might be involved in this process.

Extracellular catalases are detected in the culture medium of *P. chrysogenum* as it has been reported for other fungi, such as *Claviceps purpurea*. This microorganism secretes a catalase during infection of rye and the putative role in pathogenicity and suppression of host defense has been discussed (Garre *et al.*, 1998). It has also been reported that *Blumeria graminis* secretes an extracellular catalase during infection of barley (Zhang *et al.*, 2004). The production of extracellular phospholipases has also been shown to be important in the pathogenesis, since they cause damage to host cell

membranes and allow tissue invasion (Meyers *et al.*, 1992). Phospholipases are also produced by certain fungi, including *Candida albicans* and *P. notatum* (Saito *et al.*, 1991; Ibrahim *et al.*, 1995) and could represent a virulence determinant in *Aspergillus fumigatus* (Birch *et al.*, 1996).

■ **Relevance for the food industries**

Some proteins identified in the culture medium of *P. chrysogenum* are specially relevant because of their interest for food industry. This is the case of a probable isoamyl alcohol oxidase, an extracellular enzyme in *A. oryzae* catalyzing the formation of isovaleraldehyde -the main component of mureka that gives to the drink sake an off-flavor (Yamashita *et al.* 1999)- or sulphydryl oxidase. The treatment with sulphydryl oxidase has been reported of value in the removal of a burnt flavour from Ultra-High Temperature (UHT) sterilized milk (Swaigood *et al.*, 1987). Therefore, this enzyme may be used for treatment of bakery products or for removal of off-flavour from milk or beer. Another relevant enzyme for its application in food industry is a probable dihydroxy-acid dehydratase Ilv3, which is related to the biosynthesis of branched-chain amino acids. This enzyme is important to remove vicinal diketones formed by non-enzymatic reactions from intermediaries of the biosynthesis of valine and isoleucine. When vicinal diketones are present above certain threshold, they give foods the flavour of diacetyl (butter flavor or sweaty flavor in beer, “tsuwari-ka” or nauseating flavor in sake), a representative off-flavor in brewed alcoholic beverages such as beer, sake and wine.

Amplification of the *ilv3* gene encoding the dihydroxyacid dehydratase of *S. cerevisiae* has been patented to produce alcoholic beverages with superior flavor (Kodama *et al.*, 2009). Related to this finding is the identification of a probable ketol-acid reductoisomerase Ilv2, which is also involved in the biosynthesis of branched-chain amino acids isoleucine and valine. Also related to these two previous enzymes is the presence of a probable branched-chain-amino-acid aminotransferase Bat2p. During alcoholic fermentation, *S. cerevisiae* produces higher alcohols that can influence the flavour of the end-product, such as the taste of wines (Rankine, 1967). The higher alcohols produced by yeast can originate from the degradation of imported branched-chain amino acids or from endogenous biosynthesis (Didion *et al.*, 1996).

The *P. chrysogenum* Bat2p ortholog is predicted to possess a signal peptide (Supplementary Table 4 in the appendix section) and it has been found in the extracellular proteome. Other proteins that also have an important application for beverage industries are pectinolytic enzymes (see below).

A full arsenal of proteins involved in plant cell wall degradation is identified in the extracellular proteome of *P. chrysogenum*. Among these enzymes, pectinolytic enzymes represented by polygalacturonases or pectate lyases are important for beverage industries and preparation of juices. Other important enzymes are ferulic acid esterases. They hydrolyze the ester linkages of ferulic and diferulic acids present in plant cell walls releasing β -glucan and pentosans from the cell walls, which increases the susceptibility to further enzymatic attack (Bartolomé *et al.*, 1995).

■ *Extracellular proteins as allergens*

Penicillium species are important indoor allergens (Kurup *et al.*, 2009) and a limited number of allergens have been reported for these microorganisms (Su *et al.*, 1999; Chow *et al.*, 1999; Shen *et al.*, 1997; 2000; Chou *et al.*, 2002; Tang *et al.*, 2005). Among the *Penicillium* allergens, alkaline or vacuolar serine proteases have been described as the major allergen of *P. citrinum*, *Penicillium brevicompactum*, *P. chrysogenum* and *Penicillium oxalicum* (Shen *et al.*, 2007). Two proteins that have been shown to possess antigenic properties in the filamentous fungus *P. citrinum* are allergen Pen c 22, which is an enolase with similar immunological characteristics to Asp f22 from *A. fumigatus* (Lai *et al.*, 2002), and allergen Pen c 24, which shows sequence homology with elongation factor 1-b from *A. fumigatus* (Tang *et al.*, 2005). Orthologs of all these proteins are found in the extracellular proteome of *P. chrysogenum*.

■ *Modifications of the extracellular proteome during the industrial strain improvement program*

Comparison of the extracellular proteins of *P. chrysogenum* strains NRRL 1951, Wis 54-1255 and AS-P-78, which represent three different stages of the industrial strain improvement program, represents a further step in the characterization of the protein modifications that occurred during this process (Jami *et al.*, 2010). In general, it seems

that enzymes related to pathogenesis, tissue invasion and infectivity were diminished during the improvement of the wild-type strain, result that correlates well with the fact that the wild-type strain was isolated from an infected cantaloupe. The use of precursors and energy and the diversion of the metabolic fluxes away from the penicillin biosynthetic pathway for these virulence metabolic reactions may be one of the reasons for the very low amount of penicillin produced by the wild-type strain. It is also interesting that proteins involved in stress defense mechanisms are predominant in the Wis 54-1255 strain. Antibiotic production can be considered as a defense mechanism against competing microorganisms. Therefore, it cannot be excluded that the biosynthesis of different proteins involved in defense mechanisms against different agents (oxidative stress, microorganisms) have also been enhanced during the industrial improvement process. In fact, it was observed in our previous work, that one the main changes observed in the high producer strains were increases of stress response proteins (Jami *et al.*, 2010). The amount of pectinolytic enzymes seems to have been increased during the improvement program, specially polygalacturonases, because of the amount and intensity of the isoforms found in the AS-P-78 strain. As it has been discussed above this enzymes are of relevant importance for beverage industries. Other proteins following this pattern are proteases, which are predominant in the culture broths of the high producer AS-P-78.

■ *P. chrysogenum as a cell factory for white biotechnology*

Fungi have been described as nature's toolbox for white biotechnology (Bouws *et al.*, 2008), although there are other systems for the production of useful proteins, such as bacteria, insects, mammalian cell culture, transgenic animal, plant cell cultures and transgenic plants. Each system offers some unique advantages and features regarding yield and purity of the proteins overproduced (Desai *et al.*, 2010). In general, overproduction of proteins in bacteria is a scalable low-cost process, which reaches high yields and purity. The main drawback of this system is protein solubility, together with the presence of minimal post-translational modifications that may be necessary for the correct expression of functional eukaryotic proteins. Therefore, prokaryotic cells can be used only for proteins that do not require post-translational changes for biological activity. Eukaryotic systems are capable of a variety of post-translational modifications,

but are expensive to operate and have longer growth period. Insect expression of proteins show several advantages, such as moderate yields (up to 500 mg/l) and production of proteins with post-translational modifications present in eukaryotic cells, although this system has more demanding culture conditions. Production of useful proteins in yeasts is an easy and less expensive system compared to insect cells that reaches high yields (g/l) and shows the advantage to perform many post-translational modifications of eukaryotic proteins, although growth conditions may require optimization. Yeast expression systems are ideally suited for large-scale production of recombinant eukaryotic proteins. Mammalian host organisms provide highest levels of correct post-translational modifications, but this system provides low yields (mg/l) and has more demanding culture conditions. Plants have emerged as an attractive option for production of heterologous proteins. Plant-based expression system can replace mammalian cell culture and microbial systems for large scale production of recombinant therapeutics, but the expression levels of proteins with this system is still low.

Filamentous fungi are also able to produce and secrete high amounts of homologous and heterologous proteins (g/l), although the levels obtained for heterologous proteins were always much lower than those for fungal proteins (Punt *et al.*, 2002). Secreted proteases by fungal host strains (partially solved by improved protease-deficient host strains) and low secretion levels (improved by the gene-fusion approach or fusion of a secreted fungal protein to the heterologous protein for more efficient secretion), are the major drawbacks for this system. The availability of fungal genomes has allowed a detailed analysis of several fungal secretomes, highlighting the potential of these microorganisms for white biotechnology (Bouws *et al.*, 2008). The versatility of *P. chrysogenum*, its plasticity, its ability to grow on different media and conditions and the availability of several mutants and tools for its genetic manipulation, may confer some advantages on *P. chrysogenum* over other fungi. Due to this potential, the improvement of this microorganism, as it occurred in the past with antibiotic production, for other biotechnological purposes such as the development of optimized enzyme cocktails, is possible.

CONCLUSIONS

- A method for 2-DE analysis of the intracellular and extracellular proteins of the filamentous fungus *P. chrysogenum* has been developed and optimized.
- Data provided by PMF and MS/MS allowed the identification of most of the proteins present in the 2-DE gel, thus confirming the accuracy of the *P. chrysogenum* genome annotation.
- The proteomics and transcriptomics of β -lactam biosynthesis is an excellent model for the study of other poorly-known fungal secondary metabolites.
- The increase in penicillin production along the industrial strain improvement program is a consequence of complex metabolic reorganizations.
- Functional analysis of the *lovC* and *est* genes, encoding two proteins differentially represented in low and high-producing strains of *P. chrysogenum*, allowed us to validate the proteomics results and revealed that these two proteins play a direct role in penicillin biosynthesis.
- The versatility of *P. chrysogenum* for white biotechnology is high due to the ability of this fungus to secrete a variety of enzymes of biotechnological interest.
- The importance of *P. chrysogenum* in the immunological response as indoor allergen has been confirmed after the identification of several proteins with antigenic properties in the culture medium.
- According to our proteomics data, improvement of *P. chrysogenum* to produce higher penicillin titers seems to be still possible by manipulation of specific genes different from those encoding the penicillin biosynthetic enzymes.

PUBLICATIONS FROM THIS THESIS

1- **Jami, M. S.**, García-Estrada, C., Barreiro, C., Cuadrado, A.A., Salehi-Najafabadi, Z., Martín, J. F. (2010) The *Penicillium chrysogenum* extracellular proteome. Conversion from a food-rotting strain to a versatile cell factory for white biotechnology. Molecular & Cellular Proteomics, 2010 Sep 7. [Epub ahead of print]

2- **Jami, M. S.**, Barreiro, C., García-Estrada, C., and Martín, J. F. (2010) Proteome analysis of the penicillin producer *Penicillium chrysogenum*: Characterization of protein changes during the industrial strain improvement. Molecular & Cellular Proteomics 9.6, 1182-1198.

RESUMEN

OBJETIVOS

Ochenta años después del descubrimiento de la penicilina, el interés de este antibiótico β -lactámico a nivel industrial sigue siendo notable. Sin embargo, a pesar del conocimiento que se tiene del hongo productor de este antibiótico, *Penicillium chrysogenum*, poco se sabe sobre cómo este microorganismo se convirtió en un superproductor de penicilina tan bueno, siendo aún desconocidas gran parte de las bases moleculares responsables de la mejora de la productividad. Se ha arrojado algo de luz sobre esta cuestión después de la reciente publicación del genoma de *P. chrysogenum* (van den Berg *et al.*, 2008). En este trabajo se describe que la transcripción de los genes implicados en la biosíntesis de los aminoácidos precursores de la penicilina, así como de los genes que codifican proteínas de los microcuerpos, es mayor en la cepa DS17690 de alta producción. Sin embargo, para conseguir explotar completamente a *P. chrysogenum* se requiere la integración de los conocimientos de otras-ómicas, como la proteómica.

En este trabajo, se han optimizado los métodos para extraer proteínas citosólicas (proteoma) del *P. chrysogenum*, así como proteínas secretadas al medio de cultivo (secretoma). Como resultado, se han desarrollado los mapas de referencia del proteoma citosólico y secretoma de este hongo filamentoso. También se han caracterizado las modificaciones producidas durante el programa de mejora industrial de cepas, mediante el análisis del proteoma de tres cepas de *P. chrysogenum*: la cepa silvestre NRRL 1951 aislada en Peoria, IL (USA), la cepa de referencia del proyecto genoma Wisconsin 54-1255 (una cepa mejorada pero aún de baja producción de antibiótico) y la cepa de alta producción AS-P-78 desarrollada por Antibióticos SA (León, España). Por último, para completar este trabajo y validar los resultados obtenidos, también se ha realizado un análisis funcional de dos genes que codifican dos de las proteínas que muestran una expresión diferencial entre las tres cepas anteriormente mencionadas.

INTRODUCCIÓN

Penicillium chrysogenum

Penicillium chrysogenum es un hongo filamentosos que se encuentra ampliamente distribuido en la naturaleza, viviendo a menudo sobre los alimentos y en los ambientes interiores. A partir de este hongo, se obtiene a gran escala en fermentadores industriales el antibiótico penicilina. La penicilina actúa sobre las bacterias Gram-positivas, como *Staphylococcus* y *Streptococcus pneumoniae* mediante la interrupción de la síntesis de la pared celular bacteriana (Samson *et al.*, 1977). Raramente se ha asociado a *P. chrysogenum* con enfermedades humanas, pero las esporas de *P. chrysogenum* liberadas en el ambiente constituyen importantes alérgenos humanos. Las principales proteínas que han sido implicadas como los principales alérgenos son las proteasas vacuolares y las serina proteasas (Shen *et al.*, 2003). *P. chrysogenum* se caracteriza por la producción de diversos tipos de metabolitos secundarios, entre los que se incluyen varias penicilinas diferentes, roquefortina C, meleagrina, crisogenina, xantocilinas, ácido secalónico, sorrentanona, sorbicilina, y toxina PR (De Hoog *et al.*, 2000). Por ello, *P. chrysogenum* se ha utilizado industrialmente para producir penicilina y xantocilina X, para tratar los residuos de celulosa, y para producir las enzimas poliamina oxidasa, fosfo-gluconato deshidrogenasa y glucosa oxidasa (Raper *et al.*, 1949, De Hoog *et al.* 2000).

Penicillium es un miembro de la familia de los deuteromicetos, que incluye hongos sin ciclo sexual conocido. Sin embargo, recientemente se han descrito tipos de individuos opuestos para el apareamiento en los que ambos tipos han conservado activos los genes que codifican una feromona y un receptor de feromonas necesario para la reproducción sexual (Hoff *et al.*, 2008). Algunas otras especies de *Penicillium* también tienen un estado sexual adicional y están incluidas en la división Ascomycota del orden Eurotiales (Samson *et al.*, 1977).

Mejora Industrial de *P. chrysogenum*

Debido a la baja cantidad de penicilina (1,2 µg/ml) producida por la cepa original de Fleming *Penicillium notatum* (NRRL1249B21), el aislamiento de nuevas cepas se convirtió

en algo fundamental. La mejora de la cepa original se produjo en 1943 con el aislamiento de *P. chrysogenum* NRRL 1951 a partir de un melón en Peoria, Illinois, EE.UU. (Raper *et al.*, 1944). Este microorganismo era más adecuada que *P. notatum* para la producción de penicilina (60 µg/ml a 150 µg/ml) en cultivos sumergidos, por lo que fue sometido a tratamiento con rayos X por el Dr. Demerec (Carnegie Institution en Cold Spring Harbor, Nueva York, 1955), dando lugar al mutante X-1612. Este mutante era capaz de producir 300 µg/ml de penicilina, siendo nuevamente sometido a mutación ultravioleta en la Universidad de Wisconsin (USA). Después de varias rondas de mutagénesis clásica se obtuvo la cepa Q-176, que producía 550 µg/ml de penicilina. Esta cepa fue adoptada por la industria y es el antepasado original de la línea de las cepas Wisconsin, como la cepa de baja producción Wisconsin 54-1255 (Elander, 1983) y las cepas posteriores (Elander, 2002). Estas cepas dieron origen a las cepas de alta producción de penicilina, como la cepa P2 (Panlabs, Taiwán) (Lein, 1986), la cepa DS04825 (DSM, Holanda) (Gouka *et al.*, 1991) o las cepas E1 y AS-P-78, ambas obtenidas por la empresa Antibióticos SA (León, España). Algunas de estas cepas son los ancestros de las utilizadas actualmente para la producción industrial de penicilina, que alcanza títulos de más de 50 mg/ml.

Las numerosas rondas de mutagénesis realizadas con las cepas de *P. chrysogenum* han conducido a la introducción de varias modificaciones. Los análisis bioquímicos y genéticos han permitido la identificación de algunas de ellas. Es bien sabido que la ruta del homogentisato para el catabolismo del ácido fenilacético (el precursor de la cadena lateral en la biosíntesis de bencilpenicilina) se ve disminuida en la cepa Wisconsin 54-1255 y, presumiblemente, también en las cepas derivadas de ésta. Esto se debe a modificaciones en la monooxigenasa citocromo P450 microsomal (codificada por el gen *phacA*), la cual está implicada en la 2-hidroxilación del ácido fenilacético. *P. chrysogenum* Wisconsin 54-1255 tiene una mutación L181F en el gen *phacA*, que conduce a una reducción de la oxidación del ácido fenilacético y a la sobreproducción de penicilina (Rodríguez-Sáiz *et al.*, 2001).

Otra modificación resultante del proceso de mejora industrial que está bien caracterizada es la amplificación de la región génica que incluye los tres genes de biosíntesis de penicilina. Estos genes, *pcbAB*, *pcbC* y *penDE*, están agrupados en una región de ADN que se amplifica en tándem en las cepas de alta producción de penicilina y

que se encuentra delimitada por el hexanucleótido conservado TGTAAA/T. La unidad que se amplifica tiene una longitud de 106,5 kb o 56,8 kb, según la cepa y está presente como copia única en el genoma de las cepas NRRL 1951 y Wisconsin 54-1255. Sin embargo, muchas de las cepas mejoradas superproductoras de penicilina contienen varias copias de la región amplificada, como la cepa AS-P-78, que contiene 5 ó 6 copias (Fierro *et al.*, 1995; Newbert *et al.*, 1997).

Tras la secuenciación y análisis bioinformático de la región amplificada de 56,8 kb se encontraron un total de 15 ORFs en la cepa Wisconsin 54-1255 (van den Berg *et al.*, 2007), y 16 ORFs en la cepa AS-P-78 (Fierro *et al.*, 2006). Tres de ellos corresponden a los genes de biosíntesis de penicilina (*pcbAB*, *pcbC* y *penDE*), mientras que el resto de ORFs no tienen una función clara, ya que ninguna de los mismos está relacionado con la formación de precursores de la penicilina, la propia penicilina o el transporte y la regulación de la biosíntesis de la misma. Sorprendentemente, varios genes conocidos por jugar una función importante en la biosíntesis de antibióticos en los hongos no están incluidos en esta región, como el gen *phl* (que codifica la fenilacetil-CoA ligasa) (Lamas-Maceiras *et al.*, 2006) o la fosfopanteteinil transferasa (PPTase) codificada por el gen *ppt* (García-Estrada *et al.*, 2008a).

También se sabe que los microcuerpos (peroxisomas), unos orgánulos en los que tienen lugar las últimas etapas de la ruta de biosíntesis de la penicilina (activación de la cadena lateral a través de la fenilacetil-CoA ligasa y sustitución, mediante el concurso de la aciltransferasa, de la cadena lateral α -aminoadipil de la isopenicilina N (IPN) por dicha cadena lateral activada), están presentes en mayor número en las cepas de alta producción (Muller *et al.*, 1991; van den Berg *et al.*, 2008).

La estructura de los antibióticos β -lactámicos

Los antibióticos β -lactámicos, como muchos otros metabolitos secundarios, tienen una estructura química inusual. Todos los antibióticos β -lactámicos contienen un anillo β -lactámico de cuatro miembros cerrado por un enlace amida.

Las penicilinas contienen un núcleo bicíclico “penam” formado por fusión de los anillos β -lactámico y tiazolidínico y una cadena lateral acilo unida al grupo amino en C-6 y

son producidas por unas pocas especies de los géneros *Penicillium* y *Aspergillus* (Aharonowitz *et al.*, 1992). Recientemente se ha encontrado que las penicilinas también se producen por algunos otros deuteromicetos (Laich *et al.*, 1999; 2002; 2003). Un segundo compuesto de antibióticos β -lactámicos, la cefalosporina C, es producida por los hongos *Acremonium chrysogenum*, *Paecilomyces persicinus*, *Kallichroma tethys* (Kim *et al.*, 2003) y algunos otros deuteromicetos. Este antibiótico contiene el núcleo cephem (un anillo de seis miembros dihidrotiazínico fusionado al anillo β -lactámico) y una cadena D- α -aminoadipil unida al grupo amino en C-7. Las penicilinas y cefalosporinas son de gran interés clínico como inhibidores de la biosíntesis del peptidoglicano en las bacterias. Dos grupos de cefalosporinas modificadas, las cefamicinas y la cefabacinas se producen, respectivamente, por varios actinomicetos Gram-positivos y algunas bacterias Gram-negativas (Aharonowitz *et al.*, 1992). En las cefamicinas, el núcleo cephem contiene, además de la cadena lateral α -aminoadipil, un grupo metoxi en C-7, lo que hace que estos compuestos sean resistentes a la mayoría de las β -lactamasas (Liras *et al.*, 1998).

Todos estos compuestos β -lactámicos (penicilinas, cefalosporinas, cefamicinas y cefabacinas) comparten un modo de acción común y se sintetizan a partir de precursores similares a través de rutas biosintéticas que comparten algunas etapas en común.

Biosíntesis de penicilina. Compartimentalización de los intermediarios y las enzimas

La ruta de biosíntesis de penicilina tiene lugar en diferentes compartimentos celulares, lo que permite la separación espacial de los precursores y las enzimas y la regulación y optimización de los procesos involucrados. La distribución de la biosíntesis de penicilina en diferentes compartimentos subcelulares implica el transporte de enzimas, precursores, intermediarios y productos a través de estos compartimentos. Por tanto, cada etapa enzimática se lleva a cabo en un orgánulo que tiene sus propias condiciones ambientales óptimas. La ruta de biosíntesis de penicilina se inicia con la condensación no ribosómica de los aminoácidos L- α -aminoadípico, L-cisteína y L-valina para formar el tripéptido δ -(L- α -aminoadipil)-L-cisteinil-D-valina (ACV). Este paso se cataliza por la

ACV sintetasa, que es una enzima de ~420 kDa (Díez *et al.*, 1990; Byford *et al.*, 1997; Martín, 2000a). En estudios iniciales, se encontró que la ACV sintetasa estaba asociada a la membrana de estructuras que se identificaron como orgánulos parecidos al aparato de Golgi (Kurylowicz *et al.*, 1987). Experimentos adicionales de fraccionamiento celular ubicaron a la ACV sintetasa junto con o dentro de las vacuolas (Müller *et al.*, 1991; Lendenfeld *et al.*, 1993). Sin embargo, el pH óptimo para la actividad de la ACV sintetasa *in vitro* (pH = 8,4, el cual es mayor que el del pH vacuolar), el requisito de co-factores y la sensibilidad a proteasas, indicaron que esta enzima era una enzima citosólica. Dicha localización citosólica fue confirmada más tarde por técnicas de microscopía electrónica (van der Lende *et al.*, 2002). La ACV sintetasa se sintetiza en forma de apoproteína inactiva que se activa (forma holo) por medio de una fosfopanteteinil transferasa (PPTase) independiente, la cual une covalentemente el grupo 4'-fosfopanteteína (derivado de la coenzima A) a la ACV sintetasa (Baldwin *et al.*, 1991a; Lambalot *et al.*, 1996). Por lo tanto, las enzimas PPTasas son necesarias para la biosíntesis de la penicilina, como se vio inicialmente en *A. nidulans* (Lambalot *et al.*, 1996; Keszenman-Pereyra *et al.*, 2003; Márquez-Fernández *et al.*, 2007) y en *P. chrysogenum* (García-Estrada *et al.*, 2008a). Este tipo de PPTasas deben encontrarse en el citosol debido a su función en la activación de la ACV sintetasa y de la α -aminoadipato reductasa. Esta última enzima, que participa en la ruta biosintética de lisina en hongos, convierte el α -aminoadipato en α -aminoadipato semialdehído (Casqueiro *et al.*, 1998; Ehmann *et al.*, 1999; Guo *et al.*, 2001), y se sintetiza en el citosol (Bhattacharjee, 1985; Matsuyama *et al.*, 2006). Se ha observado que mutantes de *P. chrysogenum* defectuosos en la PPTasa, son auxótrofos de lisina debido a la falta de activación de la α -aminoadipato reductasa (García-Estrada *et al.*, 2008a).

Los aminoácidos precursores del tripéptido ACV se proporcionan a través de la síntesis *de novo*, aunque también pueden ser captados del medio de cultivo. El ácido L- α -aminoadípico, un aminoácido no proteinogénico, se capta del medio de cultivo a través de la permeasa de aminoácidos ácidos y a través de la permeasa general de aminoácidos (Viaje *et al.*, 2004). A diferencia de la L-cisteína y la L-valina, que son productos finales del metabolismo primario, el ácido L- α -aminoadípico es un intermediario clave en la ruta del metabolismo de la lisina en hongos (Bhattacharjee, 1985; Bañuelos *et al.*, 1999; 2000;

Nishida y Nishiyama, 2000; Zabriskie y Jackson, 2000). Las mitocondrias son orgánulos esenciales para la síntesis de este aminoácido precursor, ya que las enzimas responsables de la biosíntesis del ácido α -aminoadípico, tales como la homocitrato sintetasa y la homoisocitrato deshidrogenasa, se encuentran en la matriz mitocondrial (Jaklitsch y Kubicek, 1990; Kubicek *et al.*, 1990). Una vez que estos aminoácidos precursores son sintetizados, se almacenan en las vacuolas (Lendenfeld *et al.*, 1993). Estos orgánulos regulan los niveles de los aminoácidos L- α -aminoadipato y cisteína, que son tóxicos a concentraciones moderadas (Klionsky *et al.*, 1990; Kubicek-Pranz y Kubicek, 1991).

Tras la formación de ACV en el citosol, este compuesto actúa como sustrato de la IPN sintasa (38 kDa), que cicla el tripéptido para formar IPN, un intermediario que ya contiene el núcleo β -lactámico y posee actividad antibiótica (Ramos *et al.*, 1985; Carr *et al.*, 1986; Baldwin *et al.*, 1987; 1991b; Roach *et al.*, 1997). La IPN sintasa se comporta como una enzima soluble (Abraham *et al.*, 1981), aunque su actividad en los extractos libres de células parece ser estimulada por la adición de Triton X-100 o la sonicación (Sawada *et al.*, 1980). Esta proteína está localizada en el citosol junto con la ACV sintetasa, tal y como se confirmó por microscopia electrónica (Müller *et al.*, 1991; Van der Lende *et al.*, 2002).

La última etapa de la biosíntesis de penicilina implica la sustitución de la cadena lateral L- α -aminoadipil de la IPN por una cadena lateral más hidrofóbica. Esta reacción se lleva a cabo por la acil-CoA:IPN aciltransferasa o IAT (Álvarez *et al.*, 1987; Barredo *et al.*, 1989; Tobin *et al.*, 1990; Whiteman *et al.*, 1990). La IAT se expresa como un precursor de 40 kDa (proIAT) que sufre un procesamiento autocatalítico entre los residuos Gly102-Cys103 en *P. chrysogenum*. La forma activa está formada por dos subunidades; α (11 kDa, correspondiente al fragmento N-terminal) y β (29 kDa, correspondiente al fragmento C-terminal) (Barredo *et al.*, 1989; Veenstra *et al.*, 1989; Tobin *et al.*, 1990; 1993; Whiteman *et al.*, 1990). La IAT cataliza *in vivo* seis reacciones diferentes relacionadas con la rotura de la IPN (lo que libera ácido 6-aminopenicilánico o 6-APA) y la sustitución de la cadena lateral (Álvarez *et al.*, 1993) para la biosíntesis de penicilinas hidrofóbicas (bencilpenicilina o fenoximetilpenicilina), las cuales muestran un aumento significativo en su actividad antimicrobiana en comparación con la IPN. Las IATs de *P. chrysogenum* y

Aspergillus nidulans contienen en su extremo C-terminal una señal funcional de localización peroxisomal de tipo PTS1 (ARL y ANI, respectivamente). La localización de la IAT de *P. chrysogenum* en el interior de estos orgánulos se ha confirmado mediante microscopía electrónica (Müller et al., 1991; 1992; 1995; García-Estrada et al., 2008b). Además, se ha demostrado que el transporte de la IAT al interior peroxisomal no depende del estado de procesamiento de la enzima, ya que mutantes de este enzima incapaces de procesarse, se localizan correctamente dentro de los peroxisomas, aunque carezcan de actividad (García-Estrada et al., 2008b).

La activación de la cadena lateral (ácido fenilacético para la bencilpenicilina o ácido fenoxiacético para la fenoximetilpenicilina) mediante la formación de un tioéster con coenzima A (fenilacetil-CoA o fenoximetil-CoA) es un prerequisite para la biosíntesis de penicilina (Álvarez et al., 1993). Esta reacción está catalizada por aril-CoA ligasas, las cuales activan ácidos acilo o arilo usando ATP, para formar acil-AMP o acil-AMP, respectivamente. Tras la activación, el AMP se libera y el grupo carboxilo se transfiere al grupo tiol de la coenzima A formándose un tioéster. Se ha detectado al menos una aril-CoA ligasa implicada en la activación del ácido fenilacético en *P. chrysogenum* (Lamas-Maceiras et al., 2006) aunque podrían existir otras (Wang et al., 2007). De igual modo que la IAT, esta fenilacetil-CoA ligasa contiene una señal de localización peroxisomal en su extremo C-terminal y se localiza en los microcuerpos (Gledhill et al., 1997). La localización de la IAT y la fenilacetil-CoA ligasa en los peroxisomas se ha confirmado recientemente mediante el aislamiento físico de estos orgánulos y la identificación de las proteínas peroxisomales mediante espectrometría de masas (Kiel et al., 2009).

Proteómica

La proteómica es una de las herramientas más poderosas para evaluar el resultado final de la expresión génica, siendo la electroforesis bidimensional (2-DE) la técnica de elección para la obtención de una visión global del mapa de referencia de un proteoma.

Esta técnica ha sido aplicada con éxito en varios hongos ascomicetos, como *Schizosaccharomyces pombe* (Hwang et al., 2006), algunos *Aspergillus* (Melin et al., 2002;

Kniemeyer *et al.*, 2006; Vödisch *et al.*, 2009) o *Saccharomyces cerevisiae* (Shevchenko *et al.*, 1996; Bruckmann *et al.*, 2009). Sin embargo, sólo Kiel y colaboradores (2009) habían realizado un inventario de las proteínas presentes en la matriz de los microcuerpos de *P. chrysogenum*. Por lo tanto, es de gran interés la optimización y el análisis del mapa de referencia del proteoma de *P. chrysogenum*.

El uso de gradientes de pH inmovilizados en gel (IPG) y la técnica 2-DE (Görg *et al.*, 1998, Görg *et al.*, 2000) combinados con la identificación de proteínas por espectrometría de masas (Aebersold *et al.*, 2003) es actualmente el caballo de batalla de la proteómica. A pesar de la reciente aparición de otras prometedoras tecnologías alternativas/complementarias (por ejemplo, la identificación multidimensional de proteínas, el etiquetado de isótopos estables, matrices de proteínas) (Aebersold *et al.*, 2003, Washburn *et al.*, 2001), la técnica 2-DE es actualmente la única que puede ser aplicada de manera rutinaria para la cuantificación de los perfiles de expresión de grandes conjuntos de mezclas de proteínas complejas, como lisados de células enteras.

Sea cual sea la tecnología utilizada, el análisis del proteoma es técnicamente difícil, porque el número de las diferentes proteínas que se expresan en un momento dado bajo unas condiciones biológicas determinadas, está en el rango de varios miles para los organismos simples procariotas y en más de 10000 en extractos de células eucariotas.

Por otra parte, los estudios actuales de proteómica han revelado que la mayoría de las proteínas identificadas son aquellas que participan en el mantenimiento celular, mientras que proteínas como las que actúan de moléculas receptoras y que están presentes en concentraciones mucho más bajas, generalmente no se detectan. En consecuencia, se requieren mejores métodos para el enriquecimiento de las proteínas de baja abundancia, como los procedimientos de fraccionamiento previo, así como métodos de cuantificación y detección más sensibles (Görg *et al.*, 2004).

La técnica de 2-DE conjuga el isoelectroenfoque (IEF) en la primera dimensión con el SDS-PAGE en la segunda dimensión, y permite la separación de mezclas complejas de proteínas de acuerdo al PI, Mr, la solubilidad y la abundancia relativa. Dependiendo del tamaño del gel y del pH de gradiente utilizado, la técnica de 2-DE puede resolver más de 5000 proteínas simultáneamente (2000 proteínas rutinariamente) y puede detectar 1 ng de

proteína por punto (spot). Además, ofrece un mapa de las proteínas intactas, lo que refleja cambios en el nivel de expresión de éstas, la presencia de isoformas o la existencia de modificaciones post-traduccionales. Esto contrasta con los métodos basados en LC-MS/MS, los cuales realizan análisis de péptidos perdiéndose la información del Mr y pI, además de que requieren un marcado estable con isótopos para el análisis cuantitativo.

Una de las mayores ventajas de la técnica 2-DE es su capacidad para estudiar las proteínas que han sufrido alguna modificación post-traducciona (como fosforilaciones, glicosilaciones o proteólisis limitada) y que pueden, en muchos casos, ser fácilmente ubicadas en geles 2-DE, ya que aparecen como series de puntos distintos en la horizontal y/o vertical del gel. Además, la técnica 2-DE no sólo proporciona información sobre las modificaciones de las proteínas y/o los cambios en sus niveles de expresión, sino que también permite el aislamiento de cantidades de proteínas (μg) para efectuar análisis estructurales adicionales por MALDI-TOF MS, MS-ESI o microsecuenciación de Edman (Beranová-Giorgianni *et al.*, 2003, Celis *et al.*, 1999, Rabilloud 2002).

Las limitaciones iniciales con respecto a la reproducibilidad, la resolución, la separación de proteínas muy ácidas y/o muy básicas y la capacidad de carga de las muestras, se han superado en gran medida por la introducción de IPGs comerciales para la primera dimensión. La superposición de gradientes de pH de rango estrecho ha proporcionado una mayor resolución ($\text{dpi} = 0,001$) (Görg *et al.*, 1985) y favorecido la detección de las proteínas de baja abundancia (al Westbrook *et al.*, 2001), mientras que la separación de proteínas alcalinas de hasta un $\text{pH} = 12$ se ha conseguido bajo condiciones de equilibrio dinámico (Drews *et al.*, 2004).

Las principales etapas de la técnica de 2-DE-MS incluyen: (i) la preparación de muestras y solubilización de proteínas; (ii) la separación de proteínas por 2-DE, (iii) la detección y cuantificación de proteínas, (iv) el análisis asistido por ordenador de los patrones obtenidos por 2-DE, (v) la identificación y caracterización de proteínas, (vi) la construcción de bases de datos con las proteínas identificadas por 2-DE (Dunn *et al.*, 1993).

MATERIALES Y MÉTODOS

Cepas y condiciones de crecimiento

Para la realización de este trabajo se emplearon tres cepas de *P. chrysogenum*: la cepa silvestre NRRL 1951, la cepa Wisconsin 54-1255 (cepa de referencia para el proyecto de secuenciación del genoma) y la cepa de alta producción AS-P-78 (amablemente cedida por Antibioticos S.A., León, España). Estas cepas fueron cultivadas en medio sólido Power durante siete días a 28 °C. Los conidios procedentes de una placa de Petri se recolectaron y se inocularon en un matraz con 100 ml de medio definido PMMY. Los cultivos se incubaron durante 40 h (para los experimentos de comparación de los proteomas intracelulares de varias cepas), 48 h (para el mapa de referencia del proteoma intracelular) o 68 h (para el mapa de referencia del proteoma extracelular y experimentos de comparación de los proteomas extracelulares de varias cepas) a 25 °C y 250 rpm.

Obtención de extractos proteicos extracelulares e intracelulares para el análisis proteómico

Para la extracción de las proteínas extracelulares, las muestras procedentes de los cultivos se filtraron a través de una membrana Nytal. El medio que contiene las proteínas extracelulares se centrifugó inmediatamente a 4 °C y 4500 rpm durante 5 min para descartar cualquier resto de micelio. El sobrenadante se filtró en hielo con filtros de membrana (Millipore) de 0,45 µm de poro y una bomba de vacío. El sobrenadante filtrado se mezcló con un volumen igual de una solución al 20% (p/v) de ácido 2,2,2-tricloroacético (TCA)/acetona previamente enfriada que contenía 0,14% (p/v) de 1,4-ditiotreitol (DTT). La mezcla se incubó durante toda la noche a -20 °C. Las proteínas precipitadas se separaron del sobrenadante mediante centrifugación a 4500 rpm durante 5 minutos y 4 °C. El precipitado se lavó dos veces con acetona fría que contenía 0,07% (p/v) de DTT, y una vez con una solución al 80% (v/v) de acetona/agua-MQ. La concentración de proteínas se determinó según el método de Bradford. El precipitado final se resuspendió en tampón de muestra: 8 M de urea, 2% (w/v) de 3-[(3-cholamidopropyl) dimethylammonio]-1-

propanesulfonate (CHAPS), 0,5% (v/v) de anfólitos, 25 mM de DTT y 0,002% de azul de bromofenol) y se almacenó a -80 °C.

Para la extracción de proteínas intracelulares, los micelios congelados se lavaron dos veces con una solución al 0,9% (p/v) de NaCl y se molieron hasta la obtención de un polvo fino en un mortero previamente enfriado con nitrógeno líquido. Dos gramos de este polvo se resuspendieron en 10 ml de tampón fosfato potásico 10 mM (K_2HPO_4 : KH_2PO_4) (pH 7,4) (1g/5ml) que contenía 0,07% (p/v) de 1,4-ditiotreitol (DTT) y pastillas (una por cada 10 ml de solución tampón) del cóctel inhibidor de proteasas Complete™ (Roche). Se agitó la mezcla a 4 °C durante 2 h y el extracto se clarificó dos veces por centrifugación a 13200 rpm durante 5 min. Las proteínas se precipitaron durante 1 hora a -20 °C tras la adición de 1 volumen de TCA al 20% en acetona que contenía 0,14% (p/v) de DTT. El precipitado final se lavó dos veces con acetona, seguido de un lavado final con 80% de acetona y se solubilizó en 500 µl de tampón de muestra. La fracción insoluble se descartó por centrifugación a 13200 rpm durante 5 min. El sobrenadante se recogió y la concentración de proteínas se determinó según el método de Bradford.

Electroforesis en geles bidimensionales (2-DE)

Para realizar la electroforesis bidimensional se cargó una solución que contenía 350 µg de proteínas (solubilizadas en el tampón de muestra) en tiras IPG de 18 cm (GE Healthcare) con un rango de pH no lineal de 4-7 ó 3-10, según los experimentos. Las proteínas se enfocaron a 20 °C según el siguiente programa: 1 h 0 V, 12 h 30 V (rehidratación), 2 h 60 V, 1 h 500 V, 1 h 1000 V, 30 min de gradiente hasta 8000 V y hasta 7 h a 8000 V hasta alcanzar 50 kVh. Tras el isoelectroenfoque, las tiras fueron equilibradas dos veces durante 15 min en una solución que contiene 50 mM Tris HCl (pH 8.8), 6 M de urea, 30% (v/v) de glicerol, 2% (p/v) de sodio dodecilsulfato (SDS), 0.002% de azul de bromofenol y 1% (p/v) de DTT. Para la segunda etapa de equilibrado, el DTT se sustituyó por 4.0% (p/v) de yodoacetamida. La segunda dimensión se llevó a cabo por SDS-PAGE en geles de poliacrilamida a una concentración del 15% (para el mapa de referencia intracelular) o del 12,5% (para el resto de experimentos). Para ello se empleó un aparato Ettan Dalt Six (GE Healthcare) durante 45 min con una potencia de 3 W por gel, para a

continuación, aplicar durante 4 horas 18 W por gel. Como marcador de pesos moleculares se empleó el preparado comercial “Precision Plus Protein Standards” (Bio-Rad). Los geles se tiñeron con plata mediante un protocolo compatible con la espectrometría de masas o con Coomassie coloidal "Blue Silver" utilizando 0.12% de azul de Coomassie G-250 (Sigma), 10% de sulfato de amonio, 10% de ácido fosfórico y 20% de metanol.

Análisis de la expresión diferencial de proteínas

Las imágenes 2D fueron obtenidas tras escanear los geles teñidos con un aparato ImageScanner II (GE Healthcare), previamente calibrado mediante el uso de un marcador de escala de grises (Kodak) y digitalizadas con el programa informático Labscan 5,00 (v 1.0.8) (GE Healthcare). El análisis de las imágenes se llevó a cabo con el programa informático 2D ImageMaster™ Platino v 5.0 (GE Healthcare).

Identificación de proteínas por MALDI-TOF MS y MS/MS

Los puntos (spots) que contenían las proteínas de interés fueron extraídos del gel, colocados en un tubo Eppendorf y lavados dos veces con ddH₂O. Las proteínas fueron digeridas según el método de Havlis y colaboradores (2003) y las muestras fueron analizadas en un espectrómetro de masas 4800 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems). Para la identificación de proteínas, tanto los espectros de MS como los de MS/MS se buscaron automáticamente usando una licencia local de Mascot v 2.2 (Matrix Science) a través del servidor de proteínas global (GPS) v 3.6 (Applied Biosystems).

Predicción del mecanismo de secreción

La predicción de la presencia de motivos de señal de secreción se llevó a cabo mediante los programas informáticos SignalP (para motivos clásicos de señales de secreción) o SecretomeP (para motivos de señales no clásicos de secreción) (Bendtsen et al., 2004a; b).

Construcción de vectores de silenciamiento y transformación

Los plásmidos pJL43RNAi-*lovC* o pJL43RNAi-*est* fueron diseñados para generar mutantes silenciados basándonos en la maquinaria de producción de ARN pequeños interferentes (siRNA), los cuales desencadenan el mecanismo de silenciamiento génico.

Los oligonucleótidos LOV-NcoI-F y LOV-NcoI-R se utilizaron para la amplificación por PCR de 452 bp del gen *lovC* a partir de la cepa *P. chrysogenum* Wisconsin 54-1255. Para la amplificación de las primeras 431 pb del gen *est*, se utilizaron los oligonucleótidos EST-NcoI-F y EST-NcoI-R. El sitio de restricción *NcoI* se añadió a la secuencia de cada oligonucleótido para la posterior digestión enzimática y clonación de las bandas amplificadas. Éstas fueron digeridas con *NcoI* y se ligaron al plásmido de silenciamiento pJL43RNAi, previamente digerido con *NcoI*, para dar lugar a las construcciones finales pJL43RNAi-*lovC* (6459 pb) y pJL43RNAi-*est* (6438 pb).

Un total de 2×10^7 protoplastos de *P. chrysogenum* Wisconsin 54-1255 se transformaron con 10 µg de DNA (plásmidos pJL43RNAi-*lovC* o pJL43RNAi-*est*). Hasta 20 transformantes resistentes a fleomicina fueron seleccionados en cada transformación y analizados. El ADN genómico de todos los transformantes se extrajo y se verificó por PCR utilizando los primers FP-pcbC y RP-gpd2 diseñados a partir de la secuencia del vector. Los transformantes positivos fueron analizados por la técnica de Southern y los transformantes confirmados fueron estudiados en detalle mediante RT-PCR (para el análisis de la transcripción de genes) y bioensayo (para el análisis de la producción de penicilina).

RESULTADOS Y DISCUSIÓN

Uno de los esfuerzos más importantes realizados para modificar microorganismos de interés biotecnológico ha sido llevado a cabo por la industria de los antibióticos. *P. chrysogenum* ha sido ampliamente mutado en las últimas décadas para aumentar los títulos de penicilina, pero a pesar de la importancia de este microorganismo, las bases bioquímicas que han sido modificadas durante el proceso de mejora de este microorganismo aún no se conocen bien. En este estudio, hemos llevado a cabo por primera vez un amplio estudio de los mapas de referencia bidimensionales de los proteomas intracelulares y extracelulares del hongo productor de la penicilina *P. chrysogenum* con una alta resolución y reproducibilidad, junto con un detallado estudio de los cambios proteicos producidos durante el programa de mejora industrial de cepas.

Análisis del proteoma intracelular de *P. chrysogenum* y caracterización de los cambios sufridos en las proteínas citosólicas durante el desarrollo de cepas de alta producción

Como primera parte de este trabajo, se llevó a cabo el análisis del proteoma intracelular, identificándose un gran número de proteínas (950 que representan 549 proteínas diferentes) que fueron clasificadas según su función con una distribución similar a la observada para el ascomiceto *Schizosaccharomyces pombe* (Hwang *et al.*, 2006). Ya que *P. chrysogenum* es importante para la producción de metabolitos secundarios, en especial penicilinas, nos centramos en las proteínas relacionadas con la biosíntesis de estos compuestos. En este trabajo se identificaron las dos proteínas implicadas en las dos últimas etapas de la ruta de biosíntesis de penicilina (IPN sintasa e IAT). Sin embargo, la primera enzima de la ruta, la ACV sintetasa, no pudo ser identificada debido a su elevado peso molecular (425 kDa). Sólo la subunidad β de la IAT fue detectada. Como se mencionó anteriormente, la IAT es una enzima peroxisomal sintetizada como una preproteína inactiva de 40-kDa, que sufre un procesamiento autocatalítico entre los residuos Gly102-Cys103, dando lugar a un heterodímero formado por las subunidades α (11 kDa, que corresponde al fragmento N-terminal) y β (29 kDa, correspondiente a la región C-terminal) (Whiteman *et*

al., 1990; Tobin *et al.*, 1993; García-Estrada *et al.*, 2008b). Aunque la subunidad α (11-kDa) no ha sido identificado en nuestro mapa de referencia, Kiel y colaboradores (2009) encontraron mediante la técnica de nano-LC-MS/MS la subunidad α en pequeñas cantidades durante el análisis de las proteínas peroxisomales de *P. chrysogenum*. No podemos descartar que la subunidad α de 11-kDa sufra un procesamiento posterior, lo que dificultaría su identificación por PMF.

■ *Modificaciones del proteoma intracelular durante el programa de mejora industrial de cepas*

La optimización de la electroforesis bidimensional para *P. chrysogenum* nos permitió realizar un análisis comparativo detallado entre la cepa silvestre NRRL 1951, la cepa de baja producción Wisconsin 54-1255 y la cepa de alta producción AS-P-78. En la cepa silvestre, predominan las proteínas implicadas en la virulencia y la degradación de la pared celular de plantas para la obtención de nutrientes (esta cepa fue aislada de un melón y por tanto, es capaz de infectar y crecer sobre frutas) (Raper *et al.*, 1944). Una de las razones de la escasa cantidad de penicilina producida por la cepa silvestre podría ser el uso de los precursores y la energía para la biosíntesis de otros metabolitos secundarios, tales como los terpenoides (3-hidroxi-3-metilglutaril-CoA), porfirinas (coproporfirinógeno oxidasa) o pigmentos como violaxantina (zeaxantina epoxidasa) y la melanina (scytalone deshidratasa), lo cual desviaría los flujos metabólicos lejos de la ruta de biosíntesis de penicilina. Se sabe que durante los tratamientos mutagénicos desde la cepa Q-176 a la cepa BL3-D10 (precursores de la cepa Wisconsin 54-1255), se seleccionaron cepas sin el pigmento amarillo crisogenina (García-Rico *et al.*, 2008).

Las proteínas implicadas en la biosíntesis o degradación de los aminoácidos precursores están representadas en las tres cepas de modo distinto. En la cepa silvestre NRRL 1951, el aumento de los niveles de la metilmalonato semialdehído deshidrogenasa podría ser otra de las razones de la baja biosíntesis de penicilina por parte de esta cepa. Esta enzima está implicada en la “vía distal” del catabolismo de la valina (Goodwin *et al.*, 1989; Zhang *et al.*, 1996), conduciendo al agotamiento de las reservas de valina. La biosíntesis de cisteína parece ser una de las principales mejoras que se produjeron durante el proceso de selección. El flujo de producción de penicilina está fuertemente influenciado por la

disponibilidad de cisteína para la formación de ACV (Nasution *et al.*, 2008). La cisteína sintasa (encargada de la biosíntesis de cisterna a partir de serina) está más representada en la cepa Wisconsin 54-1255 que en la cepa silvestre. Esta mejora se conserva en la cepa AS-P-78 que, además, mostró mayor contenido de la enzima cistationina beta sintasa, una enzima implicada en la biosíntesis de cisteína a partir de metionina por transulfuración. Esto indica que en la cepa AS-P-78 se han mejorado las dos vías de biosíntesis de cisteína.

En la cepa Wisconsin 54-1255 son predominantes las proteínas encargadas de la obtención de energía a partir de carbohidratos. Estas proteínas pertenecen en su mayoría al ciclo de los ácidos tricarboxílicos, aunque también hay enzimas de la glicolisis y de la ruta de la fosforilación oxidativa. Es probable que durante el proceso de mejora de cepas, los mecanismos de obtención de energía fuesen beneficiosos para satisfacer la creciente demanda para la biosíntesis de penicilina.

Un hallazgo interesante es que la cepa Wisconsin 54-1255 produce mayores cantidades, en comparación con la cepa de alta producción AS-P-78, de una proteína que podría estar relacionada con la modificación o degradación de penicilina. Esta enzima es similar a una 1,4-butanodiol diacrilato esterasa BDA1 (implicada en la conversión de 1,4-butanodiol diacrilato en 4-hidroxibutil acrilato) y contiene un dominio que está conservado en la clase C de β -lactamasas y otras proteínas de unión a penicilina. Esta proteína (denominada a partir de aquí EST) muestra similitud con una supuesta transesterasa (LovD) de *Aspergillus clavatus* (85% de homología, 72% de identidad) y una β -lactamasa de *Paracoccidioides brasiliensis* (48% de homología, 32% de identidad). La transesterasa LovD añade 2-metilbutirato a monacolina J para formar lovastatina. Por lo tanto, la proteína EST podría unirse al núcleo de la penicilina contribuyendo a su modificación o degradación. La disminución de los niveles de esta proteína en la cepa AS-P-78 en comparación con las cepas silvestre y Wisconsin 54-1255 podría ser uno de los mecanismos que han contribuido al aumento de los títulos de la penicilina en esta cepa industrial. Los resultados del análisis funcional del gen que codifica esta proteína (*est*) mostraron una correlación inversa entre el nivel de la expresión del gen *est* y la producción de penicilina, lo que sugiere que la proteína EST podría actuar como una β -lactamasa.

Se sabe que durante la generación de la cepa de alta producción de penicilina AS-P-78, se produjo la amplificación del grupo de genes de biosíntesis de penicilina (*pcbAB-pcbC-penDE*) (Fierro *et al.*, 1995). Sin embargo, esta amplificación no supuso el aumento correlativo de la síntesis de las correspondientes proteínas a pesar de haber habido un ligero incremento en la transcripción de estos genes. Esto está de acuerdo con los resultados previos obtenidos para el gen *pcbC* (Kosalková *et al.*, 2007). De hecho, se ha descrito que no existe una correlación lineal entre el aumento del número de copias de este grupo de genes y el incremento en los títulos de penicilina (Newbert *et al.*, 1997). Estos resultados indican que, a pesar de que la amplificación de los genes biosintéticos tiene un efecto positivo sobre la producción de penicilina, éste no es el principal mecanismo que transformó la cepa Wisconsin 54-1255 en un gran productor de penicilina.

Otra modificación que podría haber servido de base para el aumento de los títulos de penicilina en la cepa AS-P-78 es la sobreproducción de enzimas de la ruta de las pentosas fosfato. Las enzimas de la fase no oxidativa de esta ruta, como la ribosa-5-fosfato isomerasa o la transcetolasa, son predominantes en la cepa de alta producción. Además, algunas enzimas que intervienen en la síntesis *de novo* de pirofosfato de tiamina, se encuentran más representadas en la cepa de alta producción. El pirofosfato de tiamina es un grupo prostético presente en numerosas enzimas. Una de estas enzimas es la transcetolasa (Shreve *et al.*, 1983), que conecta la ruta de las pentosas fosfato con la glicolisis mediante la introducción del exceso de azúcares fosfato en las principales rutas metabólicas de carbohidratos. Es probable que se originen altas concentraciones de ribulosa-5-fosfato (junto con poder reductor en forma de NADPH) en la cepa de alta producción a través de la fase oxidativa de la ruta de las pentosas fosfato, por lo que el exceso de este azúcar fosfato sería convertido por la ribosa-5-fosfato isomerasa en ribulosa-5-fosfato (precursora de nucleótidos y ácidos nucleicos) y por la transcetolasa en precursores para la glicolisis. El aumento en los niveles de NADPH se ha correlacionado positivamente con la producción de antibióticos β -lactámicos (Jørgensen *et al.*, 1995; Henriksen *et al.*, 1996; van Gulik *et al.*, 2000). Se sabe que la producción de penicilina en las cepas de alta producción implica una gran suministro de NADPH (Kleijn *et al.*, 2007) (la biosíntesis de un mol de penicilina requiere 8.10 moles de NADPH). Además, se ha descrito que la producción de penicilina

está fuertemente influenciada por el nivel de ATP y la concentración de cisteína. Ya que el NADPH es necesario para la biosíntesis de este aminoácido, existe una relación positiva entre los niveles de cisteína y NADPH y la producción de penicilina (Nasution *et al.*, 2008). El NADPH también es necesario para reducir el glutatión oxidado que se deriva de las tasas de aireación intensa durante el estrés oxidativo (al Díez *et al.*, 1998). Los resultados obtenidos en este trabajo demostraron que varias proteínas implicadas en la respuesta al estrés oxidativo estaban más representadas en la cepa AS-P-78.

Algunas diferencias observadas en la cepa AS-P-78 se corresponden con los datos proporcionados por la respuesta transcripcional de una cepa de *P. chrysogenum* modificada para producir ácido adipil-7-amino-3-carbamoiloximetil-3-cefem-4-carboxílico (ad7-ACCCA). Esto puede ser debido a que la cepa de *P. chrysogenum* utilizada en este estudio era una cepa superproductora de penicilina. Esta respuesta incluía una regulación positiva de la transcripción de una supuesta aflatoxina B1 aldehído reductasa, dos glutatión S-transferasas, una citocromo p450 monooxigenasa y una quinona oxidorreductasa de NADPH (Harris *et al.*, 2009). Estos resultados apuntan a que la respuesta al estrés oxidativo constituye un mecanismo de adaptación a la superproducción de penicilina.

Una visión global de las proteínas expresadas diferencialmente en estas tres cepas reveló que la representatividad de algunas rutas y funciones se había ampliado durante el proceso de mejora, mientras que para otros casos éstas habían disminuido. La glucosa oxidasa es una proteína cuya representatividad está aumentada en la cepa silvestre en comparación con las cepas de alta producción. La escasa representación de esta enzima asociada con la infectividad de plantas en las cepas de alta producción no es sorprendente, ya que los cultivos líquidos sumergidos han sustituido al crecimiento natural sobre frutas. Probablemente, la pérdida más notable que se produjo durante la selección de estas cepas fue la que implica a algunas rutas del metabolismo secundario, lo cual potenció la biosíntesis de antibióticos β -lactámicos. Los pigmentos constituyen uno de los mejores ejemplos de metabolitos secundarios que se perdieron durante la manipulación de la cepa silvestre para obtener la cepa Wisconsin 54-1255, como es el caso del pigmento amarillo. Durante la selección de la cepa de alta producción AS-P-78, otras rutas del metabolismo secundario parecen haber sido bloqueadas, como sugiere la ausencia en la cepa AS-P-78 de

una supuesta isoflavona reductasa, la cual participa en la última parte de la ruta biosintética de medicarpina.

Por otra parte, hay proteínas involucradas en otras rutas o funciones, cuya representatividad ha aumentado durante el programa de mejora de cepas. Es curioso que una de estas proteínas (enoilreductasa LovC, Pc20g05830), que participa en la biosíntesis del precursor de lovastatina, dihidromonacolina L, esté más representada en la cepa Wisconsin 54-1255 comparado con la cepa silvestre y también en la cepa AS-P-78 en comparación con la cepa Wisconsin 54-1255. También se ha descrito que existe una regulación positiva del gen que codifica la enoilreductasa LovC, como una de las respuestas transcripcionales de la cepa de *P. chrysogenum* que produce ad7-ACCCA (Harris *et al.*, 2009). Queda por establecer si esta proteína está verdaderamente implicada en la biosíntesis de lovastatina o si es una proteína indirectamente relacionada con la biosíntesis de penicilina. Aunque existen otros genes relacionados con la biosíntesis de lovastatina en el genoma de *P. chrysogenum*, no están agrupados, por lo que es poco probable que este gen sirva para la biosíntesis de lovastatina. Los resultados del análisis funcional del gen *lovC* de *P. chrysogenum* reflejaron una relación directa entre el nivel de transcripción de este gen y la producción de penicilina, lo que confirma su papel en la biosíntesis de este antibiótico. La proteína LovC de *P. chrysogenum* tiene un dominio alcohol deshidrogenasa de tipo GroEs (entre los residuos 25-89) y un dominio deshidrogenasa de unión a Zinc (residuos 161-242). La secuencia de aminoácidos de la proteína LovC de *P. chrysogenum* posee una similitud del 84% (74% de identidad) con una supuesta oxidorreductasa de unión a zinc ToxD de *A. clavatus* NRRL 1 y una similitud del 54% (38% de identidad) con una enoilreductasa LovC de *A. terreus*. En *Aspergillus* sp. LovC participa en la biosíntesis del policétido lovastatina. La agrupación génica para la biosíntesis de lovastatina en *Aspergillus* sp. contiene dos genes policétido sintasa de tipo I. La síntesis del principal esqueleto derivado del nonacétido requiere una lovastatina nonacétido sintasa reiterativa (LNKS), además de al menos una proteína adicional (LovC) que interactúa con LNKS y es necesaria para el correcto procesamiento de la cadena policétida creciente y la producción de dihidromonacolina L. La lovastatina dicétido sintasa (LDKS) no reiterativa es una enzima que especifica la formación de 2-metilbutirato e

interactúa estrechamente con un transesterasa adicional (LovD) responsable del ensamblado de la lovastatina a partir de este policétido y monacolina J (Kennedy *et al.*, 1999). Un mecanismo de acción similar se ha postulado para ToxD, que participa en la biosíntesis de péptidos cíclicos en el hongo fitopatógeno *Cochliobolus carbonum*, el cual produce la toxina HC (Pedley *et al.*, 2001). Teniendo en cuenta las similitudes entre ToxD, LovC y la proteína LovC de *P. chrysogenum* y debido a la correlación positiva existente entre esta proteína y la producción de penicilina, no podemos descartar que LovC esté implicado en la biosíntesis y/o procesamiento del tripéptido ACV. Además, el hecho de que los mutantes silenciados en la expresión de *lovC* muestren una disminución en el color verde de las conidias, indica que la proteína LovC podría estar implicada en diferentes rutas del metabolismo secundario. La naturaleza y la función del pigmento verde es desconocida, pero puede ser un policétido cuya síntesis y procesamiento también se vea modificada por LovC. La naturaleza policétido de este pigmento se ve apoyada por el hecho de que los mutantes defectuosos en la enzima fosfopanteteinil transferasa (necesaria para la activación de las policétido sintetas y péptido sintetasas no ribosomales), o en el regulador global del metabolismo secundario LaeA, hayan perdido este pigmento verde (García -Estrada *et al.*, 2008a; Kosalková *et al.*, 2009).

Análisis del proteoma extracelular (secretoma) de *P. chrysogenum* y caracterización de las modificaciones sufridas en las proteínas extracelulares durante el proceso de mejora industrial de cepas

En una segunda fase de este trabajo nos propusimos analizar la fracción de proteínas secretadas por *P. chrysogenum* al medio de cultivo. Como resultado, presentamos por primera vez, el mapa de referencia del proteoma extracelular de este hongo filamentoso. De un total de 131 proteínas diferentes identificadas en el proteoma extracelular de *P. chrysogenum*, 102 contenían secuencias correspondientes a señales clásicas o no clásicas de secreción, tal y como predijeron los programas SignalP y SecretomeP. Por tanto, las proteínas sin señales de secreción (29 de 131) constituyen un bajo porcentaje. La presencia de proteínas sin señales de secreción también se ha descrito en el hongo filamentoso *Botrytis cinerea* (Shah *et al.*, 2009), donde se sugiere que estas proteínas podrían ser

proteínas multifuncionales realmente secretadas que tendrían diferentes actividades en función de su localización intracelular o extracelular, un hecho que ha sido confirmado en otros organismos (McGoldrick *et al.*, 2005). Por lo tanto, no podemos descartar una doble función para las 29 proteínas identificadas en *P. chrysogenum*, sobre todo cuando no se observaron procesos de lisis celular al momento de extraer estas proteínas (68h).

Curiosamente, Kiel y colaboradores (2009) también identificaron algunas de estas proteínas extracelulares en la matriz de los microcuerpos (peroxisomas) de *P. chrysogenum*, incluso las que supuestamente tenían una localización citosólica, mitocondrial o vacuolar. Por tanto, una explicación alternativa podría ser la denominada degradación selectiva autofágica de los peroxisomas (pexofagia). Se sabe que en la última etapa de los cultivos, los peroxisomas se integran en vacuolas mediante el fenómeno de la pexofagia (Sakai *et al.*, 2006), pudiéndose liberar su contenido al medio de cultivo. La integración de los peroxisomas en las vacuolas y la posterior degradación podría conducir a la secreción de las proteínas localizadas en la matriz peroxisomal por exocitosis, un mecanismo que ha sido propuesto como una ruta alternativa para la liberación de la penicilina al medio de cultivo a partir de los peroxisomas (Martín *et al.* 2010).

La presencia de 29 proteínas sin señales clásicas o no clásicas de secreción también se puede atribuir a mecanismos de lisis celular. Esto es muy poco probable porque las proteínas que se han identificado como las más abundantes en el proteoma intracelular de *P. chrysogenum*, como es el caso de una flavohemoglobina, un hipotético homólogo ECM33 SPCC1223.12c, una manganeso superóxido dismutasa o una metiltransferasa, no se detectaron en el proteoma extracelular. Sólo se detectaron trazas de una probable gliceraldehído-3-fosfato deshidrogenasa (Pc21g14560), que también es relativamente abundante en el proteoma intracelular de *P. chrysogenum*, lo que sugiere que la contribución de las proteínas provenientes de la lisis celular al proteoma extracelular es mínima. El caso de esta última proteína es especialmente interesante, ya que parece ser una proteína multifuncional que se ha encontrado en varias localizaciones dentro de las células eucariotas mostrando funciones no relacionadas con la glicolisis, tales como el transporte de membrana, así como el transporte nuclear de ARN (Singh y Green, 1993; Sirover, 1999). En algunos hongos patógenos, esta enzima se ha encontrado en la pared celular,

donde podría desempeñar diversas funciones relacionadas con la interacción hospedador-patógeno (Gil-Navarro *et al.*, 1997; Gozalbo *et al.*, 1998; Barbosa *et al.*, 2006). Por tanto, no se puede descartar que esta enzima juegue un papel similar en el medio de cultivo al ser secretada por *P. chrysogenum*.

Para descartar que la presencia de ciertas proteínas a 68 h se debiese a procesos de lisis celular y que el secretoma pudiese estar contaminado con dichas proteínas, se analizó el proteoma extracelular a las 40 h y se buscaron en el mismo las proteínas detectadas a las 68 h que carecían de una secuencia clásica de secreción (58 proteínas diferentes incluídas en 118 puntos). Los resultados indicaron que 32 de los 118 puntos no estaban presentes a las 40 h. Esto podría indicar que 32 de los 279 puntos analizados en el secretoma (11,47%) podrían estar “contaminando” el mapa de referencia extracelular a 68 h. Aunque algunos de los puntos que incluyen una proteína específica no se detectaron a las 40 h, otros puntos que incluyen isoformas de esa misma proteína sí que se detectaron a ese tiempo (por ejemplo, Pc16g11860), lo que indica que la presencia de dicha proteína a 68 h podría no ser debida a procesos de lisis celular. Además, 15 de los 32 puntos que no se detectaron a las 40 h, incluían isoformas de 10 proteínas diferentes que podrían secretarse a través de una vía no clásica, tal y como se predijo mediante el programa SecretomeP. Por lo tanto, podríamos considerar que sólo 17 de los 279 puntos analizados (6,09%) corresponderían a contaminación procedente de proteínas intracelulares, lo que supone un bajo porcentaje y confirma la autenticidad del mapa de referencia extracelular.

■ *Muchas de las enzimas extracelulares están claramente relacionadas con el crecimiento saprofítico del hongo sobre material vegetal*

El principal componente estructural de la pared fúngica es el β -1,3-glucano, que sirve como punto de anclaje para otros polisacáridos, como la quitina y el galactomanano (Fontaine *et al.*, 1997). Tras la biosíntesis de β -1,3-glucano, este compuesto sufre un procesamiento posterior que conduce a una extensa ramificación y entrecruzamiento con otros componentes de la pared celular (Klis *et al.*, 2002). Las β -1,3-glucanosiltransferasas son enzimas clave implicadas en este procesamiento y desempeñan una función activa en la biosíntesis de la pared celular fúngica y la morfogénesis. Estas enzimas también están relacionadas con la virulencia, ya que uno de los factores claves subyacentes del ataque

fúngico es la capacidad de crecer en forma de hifas (Madhani y Fink, 1998), un proceso que requiere la biogénesis polarizada de la pared celular en el extremo apical (Harris y Momany, 2004). Las β -1,3-glucanosiltransferasas se detectaron en el proteoma extracelular de *P. chrysogenum*, junto con quitinasas y quitosanasas. *Coccidioides immitis* secreta, tanto en las fase sapróbica como parasitaria, un antígeno de 48 kDa que actúa como una quitinasa (Johnson y Pappagianis, 1992). Se ha sugerido que la presencia de una quitinasa activa está asociada con la autólisis de la pared celular durante la endosporulación en las etapas tempranas del ciclo reproductivo asexual (Cole *et al.*, 1993).

Las glucosa oxidasas son enzimas que intervienen en la virulencia, puesto que el ácido glucónico y la glucosa oxidasa se han asociado con la patogenicidad de *Penicillium expansum* en manzanas (Hadas *et al.*, 2007). Una localización extracelular de la actividad glucosa oxidasa implica una producción extracelular de peróxido de hidrógeno. El secuestro de este compuesto tóxico evita cualquier daño a la célula, pudiendo estar implicadas en este proceso las catalasas extracelulares. Estas enzimas se detectaron en el medio de cultivo de *P. chrysogenum*, así como en otros hongos como *Claviceps purpurea*. Este microorganismo segrega una catalasa durante la infección del centeno, habiéndose discutido su posible función en la patogenicidad y en la supresión de la defensa del hospedador (Garre *et al.*, 1998). También se ha descrito que *Blumeria graminis* secreta una catalasa extracelular durante la infección de la cebada (Zhang *et al.*, 2004).

También se ha demostrado que la producción de fosfolipasas extracelulares es importante en la patogénesis, ya que estas enzimas causan daño a las membranas celulares de los organismos hospedadores y permiten la invasión tisular (Meyers *et al.*, 1992). Estas enzimas se detectaron en el secretoma de *P. chrysogenum* y también son producidas por ciertos hongos, como *Candida albicans* y *P. notatum* (Saito *et al.*, 1991; Ibrahim *et al.*, 1995) y podría representar un factor de virulencia en *Aspergillus fumigatus* (Birch *et al.*, 1996).

■ Importancia para la industria alimentaria

Algunas proteínas identificadas en el medio de cultivo de *P. chrysogenum* son especialmente relevantes por su interés para la industria alimentaria. Este es el caso de una probable alcohol isoamílico oxidasa, una enzima extracelular en *A. oryzae* que cataliza la

formación de isovaleraldehído -el principal componente del mureka que da mal sabor al sake (Yamashita *et al.* 1999)- o de una sulfhidrilo oxidasa. El tratamiento con la sulfhidrilo oxidasa tiene gran valor en la eliminación del sabor a quemado generado en la leche esterilizada al aplicarle tratamientos UHT (ultra high temperature) (Swaigood *et al.*, 1987). Por lo tanto, esta enzima podría usarse para el tratamiento de productos de panadería o para la eliminación de mal sabor de la leche o la cerveza. Otra enzima importante por su papel en la industria alimentaria es una probable dihidroxiácido deshidratasa Ilv3, que está relacionada con la biosíntesis de aminoácidos de cadena ramificada. Esta enzima es importante para eliminar las dicetonas contiguas unidas a dos átomos de carbono adyacentes formadas a través de reacciones no enzimáticas a partir de intermediarios de la biosíntesis de valina e isoleucina. Cuando estas dicetonas están presentes por encima de cierto umbral, proporcionan a los alimentos el sabor “diacetil” (sabor a mantequilla, sabor “sweaty” de la cerveza o "tsuwari-ka" o sabor nauseabundo del sake), un mal sabor presente en las bebidas alcohólicas elaboradas, como la cerveza, el sake o el vino. La amplificación del gen *ilv3* que codifica la dihidroxiácido deshidratasa de *S. cerevisiae* ha sido patentada para producir bebidas alcohólicas con un sabor superior (Kodama *et al.*, 2009). Relacionado con este tema, se identificó una probable cetol-ácido reductoisomerasa Ilv2, que también participa en la biosíntesis de los aminoácidos de cadena ramificada isoleucina y valina. También, en relación con estas dos enzimas anteriores, se identificó una probable aminotransferasa de aminoácidos de cadena ramificada Bat2p. Durante la fermentación alcohólica, *S. cerevisiae* produce alcoholes de cadena larga que pueden influir en el sabor del producto final, como es el caso del vino (Rankine, 1967). Estos alcoholes producidos por la levadura pueden provenir de la degradación de aminoácidos de cadena ramificada importados o provenientes de la biosíntesis endógena (Didion *et al.*, 1996). El ortólogo de Bat2p se encontró e identificado en el proteoma extracelular de *P. chrysogenum* y posee una señal de secreción.

Otras proteínas que también tienen una aplicación importante en la industria alimentaria son las enzimas pectinolíticas. En el proteoma extracelular de *P. chrysogenum* se identificó un arsenal completo de proteínas implicadas en la degradación de la pared celular de plantas. Entre estas enzimas, las enzimas pectinolíticas, representadas por

poligalacturonasas o pectato liasas, son importantes para la industria de bebidas y elaboración de zumos. Otras enzimas importantes son las esterasas del ácido ferúlico, que hidrolizan los enlaces éster de los ácidos ferúlico y diferúlico presentes en las paredes celulares de plantas liberando β -glucano y pentosanós, lo que aumenta la susceptibilidad al ataque enzimático posterior (Bartolomé *et al.*, 1995).

■ *Proteínas extracelulares con propiedades alergénicas*

Las especies de *Penicillium* son importantes alérgenos de interior (Kurup *et al.*, 2009), habiéndose descrito un número limitado de alérgenos para estos microorganismos (Su *et al.*, 1999; Chow *et al.*, 1999; Shen *et al.*, 1997; 2000; Chou *et al.*, 2002; Tang *et al.*, 2005). Entre éstos, las serina proteasas alcalinas o vacuolares constituyen el alérgeno principal de *P. citrinum*, *Penicillium brevicompactum*, *P. chrysogenum* y *Penicillium oxalicum* (Shen *et al.*, 2007). Los alérgenos Pen c 22, que es un enolasa con similares características inmunológicas que el alérgeno Asp f22 de *A. fumigatus* (Lai *et al.*, 2002), y Pen c 24, que muestra homología con el factor de elongación 1-b de *A. fumigatus* (Tang *et al.*, 2005), son dos proteínas que se ha demostrado que poseen propiedades antigénicas en el hongo filamentoso *P. citrinum*. Ortólogos de todas estas proteínas se identificaron en el proteoma extracelular de *P. chrysogenum*.

■ *Modificaciones del proteoma extracelular durante el programa de mejora industrial de cepas*

La comparación de las proteínas extracelulares de las tres cepas de *P. chrysogenum* NRRL 1951, Wisconsin 54-1255 y AS-P-78, las cuales representan tres etapas del programa industrial de mejora de cepas, supone un paso más en la caracterización de las modificaciones que se produjeron durante este proceso. En general, parece que las enzimas relacionadas con la patogénesis, la invasión tisular e infectividad sufrieron una disminución en su representatividad durante el proceso de mejora de la cepa silvestre, resultado que se correlaciona bien con el hecho de que esta cepa fuese aislada de un melón infectado. El uso de precursores y energía y la deriva de flujos metabólicos lejos de la ruta de biosíntesis de penicilina para llevar a cabo estas reacciones metabólicas de virulencia podrían explicar las bajas concentraciones de penicilina producidas por la cepa NRRL 1951. También es interesante que las proteínas implicadas en los mecanismos de defensa al estrés sean

predominantes en la cepa Wisconsin 54-1255. La producción de antibióticos puede ser considerada como un mecanismo de defensa contra microorganismos competidores. Por lo tanto, no puede excluirse que se haya mejorado la biosíntesis de diferentes proteínas que participan en mecanismos de defensa contra los diversos agentes (estrés oxidativo, microorganismos) durante el proceso industrial. De hecho, como se observó en el apartado anterior, uno de los principales cambios observados en las cepas de alta producción fue el incremento de proteínas intracelulares de respuesta al estrés.

Durante el programa de mejora, también parece haberse incrementado la cantidad de enzimas pectinolíticas, especialmente las poligalacturonasas, debido a la cantidad e intensidad de las isoformas detectadas en la cepa AS-P-78. Como ya se indicó anteriormente, estas enzimas son importantes para la industria de bebidas. Otras proteínas que siguen este patrón son las proteasas, que son predominantes en los caldos de cultivo de la cepa de alta producción AS-P-78.

■ *P. chrysogenum como una factoría celular para la biotecnología blanca*

Los hongos se han descrito como una herramienta de la naturaleza para la biotecnología blanca (Bouws *et al.*, 2008), aunque hay otros sistemas para la producción de proteínas útiles, tales como bacterias, insectos, cultivos celulares de mamíferos, animales transgénicos, cultivos de células vegetales o plantas transgénicas. Cada sistema ofrece varias ventajas y características únicas en relación con el rendimiento y la pureza de las proteínas sobreproducidas (Desai *et al.*, 2010). En general, la sobreproducción de proteínas en las bacterias es un proceso escalable de bajo coste, que alcanza altos rendimientos y pureza. El principal inconveniente de este sistema es la solubilidad de la proteína junto con la presencia de mínimas modificaciones post-translacionales que puedan ser insuficientes para la correcta actividad de las proteínas eucariotas. Los sistemas eucariotas son capaces de introducir una variedad de modificaciones post-translacionales, pero son caros y requieren períodos más largos de crecimiento. Los hongos filamentosos son también capaces de producir y secretar grandes cantidades de proteínas (g/l) homólogas y heterólogas aunque los niveles de proteínas heterólogas obtenidos son siempre muy inferiores a los de las proteínas homólogas (Punt *et al.*, 2002). Las proteasas secretadas por las cepas de hongos productores (un problema parcialmente solucionado mediante la

mejora de cepas deficientes en proteasas) y los bajos niveles de secreción (mejorado por el método de fusión génica o fusión de una proteína secretada por hongos a la proteína heteróloga para una secreción más eficiente), son los mayores inconvenientes de este sistema. La disponibilidad de los genomas de varios hongos ha permitido realizar un análisis detallado de sus secretomas, subrayando el potencial de estos microorganismos para la biotecnología blanca (Bouws *et al.*, 2008). La versatilidad de *P. chrysogenum*, su plasticidad, su capacidad para crecer en diferentes medios y condiciones y la disponibilidad de diversos mutantes y herramientas para su manipulación genética, constituyen algunas ventajas sobre otros hongos. Debido a este potencial, es posible la mejora de este microorganismo (como ocurrió en el pasado con la producción de antibióticos) para otros fines biotecnológicos como la elaboración de cócteles optimizados de enzimas.

En conclusión, hemos demostrado que el aumento de la producción de penicilina a lo largo del programa industrial de mejora de cepas es una consecuencia de complejas reorganizaciones metabólicas. Aunque el análisis del proteoma y secretoma nos ha permitido descifrar algunas de los mecanismos que condujeron a la producción de alta títulos de penicilina, muchos otros procesos quedan aún por esclarecerse, como la función de los transportadores asociados a membrana en los flujos de penicilina, precursores de la penicilina o intermediarios (Martín *et al.*, 2010). Los datos aportados aquí demuestran que a pesar de las rondas de mutagénesis clásica y la selección artificial sufridas por este microorganismo exclusivamente para aumentar la producción de antibióticos β -lactámicos, la versatilidad de este hongo filamentoso es aún alta. Esta afirmación está apoyada en la capacidad de este microorganismo para secretar una gran variedad de enzimas de interés relevante para la biotecnología blanca. Además, no debemos olvidarnos de la importancia que tiene este hongo filamentoso en la respuesta inmunológica como alérgeno de interior, lo que se ha confirmado tras la identificación en el medio de cultivo de varias proteínas con propiedades antigénicas.

CONCLUSIONES

- Se ha desarrollado y optimizado un método para el análisis, mediante la técnica de electroforesis bidimensional (2-DE), de las proteínas intracelulares y extracelulares del hongo filamentoso *P. chrysogenum*.

- El estudio de la huella peptídica y el análisis de los resultados proporcionados por la espectrometría de masas en tándem permitió la identificación de la mayoría de las proteínas presentes en los geles bidimensionales, lo que confirma la exactitud de la anotación del genoma de *P. chrysogenum*.

- La proteómica y transcriptómica de la biosíntesis de los antibióticos β -lactámicos constituye un excelente modelo para el estudio de otros metabolitos secundarios fúngicos poco conocidos.

- El aumento de la producción de penicilina a lo largo del programa industrial de mejora de cepas es consecuencia de complejas reorganizaciones metabólicas.

- El análisis funcional de los genes *lovC* y *est*, los cuales codifican dos proteínas cuyo nivel de síntesis es diferente en las cepas de *P. chrysogenum* de baja o alta producción, nos permitió validar los resultados obtenidos mediante el análisis proteómico y reveló que estas dos proteínas juegan un papel directo en la biosíntesis de penicilina

- Debido a la secreción de una gran variedad de enzimas de interés biotecnológico, la versatilidad de *P. chrysogenum* para la biotecnología blanca es alta.

- La importancia de *P. chrysogenum* en la respuesta inmunológica, como alérgeno de interior, se vio confirmada tras la identificación en el medio de cultivo de varias proteínas con propiedades antigénicas.

- En función de los datos de proteómica obtenidos en este trabajo, la mejora de *P. chrysogenum* para producir mayores títulos de penicilina parece ser aún posible mediante la manipulación de genes específicos diferentes de los que codifican las enzimas biosintéticas de penicilina.

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APPENDIX

Supplementary Table 1. Proteins identified in the cytosolic reference map of *Penicillium chrysogenum* Wisconsin 54-1255

Spot	ORF name	Accession Number	Description	Mass (kDa)	pI	Mass (kDa)	pI	Matched	Non-Matched	Protein coverage (%)	Score	Functional Category
1	Pc22g022640	gi 211593175	strong similarity to hypothetical protein An04g02910 - <i>Aspergillus niger</i>	30	6.1	282	7.2	5	60	21	113	Unclassified
2	Pc22g05690	gi 211591618	strong similarity to hypothetical protein contig12.tfa_1730cg - <i>Aspergillus fumigatus</i>	60	4.9	220	4.3	15	50	26	129	Unclassified
3	Pc22g05690	gi 211591618	strong similarity to hypothetical protein contig12.tfa_1730cg - <i>Aspergillus fumigatus</i>	60	4.9	215	4.3	15	50	25	84	Unclassified
5	Pc06g00710	gi 211581297	strong similarity to 150 kDa oxygen regulated protein ORP150 - <i>Rattus norvegicus</i>	108	5.5	135	5.6	20	45	27	402	Unclassified
6	Pc13g07230	gi 211583774	strong similarity to pyruvate carboxylase pyc - <i>Aspergillus niger</i>	132	6.1	131	7.1	34	31	35	356	Metabolism and Energy
8	Pc16g01190	gi 211585160	strong similarity to actin-binding protein like protein An03g06960 - <i>Aspergillus niger</i>	84	4.9	123	4.3	21	44	33	590	Subcellular localization
10	Pc22g04770	gi 211591532	strong similarity to oxoglutarate dehydrogenase (lipoamide) Kgd1 - <i>Saccharomyces cerevisiae</i>	120	6.2	119	6.7	19	45	20	94	Metabolism and Energy
12	Pc12g06650	gi 211582124	strong similarity to translation elongation factor 3 Yef3 - <i>Saccharomyces cerevisiae</i>	118	6.0	118	6.8	30	34	38	522	Protein synthesis
13	Pc22g04770	gi 211591532	strong similarity to oxoglutarate dehydrogenase (lipoamide) Kgd1 - <i>Saccharomyces cerevisiae</i>	120	6.2	119	6.9	41	22	46	802	Metabolism and Energy
14	Pc12g06650	gi 211582124	strong similarity to translation elongation factor 3 Yef3 - <i>Saccharomyces cerevisiae</i>	118	6.0	118	7.2	32	31	40	600	Protein synthesis
15	Pc18g02900	gi 211586864	lysophospholipase phospholipase B plb1	69	4.4	113	3.9	10	53	20	306	Metabolism and Energy
17	Pc22g04770	gi 211591532	strong similarity to oxoglutarate dehydrogenase (lipoamide) Kgd1 - <i>Saccharomyces cerevisiae</i>	120	6.2	115	7.1	39	25	44	533	Metabolism and Energy
18	Pc21g16930	gi 211590404	strong similarity to 100 kDa coactivator snd1 - <i>Homo sapiens</i>	98	5.7	114	6.5	25	40	39	431	Transcription
19	Pc21g02440	gi 211589019	strong similarity to ubiquitin activating protein Uba1 - <i>Saccharomyces cerevisiae</i>	115	5.0	111	4.7	20	44	30	111	Protein fate
20	Pc21g19270	gi 211590629	strong similarity to valosin-containing protein like AAA-ATPase Cdc48 - <i>Saccharomyces cerevisiae</i>	91	5.0	111	5.4	32	27	43	679	Protein fate
21	Pc21g19270	gi 211590629	strong similarity to valosin-containing protein like AAA-ATPase Cdc48 - <i>Saccharomyces cerevisiae</i>	91	5.0	104	4.6	31	32	47	528	Protein fate
22	Pc18g02580	gi 211586833	strong similarity to acid phosphatase AFPhoA - <i>Aspergillus ficuum</i>	50	5.0	87	4.4	9	56	18	379	Metabolism and Energy
23	Pc21g19270	gi 211590629	strong similarity to valosin-containing protein like AAA-ATPase Cdc48 - <i>Saccharomyces cerevisiae</i>	91	5.0	104	4.7	29	33	39	452	Protein fate
25	Pc22g08680	gi 211591897	strong similarity to myb-related protein cdc5p - <i>Schizosaccharomyces pombe</i>	88	6.1	98	7.3	20	38	31	161	Cell cycle and DNA processing
27	Pc16g09030	gi 211585845	strong similarity to polyadenylate-binding protein Pabp - <i>Saccharomyces cerevisiae</i>	80	5.5	96	5.4	16	49	26	153	Protein synthesis
28	Pc16g09030	gi 211585845	strong similarity to polyadenylate-binding protein Pabp - <i>Saccharomyces cerevisiae</i>	80	5.5	96	7.7	28	37	37	405	Protein synthesis
29	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	95	5.1	18	47	34	152	Cell rescue, defense and virulence

30	Pc22g05050	gj 211591558	strong similarity to N-acetylglucosaminyltransferases chain like protein An01g07200 - <i>Aspergillus niger</i>	95	6.8	95	8.1	23	41	31	218	Metabolism and Energy
33	Pc13g12990	gj 211584333	strong similarity to heat shock protein Hsp70 ss1p - <i>Schizosaccharomyces pombe</i>	79	5.1	92	4.7	28	36	37	493	Protein fate
34	Pc22g09990	gj 211592025	strong similarity to carboxypeptidase S1 - <i>Penicillium janthinellum</i>	62	4.7	91	4.1	12	53	26	262	Protein fate
35	Pc18g02580	gj 211586833	strong similarity to acid phosphatase AFPhoA - <i>Aspergillus ficuum</i>	50	5.0	85	4.5	6	59	16	410	Metabolism and Energy
36	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	91	5.2	27	37	38	500	Cell rescue, defense and virulence
37	Pc15g00510	gj 211584900	strong similarity to 3-isopropylmalate dehydratase leu1 - <i>Rhizopus niveus</i>	85	5.6	93	5.9	16	43	25	160	Metabolism and Energy
38	Pc16g09030	gj 211585845	strong similarity to polyadenylate-binding protein Pabp - <i>Saccharomyces cerevisiae</i>	80	5.5	92	6.1	23	42	30	239	Protein synthesis
39	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	91	5.3	23	42	39	342	Cell rescue, defense and virulence
40	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	85	3.2	16	49	32	96	Cell rescue, defense and virulence
41	Pc15g03220	gj 211585311	strong similarity to hypothetical protein An09g03750 - <i>Aspergillus niger</i>	85	5.7	86	6.0	35	30	46	369	Unclassified
42	Pc18g02580	gj 211586833	strong similarity to acid phosphatase AFPhoA - <i>Aspergillus ficuum</i>	50	5.0	83	4.3	8	56	16	382	Metabolism and Energy
43	Pc15g03220	gj 211585311	strong similarity to hypothetical protein An09g03750 - <i>Aspergillus niger</i>	85	5.7	85	6.2	33	30	45	407	Unclassified
45	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	83	5.0	28	36	46	672	Cell rescue, defense and virulence
46	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	83	5.0	22	42	37	315	Cell rescue, defense and virulence
47	Pc18g02580	gj 211586833	strong similarity to acid phosphatase AFPhoA - <i>Aspergillus ficuum</i>	50	5.0	83	4.2	9	56	16	497	Metabolism and Energy
48	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	82	5.1	30	35	41	585	Cell rescue, defense and virulence
49	Pc22g18630	gj 211592785	strong similarity to ATP-dependent DNA helicase II subunit Ku70 - <i>Mus musculus</i>	88	6.1	83	7.2	25	39	40	409	Cell cycle and DNA processing
50	Pc20g06360	gj 211587900	strong similarity to catalase A catA - <i>Aspergillus nidulans</i>	84	6.1	84	7.4	14	49	24	72	Cell rescue, defense and virulence
51	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	81	5.2	25	38	41	577	Cell rescue, defense and virulence
52	Pc22g18630	gj 211592785	strong similarity to ATP-dependent DNA helicase II subunit Ku70 - <i>Mus musculus</i>	88	6.1	83	7.0	32	29	49	845	Cell cycle and DNA processing
53	Pc22g18630	gj 211592785	strong similarity to ATP-dependent DNA helicase II subunit Ku70 - <i>Mus musculus</i>	88	6.1	82	7.3	29	25	48	563	Cell cycle and DNA processing
54	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	83	5.6	30	33	45	826	Cell rescue, defense and virulence
55	Pc22g02000	gj 211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	86	6.0	81	6.4	21	37	33	374	Metabolism and Energy
56	Pc22g09990	gj 211592025	strong similarity to carboxypeptidase S1 - <i>Penicillium janthinellum</i>	62	4.7	82	4.1	14	51	42	447	Protein fate
57	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	82	5.3	31	31	48	816	Cell rescue, defense and virulence

58	Pc22g02000	gj 211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	86	6.0	83	6.1	24	33	41	250	Metabolism and Energy
59	Pc22g02000	gj 211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	86	6.0	82	6.6	18	47	26	293	Metabolism and Energy
60	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	82	4.6	28	37	46	632	Cell rescue, defense and virulence
61	Pc16g11860	gj 211586109	strong similarity to catalase R catR - <i>Aspergillus niger</i>	80	5.4	81	5.4	30	32	45	832	Cell rescue, defense and virulence
62	Pc22g02000	gj 211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	86	6.0	82	6.2	14	51	21	219	Metabolism and Energy
63	Pc13g12290	gj 211584265	anthranilate synthase multifunctional protein trpC- <i>Penicillium chrysogenum</i>	81	6.0	81	6.8	14	51	25	107	Metabolism and Energy
64	Pc22g09380	gj 211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - <i>Aspergillus fumigatus</i>	58	4.9	80	4.1	11	54	23	285	Biogenesis of cellular components
65	Pc22g11240	gj 211592088	Title: strong similarity to heat shock protein 70 hsp70 - <i>Ajellomyces capsulatus</i>	70	5.0	79	4.7	21	44	40	413	Protein fate
66	Pc22g09380	gj 211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - <i>Aspergillus fumigatus</i>	58	4.9	79	4.5	15	50	27	402	Biogenesis of cellular components
67	Pc22g09380	gj 211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - <i>Aspergillus fumigatus</i>	58	4.9	78	4.2	13	52	26	442	Biogenesis of cellular components
68	Pc22g09380	gj 211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - <i>Aspergillus fumigatus</i>	58	4.9	77	4.3	7	6	17	269	Biogenesis of cellular components
69	Pc12g05640	gj 211582027	strong similarity to heat shock protein sspB - <i>Aspergillus niger</i>	80	4.9	78	4.8	23	42	38	340	Protein fate
70	Pc22g09380	gj 211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - <i>Aspergillus fumigatus</i>	58	4.9	77	4.9	15	50	28	433	Biogenesis of cellular components
71	Pc22g15510	gj 211592485	strong similarity to ATP-dependent DNA helicase II subunit Ku70 - <i>Mus musculus</i>	74	5.3	78	5.5	21	44	38	306	Cell cycle and DNA processing
72	Pc22g15510	gj 211592485	strong similarity to ATP-dependent DNA helicase I subunit Ku70 - <i>Mus musculus</i>	74	5.3	78	5.3	12	29	20	208	Cell cycle and DNA processing
73	Pc12g09250	gj 211582371	strong similarity to cytosolic lysine-tRNA ligase Krs1 - <i>Saccharomyces cerevisiae</i>	72	5.7	77	6.4	27	36	40	431	Protein synthesis
74	Pc22g06680	gj 211591713	acetate-CoA ligase facA - <i>Penicillium chrysogenum</i>	75	5.9	77	6.7	26	39	36	348	Metabolism and Energy
75	Pc13g02680	gj 211583327	strong similarity to glutamine-tRNA ligase - <i>Saccharomyces cerevisiae</i>	74	6.3	77	7.5	25	39	37	264	Protein synthesis
76	Pc12g09250	gj 211582371	strong similarity to cytosolic lysine-tRNA ligase Krs1 - <i>Saccharomyces cerevisiae</i>	72	5.7	76	7.1	26	36	36	420	Protein synthesis
77	Pc22g11240	gj 211592088	Title: strong similarity to heat shock protein 70 hsp70 - <i>Ajellomyces capsulatus</i>	70	5.0	73	4.7	38	26	68	975	Protein fate
78	Pc13g12480	gj 211584284	strong similarity to phenol 2-monooxygenase - <i>Trichosporon beigelii</i>	75	5.5	76	5.8	22	43	40	218	Metabolism and Energy
79	Pc12g01650	gj 211581679	strong similarity to ras pathway interacting protein moe1p - <i>Schizosaccharomyces pombe</i>	65	5.5	75	5.9	20	43	38	241	Cell type differentiation
80	Pc16g04640	gj 211585438	similarity to L-galactonolactone oxidase Alo - <i>Saccharomyces cerevisiae</i>	55	5.9	76	6.0	14	51	39	259	Metabolism and Energy
81	Pc16g07440	gj 211585692	strong similarity to cytoplasmic proline-tRNA ligase like protein An08g02860 - <i>Aspergillus niger</i>	70	6.5	76	7.2	21	44	42	310	Protein synthesis
82	Pc22g15910	gj 211592524	strong similarity to cytoplasmic form of aminopeptidase like protein An03g04230 - <i>Aspergillus niger</i>	68	5.5	75	5.5	13	27	33	186	Protein fate

83	Pc22g02800	gi 211591341	strong similarity to calcium-binding protein precursor cnx1p - <i>Schizosaccharomyces pombe</i>	62	4.8	75	4.1	9	55	21	71	Protein fate
84	Pc22g11240	gi 211592088	strong similarity to heat shock protein 70 <i>hsp70</i> - <i>Ajellomyces capsulatus</i>	70	5.0	74	4.6	39	26	70	857	Protein fate
85	Pc22g19990	gi 211592918	strong similarity to endonuclease Scl 75 kDa subunit Ens1 - <i>Saccharomyces cerevisiae</i>	73	5.6	74	4.7	10	55	20	108	Protein fate
86	Pc22g15910	gi 211592524	strong similarity to cytoplasmic form of aminopeptidase like protein An03g04230 - <i>Aspergillus niger</i>	68	5.5	75	5.7	28	35	67	431	Protein fate
87	Pc16g04640	gi 211585438	similarity to L-galactonolactone oxidase Alo - <i>Saccharomyces cerevisiae</i>	55	5.9	74	6.2	19	41	36	727	Metabolism and Energy
88	Pc22g19990	gi 211592918	strong similarity to endonuclease Scl 75 kDa subunit Ens1 - <i>Saccharomyces cerevisiae</i>	73	5.6	70	4.9	23	40	40	328	Protein fate
	Pc22g10220	gi 211592047	strong similarity to dnaK-type molecular chaperone Ssb2 - <i>Saccharomyces cerevisiae</i>	67	5.3	70	4.9	22	43	48	170	Protein fate
89	Pc13g12450	gi 211584281	strong similarity to transketolase Tk11 - <i>Saccharomyces cerevisiae</i>	75	5.8	74	6.6	29	34	53	497	Metabolism and Energy
90	Pc13g12450	gi 211584281	strong similarity to transketolase Tk11 - <i>Saccharomyces cerevisiae</i>	75	5.8	73	6.6	24	41	46	515	Metabolism and Energy
91	Pc13g12450	gi 211584281	strong similarity to transketolase Tk11 - <i>Saccharomyces cerevisiae</i>	75	5.8	72	6.8	33	30	57	702	Metabolism and Energy
92	Pc13g12450	gi 211584281	strong similarity to transketolase Tk11 - <i>Saccharomyces cerevisiae</i>	75	5.8	73	6.9	22	42	38	434	Metabolism and Energy
93	Pc16g04640	gi 211585438	similarity to L-galactonolactone oxidase Alo - <i>Saccharomyces cerevisiae</i>	55	5.9	73	6.0	14	48	36	446	Metabolism and Energy
94	Pc21g02050	gi 211588981	strong similarity to multifunctional glutamine-proline-tRNA ligase Aats-glupro - <i>Drosophila melanogaster</i>	70	6.2	73	7.3	12	34	31	182	Protein synthesis
95	Pc21g12770	gi 211590015	strong similarity to stress-induced protein Sti1 - <i>Saccharomyces cerevisiae</i>	63	5.3	71	5.1	23	42	45	401	Protein fate
96	Pc20g07710	gi 211588029	Chain A, Crystal Structure Of Alp Sulfurylase From <i>Penicillium chrysogenum</i>	64	6.4	72	7.4	20	42	49	150	Metabolism and Energy
97	Pc20g07710	gi 211588029	Chain A, Crystal Structure Of Alp Sulfurylase From <i>Penicillium chrysogenum</i>	64	6.4	71	7.5	34	30	61	751	Metabolism and Energy
98	Pc21g11280	gi 211589871	strong similarity to protein disulfide isomerase A pdiA - <i>Aspergillus niger</i>	57	4.7	72	4.1	15	49	41	127	Protein fate
99	Pc22g10220	gi 211592047	strong similarity to dnaK-type molecular chaperone Ssb2 - <i>Saccharomyces cerevisiae</i>	67	5.3	71	4.8	24	41	56	560	Protein fate
100	Pc22g10220	gi 211592047	strong similarity to dnaK-type molecular chaperone Ssb2 - <i>Saccharomyces cerevisiae</i>	67	5.3	71	5.0	22	42	51	366	Protein fate
	Pc21g15100	gi 211590231	strong similarity to H+-transporting ATPase vma-1 - <i>Neurospora crassa</i>	67	5.2	71	5.0	29	34	45	363	Metabolism and Energy
101	Pc16g09030	gi 211585845	strong similarity to polyadenylate-binding protein Pabp - <i>Saccharomyces cerevisiae</i>	80	5.5	71	5.2	16	49	24	171	Protein synthesis
102	Pc12g12720	gi 211582702	strong similarity to arginine-tRNA ligase Msr1 - <i>Saccharomyces cerevisiae</i>	73	6.5	71	7.7	29	36	46	319	Protein synthesis
103	Pc12g16290	gi 211583046	strong similarity to heat shock protein 70 homolog Pdr13 - <i>Saccharomyces cerevisiae</i>	61	5.0	71	4.6	24	41	51	439	Protein fate
105	Pc22g23600	gi 211593268	strong similarity to ubiquitin specific protease Ubp6 - <i>Saccharomyces cerevisiae</i>	65	5.5	70	5.5	9	56	23	73	Protein fate
107	Pc16g09030	gi 211585845	strong similarity to polyadenylate-binding protein Pabp - <i>Saccharomyces cerevisiae</i>	80	5.5	70	5.5	11	29	22	74	Protein synthesis

108	Pc20g04410	gj 211587723	strong similarity to hypothetical phosphomannomutase homolog - <i>Schizosaccharomyces pombe</i>	66	5.5	70	5.6	19	45	39	255	Metabolism and Energy
109	Pc18g05270	gj 211587087	strong similarity to clathrin associated epsin 2A - <i>Homo sapiens</i>	55	5.5	69	6.1	9	55	21	100	Protein fate
110	Pc16g12680	gj 211586190	similarity to brefeldin A resistance protein Bfr1 - <i>Saccharomyces cerevisiae</i>	56	8.6	69	8.0	22	43	38	200	Cell rescue, defense and virulence
111	Pc21g14140	gj 211590141	strong similarity to acid phosphatase aph, 3-phylase phyB - <i>Aspergillus niger</i>	55	4.7	68	4.2	13	52	39	142	Metabolism and Energy
112	Pc22g09380	gj 211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanoyltransferase ge3 - <i>Aspergillus fumigatus</i>	58	4.9	68	4.3	9	56	22	72	Biogenesis of cellular components
113	Pc12g04590	gj 211581947	similarity to beta transducin-like protein het-e1 - <i>Podospira anserina</i>	64	6.5	68	7.1	14	51	34	247	Cellular communication /Signal transduction mechanism
114	Pc22g17870	gj 211592713	strong similarity to hypothetical ECM33 homolog SPOC1223.12c - <i>Schizosaccharomyces pombe</i>	41	4.6	61	3.9	17	44	47	453	Unclassified
115	Pc22g10360	gj 211588263	beta-N-acetylhexosaminidase precursor [<i>Penicillium chrysogenum</i>]	67	5.3	67	4.8	23	40	50	527	Metabolism and Energy
116	Pc22g13590	gj 211592302	strong similarity to hypothetical protein - <i>Gloeobacter violaceus</i>	70	5.9	68	6.7	23	41	48	432	Unclassified
117	Pc22g08080	gj 211591841	strong similarity to actin interacting protein like protein An02g14620 - <i>Aspergillus niger</i>	65	6.3	68	7.3	21	44	49	380	Subcellular localization
118	Pc22g13590	gj 211592302	strong similarity to hypothetical protein - <i>Gloeobacter violaceus</i>	70	5.9	67	6.8	31	34	665	556	Unclassified
119	Pc12g04590	gj 211581947	similarity to beta transducin-like protein het-e1 - <i>Podospira anserina</i>	64	6.5	68	7.3	9	56	20	83	Cellular communication /Signal transduction mechanism
120	Pc22g08080	gj 211591841	strong similarity to actin interacting protein like protein An02g14620 - <i>Aspergillus niger</i>	65	6.3	67	7.5	31	33	66	880	Subcellular localization
121	Pc22g10360	gj 211588263	beta-N-acetylhexosaminidase precursor [<i>Penicillium chrysogenum</i>]	67	5.3	67	4.7	13	52	29	210	Metabolism and Energy
122	Pc22g21370	gj 211593050	weak similarity to RNA polymerase II-associated factor Paf1 - <i>Saccharomyces cerevisiae</i>	54	5.5	68	5.5	23	39	57	449	Transcription
123	Pc22g06030	gj 211587867	strong similarity to succinate dehydrogenase Sdh1 - <i>Saccharomyces cerevisiae</i>	72	6.6	67	6.8	11	54	25	169	Metabolism and Energy
124	Pc22g19990	gj 211592918	strong similarity to endonuclease Scel 75 kDa subunit Ens1 - <i>Saccharomyces cerevisiae</i>	73	5.6	67	5.2	9	56	17	140	Protein fate
125	Pc22g19100	gj 211592830	strong similarity to 5-aminoimidazole-4-carboxamide ribotide transformylase Ade17 - <i>Saccharomyces cerevisiae</i>	65	5.9	67	6.3	22	42	45	462	Metabolism and Energy
126	Pc22g17940	gj 211592720	strong similarity to asparagine synthase Asn1 - <i>Saccharomyces cerevisiae</i>	65	5.9	67	6.3	12	53	30	158	Metabolism and Energy
127	Pc22g17940	gj 211592720	strong similarity to asparagine synthase Asn1 - <i>Saccharomyces cerevisiae</i>	65	5.9	67	6.3	17	48	29	227	Metabolism and Energy
128	Pc22g19100	gj 211592830	strong similarity to 5-aminoimidazole-4-carboxamide ribotide transformylase Ade17 - <i>Saccharomyces cerevisiae</i>	65	5.9	67	6.3	21	44	42	201	Metabolism and Energy
129	Pc21g02040	gj 211588980	strong similarity to T-complex-type molecular chaperone Tcpl - <i>Saccharomyces cerevisiae</i>	62	5.8	67	6.6	15	50	26	86	Protein fate
130	Pc22g19100	gj 211592830	strong similarity to 5-aminoimidazole-4-carboxamide ribotide transformylase Ade17 - <i>Saccharomyces cerevisiae</i>	65	5.9	67	6.6	14	51	30	86	Metabolism and Energy
131	Pc22g17940	gj 211592720	strong similarity to asparagine synthase Asn1 - <i>Saccharomyces cerevisiae</i>	65	5.9	66	6.5	14	51	36	184	Metabolism and Energy
132	Pc22g08080	gj 211591841	strong similarity to actin interacting protein like protein An02g14620 - <i>Aspergillus niger</i>	65	6.3	66	7.4	12	53	27	195	Subcellular localization

130	Pc22g08080	gij211591841	strong similarity to actin interacting protein like protein An02g14620 - <i>Aspergillus niger</i>	65	6.3	67	7.8	34	31	68	725	Subcellular localization
131	Pc12g04590	gij211581947	similarity to beta transducin-like protein het-e1 - <i>Podospora anserina</i>	64	6.5	67	8.1	20	45	44	406	Cellular communication /Signal transduction mechanism
132	Pc22g19100	gij211592830	strong similarity to 5-aminoimidazole-4-carboxamide ribotide transformylase Ade17 - <i>Saccharomyces cerevisiae</i>	65	5.9	65	6.6	30	32	66	1060	Metabolism and Energy
133	Pc12g15940	gij211583011	strong similarity to TCP-1-containing cytosolic chaperonin zeta subunit CcdZ - <i>Mus musculus</i>	59	6.2	66	7.4	14	51	35	171	Protein fate
134	Pc21g11280	gij211589871	strong similarity to protein disulfide isomerase A pdiA - <i>Aspergillus niger</i>	57	4.7	65	4.1	25	40	53	513	Protein fate
135	Pc16g11070	gij211586036	strong similarity to mitochondrial heat shock protein Hsp60 - <i>Saccharomyces cerevisiae</i>	62	5.6	66	4.9	23	41	53	359	Protein fate
136	Pc22g13590	gij211592302	strong similarity to hypothetical protein - <i>Gloeobacter violaceus</i>	70	5.9	66	7.8	29	31	51	645	Unclassified
137	Pc12g09360	gij211582382	strong similarity to phosphoenolpyruvate carboxykinase KIPck1 - <i>Kluyveromyces fragilis</i>	67	5.8	65	5.7	22	43	50	441	Metabolism and Energy
138	Pc22g10020	gij211592028	strong similarity to alcohol dehydrogenase alkJ - <i>Pseudomonas oleovorans</i>	60	5.9	65	5.9	23	42	50	430	Metabolism and Energy
139	Pc22g23150	gij211593224	strong similarity to 2-methylisocitrate lyase Icl2 - <i>Saccharomyces cerevisiae</i>	66	7.2	65	7.0	20	44	40	223	Metabolism and Energy
140	Pc13g11340	gij211584172	strong similarity to adenylate cyclase-associated protein Cap1 - <i>Saccharomyces cerevisiae</i>	56	6.3	65	7.2	16	49	31	175	Cell fate
141	Pc16g03900	gij211585370	strong similarity to hypothetical protein An18g01950 - <i>Aspergillus niger</i> [putative sequencing error]	63	5.5	65	4.7	17	48	36	255	Unclassified
143	Pc21g01230	gij211588905	strong similarity to hypothetical protein YJL145w - <i>Saccharomyces cerevisiae</i>	47	4.9	64	4.3	6	59	15	260	Unclassified
144	Pc20g01630	gij211587457	strong similarity to dihydrolipoamide acetyltransferase Lat1 - <i>Saccharomyces cerevisiae</i>	71	5.9	64	5.3	9	54	21	151	Metabolism and Energy
145	Pc16g12680	gij211586190	similarity to brefeldin A resistance protein Bfr1 - <i>Saccharomyces cerevisiae</i>	56	8.6	64	9.0	26	39	46	303	Cell rescue, defense and virulence
146	Pc22g22710	gij211593182	strong similarity to dihydroxy-acid dehydratase Iiv3 - <i>Saccharomyces cerevisiae</i>	65	6.2	63	5.4	15	50	23	127	Metabolism and Energy
147	Pc22g22710	gij211593182	strong similarity to dihydroxy-acid dehydratase Iiv3 - <i>Saccharomyces cerevisiae</i>	65	6.2	63	5.6	14	51	29	145	Metabolism and Energy
148	Pc13g01590	gij211583224	strong similarity to component of chaperonin-containing T-complex Cct7 - <i>Saccharomyces cerevisiae</i>	62	6.1	64	6.9	15	50	33	194	Protein fate
149	Pc18g05340	gij211587094	strong similarity to cytosolic phenylalanine-tRNA ligase beta subunit Frs2 - <i>Saccharomyces cerevisiae</i>	58	6.4	63	7.6	19	45	43	140	Protein synthesis
150	Pc22g17870	gij211592713	strong similarity to hypothetical ECM33 homolog SPPC1223.12c - <i>Schizosaccharomyces pombe</i>	41	4.6	60	3.5	17	48	45	506	Unclassified
151	Pc14g00170	gij211584633	strong similarity to hypothetical protein conig1490_3.tfa_630cog - <i>Aspergillus fumigatus</i>	47	4.8	62	4.2	7	58	26	303	Unclassified
152	Pc20g01630	gij211587457	strong similarity to dihydrolipoamide acetyltransferase Lat1 - <i>Saccharomyces cerevisiae</i>	71	5.9	62	5.0	24	39	36	542	Metabolism and Energy
153	Pc12g16040	gij211583021	strong similarity to phosphoglycerate mutase pgm - <i>Bacillus subtilis</i>	57	5.2	63	4.8	15	50	26	307	Metabolism and Energy
154	Pc22g03660	gij211591424	strong similarity to isocitrate lyase acuD - <i>Aspergillus nidulans</i>	60	6.5	62	7.8	19	46	35	252	Metabolism and Energy
155	Pc20g06050	gij211587869	strong similarity to cytoskeleton specific chaperonin subunit Ccd4 - <i>Saccharomyces cerevisiae</i>	58	8.1	63	8.4	14	51	31	115	Protein fate

156	Pc21g16870	gij211590398	strong similarity to hypothetical protein smik_17056 - <i>Saccharomyces mikatae</i>	46	4.6	59	3.8	14	51	51	145	Unclassified
157	Pc18g01390	gij211586717	strong similarity to phosphoglucosyltransferase pgmB - <i>Aspergillus nidulans</i>	60	5.7	61	6.0	31	34	59	454	Metabolism and Energy
158	Pc13g10140	gij211584056	strong similarity to galactokinase Gal1 - <i>Saccharomyces cerevisiae</i>	58	5.7	62	6.3	15	50	33	211	Metabolism and Energy
159	Pc21g12080	gij211589950	strong similarity to monodehydroascorbate reductase like protein An12g00600 - <i>Aspergillus niger</i>	60	7.2	62	8.1	27	38	56	625	Metabolism and Energy
160	Pc13g13980	gij211584426	strong similarity to TCP1-related protein Cct3 - <i>Saccharomyces cerevisiae</i>	60	6.0	62	6.9	10	55	21	104	Protein fate
161	Pc18g05320	gij211587092	strong similarity to IMP dehydrogenase IMH3 - <i>Candida albicans</i>	58	6.5	62	7.7	14	51	36	261	Metabolism and Energy
162	Pc22g03660	gij211591424	strong similarity to isocitrate lyase acuD - <i>Aspergillus nidulans</i>	60	6.5	62	7.9	24	39	50	464	Metabolism and Energy
163	Pc13g07730	gij211583822	strong similarity to threonine deaminase ILV - <i>Arxula adeninivorans</i>	62	5.9	62	6.3	13	52	32	269	Metabolism and Energy
164	Pc12g10310	gij211582473	strong similarity to hypothetical protein contig_1_32_scaffold_2.tfa_760wg - <i>Aspergillus nidulans</i>	49	6.4	62	7.6	19	45	47	443	Unclassified
165	Pc16g11070	gij211586036	strong similarity to mitochondrial heat shock protein Hsp60 - <i>Saccharomyces cerevisiae</i>	62	5.6	59	4.9	27	37	50	590	Protein fate
166	Pc12g03130	gij211581818	strong similarity to acetyl-CoA hydrolase Ach1 - <i>Saccharomyces cerevisiae</i>	58	6.2	61	7.5	22	43	48	446	Metabolism and Energy
167	Pc16g11070	gij211586036	strong similarity to mitochondrial heat shock protein Hsp60 - <i>Saccharomyces cerevisiae</i>	62	5.6	61	4.7	16	49	33	157	Protein fate
168	Pc12g03130	gij211581818	strong similarity to acetyl-CoA hydrolase Ach1 - <i>Saccharomyces cerevisiae</i>	58	6.2	60	7.3	23	41	56	406	Metabolism and Energy
169	Pc21g11950	gij211589937	strong similarity to UDP-N-acetylglucosamine pyrophosphorylase CaUAP1 - <i>Candida albicans</i>	56	5.4	60	5.2	24	40	55	598	Metabolism and Energy
170	Pc22g05940	gij211591643	strong similarity to aspartate aminotransferase like protein An04g05130 - <i>Aspergillus niger</i>	54	5.5	61	5.3	15	50	30	108	Metabolism and Energy
171	Pc22g05940	gij211591643	strong similarity to aspartate aminotransferase like protein An04g05130 - <i>Aspergillus niger</i>	54	5.5	60	5.5	25	40	42	520	Metabolism and Energy
172	Pc12g13060	gij211582733	strong similarity to coronin Cm1 - <i>Saccharomyces cerevisiae</i>	65	5.5	60	6.9	22	43	40	327	Subcellular localization
173	Pc12g03130	gij211581818	strong similarity to acetyl-CoA hydrolase Ach1 - <i>Saccharomyces cerevisiae</i>	58	6.2	60	7.2	26	35	57	494	Metabolism and Energy
174	Pc22g1650	gij211593078	strong similarity to tubulin alpha-1 chain tubA - <i>Aspergillus nidulans</i>	51	4.9	59	4.6	15	50	48	231	Subcellular localization
	Pc15g01880	gij211585025	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	51	5.1	59	4.6	9	56	25	157	Metabolism and Energy
175	Pc21g11950	gij211589937	strong similarity to UDP-N-acetylglucosamine pyrophosphorylase CaUAP1 - <i>Candida albicans</i>	56	5.4	60	5.0	15	50	38	244	Metabolism and Energy
	Pc16g11070	gij211586036	strong similarity to mitochondrial heat shock protein Hsp60 - <i>Saccharomyces cerevisiae</i>	62	5.6	60	5.0	16	49	37	202	Protein fate
176	Pc22g16570	gij211592588	strong similarity to serine O-acetyltransferase cysA - <i>Aspergillus nidulans</i>	58	6.5	60	6.6	13	52	24	142	Metabolism and Energy
177	Pc18g06000	gij211587160	strong similarity to pyruvate kinase <i>pkA</i> - <i>Aspergillus niger</i>	59	6.6	59	7.1	18	46	38	222	Metabolism and Energy
178	Pc18g00980	gij211586677	strong similarity to hypothetical trunk lateral cell specific gene HrtLC1 - <i>Habocynthia roretzi</i>	57	5.6	59	7.1	18	47	39	400	Cell rescue, defense and virulence

179	Pc18g06000	gil211587160	strong similarity to pyruvate kinase <i>pkA</i> - <i>Aspergillus niger</i>	59	6.6	58	7.4	26	35	55	460	Metabolism and Energy
180	Pc15g01030	gil211584950	strong similarity to 1,3-beta-glucanoyltransferase <i>gel1</i> - <i>Aspergillus fumigatus</i>	50	5.2	58	4.3	15	49	43	314	Metabolism and Energy
181	Pc22g04380	gil211591493	strong similarity to anthranilate synthase component I <i>Trp2</i> - <i>Saccharomyces cerevisiae</i>	56	5.8	59	5.7	13	52	36	181	Metabolism and Energy
	Pc15g00200	gil211584870	strong similarity to D-stereospecific aminopeptidase <i>dap</i> - <i>Ochrobactrum anthropi</i>	60	5.6	59	5.7	12	52	29	143	Protein fate
182	Pc22g09390	gil211591968	strong similarity to mannitol dehydrogenase <i>mldD</i> - <i>Pseudomonas fluorescens</i>	59	5.6	59	6.0	28	34	58	527	Metabolism and Energy
183	Pc12g04590	gil211581947	similarity to beta transducin-like protein <i>het-e1</i> - <i>Podospora anserina</i>	64	6.5	59	7.6	19	46	41	291	Cellular communication /Signal transduction mechanism
	Pc18g06000	gil211587160	strong similarity to pyruvate kinase <i>pkA</i> - <i>Aspergillus niger</i>	59	6.6	59	7.6	12	53	25	112	Metabolism and Energy
184	Pc20g03330	gil211587620	strong similarity to glucose-6-phosphate 1-dehydrogenase <i>gsdA</i> - <i>Aspergillus niger</i>	59	6.0	59	6.8	13	52	34	95	Metabolism and Energy
	Pc18g00980	gil211586677	strong similarity to hypothetical trunk lateral cell specific gene <i>HrTLC1</i> - <i>Halocynthia roretzi</i>	57	5.6	59	6.8	14	51	31	91	Cell rescue, defense and virulence
185	Pc22g21240	gil211593038	strong similarity to catalase <i>cta1p</i> - <i>Schizosaccharomyces pombe</i>	57	6.6	59	9.0	19	45	39	390	Cell rescue, defense and virulence
186	Pc21g02360	gil211589011	strong similarity to GU4 nucleic-binding protein 1 <i>Arc1</i> - <i>Saccharomyces cerevisiae</i>	48	6.4	58	7.3	16	49	43	353	Protein with binding function or cofactor requirement
	Pc20g03330	gil211587620	strong similarity to glucose-6-phosphate 1-dehydrogenase <i>gsdA</i> - <i>Aspergillus niger</i>	59	6.0	58	7.3	18	47	40	154	Metabolism and Energy
187	Pc21g02360	gil211589011	strong similarity to GU4 nucleic-binding protein 1 <i>Arc1</i> - <i>Saccharomyces cerevisiae</i>	48	6.4	57	7.5	27	35	82	711	Protein with binding function or cofactor requirement
188	Pc20g03330	gil211587620	strong similarity to glucose-6-phosphate 1-dehydrogenase <i>gsdA</i> - <i>Aspergillus niger</i>	59	6.0	57	6.9	31	33	62	679	Metabolism and Energy
189	Pc21g02360	gil211589011	strong similarity to GU4 nucleic-binding protein 1 <i>Arc1</i> - <i>Saccharomyces cerevisiae</i>	48	6.4	58	7.7	14	50	41	178	Protein with binding function or cofactor requirement
190	Pc18g02760	gil211586850	strong similarity to hypothetical aldehyde dehydrogenase <i>CAB63554.1</i> - <i>Schizosaccharomyces pombe</i>	54	5.5	57	5.3	23	42	62	659	Metabolism and Energy
191	Pc22g21240	gil211593038	strong similarity to catalase <i>cta1p</i> - <i>Schizosaccharomyces pombe</i>	57	6.6	57	7.9	9	56	14	110	Cell rescue, defense and virulence
192	Pc22g07840	gil211591817	strong similarity to actin like protein <i>Ano2g14750</i> - <i>Aspergillus niger</i>	51	5.2	57	5.0	22	43	44	562	Subcellular localization
193	Pc18g02760	gil211586850	strong similarity to hypothetical aldehyde dehydrogenase <i>CAB63554.1</i> - <i>Schizosaccharomyces pombe</i>	54	5.5	57	5.4	18	47	46	239	Metabolism and Energy
	Pc21g11360	gil211589879	strong similarity to amidotransferase/cyclase <i>His7</i> - <i>Saccharomyces cerevisiae</i> *	60	5.4	57	5.4	19	46	39	227	Metabolism and Energy
194	Pc22g00890	gil211591151	strong similarity to carboxypeptidase <i>y</i> <i>CPY</i> - <i>Candida albicans</i>	62	4.9	56	4.2	14	49	36	396	Protein fate
195	Pc22g09390	gil211591968	strong similarity to mannitol dehydrogenase <i>mldD</i> - <i>Pseudomonas fluorescens</i>	59	5.6	57	5.7	23	42	48	351	Metabolism and Energy
196	Pc21g11580	gil211589900	strong similarity to mRNA processing protein of cytochrome c oxidase <i>Mss51</i> - <i>Saccharomyces cerevisiae</i>	60	6.6	56	5.9	13	52	32	160	Transcription
	Pc22g14740	gil211592412	strong similarity to 26SA TP/ubiquitin-dependent proteinase chain <i>S4</i> - <i>Schizosaccharomyces pombe</i>	51	5.6	56	5.9	13	52	34	127	Protein fate
197	Pc18g00980	gil211586677	strong similarity to hypothetical trunk lateral cell specific gene <i>HrTLC1</i> - <i>Halocynthia roretzi</i>	57	5.6	55	6.2	23	39	48	677	Cell rescue, defense and virulence

198	Pc18g000980	gj 211586677	strong similarity to hypothetical trunk lateral cell specific gene HrTLC1 - <i>Halobacterium roretzi</i>	57	5.6	55	6.0	24	37	45	620	Cell rescue, defense and virulence
199	Pc12g16160	gj 211583033	strong similarity to GMP synthase (glutamine-hydrolyzing) Gua1 - <i>Saccharomyces cerevisiae</i>	59	5.7	56	6.2	19	45	38	388	Metabolism and Energy
	Pc18g000980	gj 211586677	strong similarity to hypothetical trunk lateral cell specific gene HrTLC1 - <i>Halobacterium roretzi</i>	57	5.6	56	6.2	19	46	45	242	Cell rescue, defense and virulence
200	Pc13g02690	gj 211583328	strong similarity to proteasome 19S regulatory particle subunit Rpl5 - <i>Saccharomyces cerevisiae</i>	52	4.8	56	4.3	23	39	46	318	Regulation of metabolism and protein function
201	Pc21g12790	gj 211590017	strong similarity to UTP-glucose-1-phosphate uridylyltransferase Ugp1 - <i>Saccharomyces cerevisiae</i>	58	6.4	55	7.6	28	37	63	833	Metabolism and Energy
202	Pc21g12790	gj 211590017	strong similarity to UTP-glucose-1-phosphate uridylyltransferase Ugp1 - <i>Saccharomyces cerevisiae</i>	58	6.4	56	7.9	8	57	22	111	Metabolism and Energy
203	Pc21g16310	gj 211590349	weak similarity to G1/S transition control protein-binding protein RbAp48 - <i>Mus musculus</i>	50	4.9	56	4.4	9	54	19	152	Cell cycle and DNA processing
204	Pc22g19990	gj 211592918	strong similarity to endonuclease Scl 75 kDa subunit Ens1 - <i>Saccharomyces cerevisiae</i>	73	5.6	56	4.5	10	55	16	112	Protein fate
205	Pc21g12790	gj 211590017	strong similarity to UTP-glucose-1-phosphate uridylyltransferase Ugp1 - <i>Saccharomyces cerevisiae</i>	58	6.4	56	7.2	13	52	33	136	Metabolism and Energy
206	Pc21g12790	gj 211590017	strong similarity to UTP-glucose-1-phosphate uridylyltransferase Ugp1 - <i>Saccharomyces cerevisiae</i>	58	6.4	56	7.8	15	50	33	354	Metabolism and Energy
207	Pc12g03370	gj 211581841	strong similarity to mitochondrial F1-ATPase alpha-subunit Atp1 - <i>Saccharomyces cerevisiae</i>	60	9.0	56	8.0	32	33	56	752	Metabolism and Energy
208	Pc13g05320	gj 211583586	strong similarity to hypothetical cystathione beta-synthase cysB - <i>Dictyostelium discoideum</i>	56	5.9	55	6.6	31	33	63	737	Metabolism and Energy
210	Pc22g21090	gj 211593023	strong similarity to hypothetical protein An12g09150 - <i>Aspergillus niger</i>	55	5.3	55	5.1	13	52	25	79	Unclassified
211	Pc12g05970	gj 211582060	strong similarity to cell division control protein CDC3 - <i>Candida albicans</i>	47	6.3	55	6.4	20	43	67	285	Cell cycle and DNA processing
212	Pc22g19730	gj 211592892	strong similarity to glucose-6-phosphate isomerase Pg1 - <i>Saccharomyces cerevisiae</i>	61	5.8	55	6.7	19	46	47	212	Metabolism and Energy
	Pc12g08900	gj 211582337	strong similarity to delta-1-pyrroline-5-carboxylate dehydrogenase p5cdh - <i>Homosapiens</i>	63	6.8	55	6.7	14	51	32	169	Metabolism and Energy
213	Pc13g05320	gj 211583586	strong similarity to hypothetical cystathione beta-synthase cysB - <i>Dictyostelium discoideum</i>	56	5.9	55	6.9	20	45	41	327	Metabolism and Energy
	Pc20g03330	gj 211587620	strong similarity to glucose-6-phosphate 1-dehydrogenase gsdA - <i>Aspergillus niger</i>	59	6.0	55	6.9	16	49	44	320	Metabolism and Energy
214	Pc21g12790	gj 211590017	strong similarity to UTP-glucose-1-phosphate uridylyltransferase Ugp1 - <i>Saccharomyces cerevisiae</i>	58	6.4	55	7.5	26	39	57	622	Metabolism and Energy
215	Pc21g12790	gj 211590017	strong similarity to UTP-glucose-1-phosphate uridylyltransferase Ugp1 - <i>Saccharomyces cerevisiae</i>	58	6.4	55	7.4	23	41	54	612	Metabolism and Energy
216	Pc22g13230	gj 211592266	similarity to hypothetical protein B7N4.10 - <i>Neurospora crassa</i>	45	6.7	55	8.0	20	45	55	274	Unclassified
217	Pc22g07050	gj 211591747	strong similarity to DNA-helicase TIP49a - <i>Rattus norvegicus</i>	50	6.3	55	7.5	20	45	57	561	Transcription
218	Pc12g09430	gj 211582389	strong similarity to alanine transaminase ALA2 - <i>Hordeum vulgare</i>	60	6.4	55	5.6	11	54	27	173	Metabolism and Energy
219	Pc12g03370	gj 211581841	strong similarity to mitochondrial F1-ATPase alpha-subunit Atp1 - <i>Saccharomyces cerevisiae</i>	60	9.0	54	8.4	31	34	53	669	Metabolism and Energy
221	Pc22g17420	gj 211592672	strong similarity to hypothetical protein contig_1_153_scaffold_12.tfa_500cg - <i>Aspergillus nidulans</i>	47	5.5	54	4.6	19	46	47	580	Unclassified
	Pc13g13470	gj 211584381	strong similarity to tubulin beta chain beta-tubulin like protein An08g03190 - <i>Aspergillus niger</i>	50	4.9	54	4.6	17	45	38	206	Subcellular localization

222	Pc12g11190	gj 211582556	strong similarity to argininosuccinate synthase Arg1 - <i>Saccharomyces cerevisiae</i>	47	5.1	55	4.9	15	50	41	279	Metabolism and Energy
	Pc22g08480	gj 211591877	strong similarity to hexokinase 1 hxx1p - <i>Schizosaccharomyces pombe</i>	55	5.2	55	4.9	18	47	40	202	Metabolism and Energy
223	Pc22g13480	gj 211592291	strong similarity to vacuolar H ⁺ -transporting ATPase subunit B Vma2 - <i>Saccharomyces cerevisiae</i>	56	5.7	54	6.0	27	34	57	775	Metabolism and Energy
224	Pc12g03370	gj 211581841	strong similarity to mitochondrial F1-ATPase alpha-subunit Atp1 - <i>Saccharomyces cerevisiae</i>	60	9.0	54	8.7	13	31	24	176	Metabolism and Energy
225	Pc20g09960	gj 211588225	strong similarity to FUSCA protein FUS6 - <i>Arabidopsis thaliana</i>	55	5.7	53	6.7	14	51	30	227	Cellular communication /Signal transduction mechanism
	Pc20g06940	gj 211587954	strong similarity to aminopeptidase like protein An02g11940 - <i>Aspergillus niger</i>	53	5.9	53	6.7	11	54	26	130	Protein fate
226	Pc20g13350	gj 211588550	strong similarity to 2-methylcitrate dehydratase PrpD - <i>Salmonella typhimurium</i>	61	8.0	53	7.3	25	38	52	603	Metabolism and Energy
227	Pc22g08480	gj 211591877	strong similarity to hexokinase 1 hxx1p - <i>Schizosaccharomyces pombe</i>	55	5.2	53	4.9	19	46	40	154	Metabolism and Energy
	Pc22g17420	gj 211592672	strong similarity to hypothetical protein contig_1_153_scaffold_12.tfa_500cg - <i>Aspergillus nidulans</i>	47	5.5	53	4.9	7	58	22	134	Unclassified
228	Pc12g05970	gj 211582060	strong similarity to cell division control protein CDC3 - <i>Candida albicans</i>	47	6.3	53	7.0	24	41	67	640	Cell cycle and DNA processing
229	Pc12g15120	gj 211582934	strong similarity to polyadenylated RNA-binding protein like protein An08g04280 - <i>Aspergillus niger</i>	54	6.8	53	7.8	15	47	25	329	Protein with binding function or cofactor requirement
230	Pc13g10770	gj 211584117	strong similarity to cAMP-dependent protein kinase regulatory subunit pkaR - <i>Aspergillus niger</i>	45	5.0	53	4.5	16	48	54	218	Regulation of metabolism and protein function
	Pc21g10070	gj 211589753	strong similarity to H ⁺ -transporting ATP synthase beta chain - <i>Neurospora crassa</i>	55	5.3	53	4.5	8	57	24	146	Metabolism and Energy
231	Pc15g00640	gj 211584913	strong similarity to GDP dissociation inhibitor in the secretory pathway Gdi1 - <i>Saccharomyces cerevisiae</i>	52	5.3	52	5.2	18	47	51	357	Cellular transport, transport facilitation and transport routes
232	Pc22g10030	gj 211592029	strong similarity to betaine-aldehyde dehydrogenase betB - <i>Escherichia coli</i>	54	5.7	53	5.9	15	50	48	216	Metabolism and Energy
233	Pc08g00180	gj 211581247	strong similarity to aldehyde dehydrogenase aldA - <i>Aspergillus niger</i>	54	5.9	53	6.4	28	37	65	752	Metabolism and Energy
234	Pc18g13280	gj 211586250	strong similarity to glutathione reductase Grl1 - <i>Saccharomyces cerevisiae</i>	51	6.2	53	7.5	16	49	36	527	Cell rescue, defense and virulence
	Pc22g19040	gj 211592824	strong similarity to hypothetical microtubule-interacting protein Ytm1 - <i>Saccharomyces cerevisiae</i>	52	6.4	53	7.5	11	54	37	162	Subcellular localization
235	Pc21g17880	gj 211590494	strong similarity to 4-aminobutyrate transaminase gata - <i>Aspergillus nidulans</i>	55	8.8	53	8.2	20	44	41	488	Metabolism and Energy
236	Pc15g00640	gj 211584913	strong similarity to GDP dissociation inhibitor in the secretory pathway Gdi1 - <i>Saccharomyces cerevisiae</i>	52	5.3	52	5.1	21	44	61	260	Cellular transport, transport facilitation and transport routes
237	Pc22g04840	gj 211591538	strong similarity to hypothetical phosphatidy synthase SPAC22A12.08c - <i>Schizosaccharomyces pombe</i>	53	5.4	52	5.1	17	48	45	210	Metabolism and Energy
	Pc20g06940	gj 211587954	strong similarity to aminopeptidase like protein An02g11940 - <i>Aspergillus niger</i>	53	5.9	53	6.9	27	37	64	683	Protein fate
238	Pc18g13280	gj 211586250	strong similarity to glutathione reductase Grl1 - <i>Saccharomyces cerevisiae</i>	51	6.2	53	7.5	19	44	51	739	Cell rescue, defense and virulence
239	Pc21g10070	gj 211589753	strong similarity to H ⁺ -transporting ATP synthase beta chain - <i>Neurospora crassa</i>	55	5.3	52	4.4	25	35	76	784	Metabolism and Energy

240	Pc06g00180	gj211581247	strong similarity to aldehyde dehydrogenase aldA - <i>Aspergillus niger</i>	54	5.9	53	5.9	15	50	45	395	Metabolism and Energy
241	Pc06g00180	gj211581247	strong similarity to aldehyde dehydrogenase aldA - <i>Aspergillus niger</i>	54	5.9	52	6.3	26	35	68	819	Metabolism and Energy
242	Pc16g13280	gj211586250	strong similarity to glutathione reductase Glr1 - <i>Saccharomyces cerevisiae</i>	51	6.2	52	7.6	22	36	59	837	Cell rescue, defense and virulence
243	Pc22g04840	gj211591538	strong similarity to hypothetical phosphatidyl synthase SPAC22A12.08c - <i>Schizosaccharomyces pombe</i>	53	5.4	51	5.4	19	45	39	172	Metabolism and Energy
244	Pc06g00180	gj211581247	strong similarity to aldehyde dehydrogenase aldA - <i>Aspergillus niger</i>	54	5.9	52	6.1	19	46	48	396	Metabolism and Energy
	Pc20g00490	gj211587347	strong similarity to cytosolic serine-tRNA ligase Ses1 - <i>Saccharomyces cerevisiae</i>	54	5.6	52	6.1	18	47	40	314	Protein synthesis
	Pc06g00180	gj211581247	strong similarity to aldehyde dehydrogenase aldA - <i>Aspergillus niger</i>	54	5.9	52	6.2	21	44	53	432	Metabolism and Energy
245	Pc21g09780	gj211589725	strong similarity to fructosyl amine oxygen oxidoreductase - <i>Aspergillus fumigatus</i>	50	5.8	52	6.2	17	48	47	236	Metabolism and Energy
246	Pc13g10770	gj211584117	strong similarity to cAMP-dependent protein kinase regulatory subunit pkaR - <i>Aspergillus niger</i>	45	5.0	52	4.6	15	48	57	345	Regulation of metabolism and protein function
247	Pc12g11540	gj211582591	similarity to isopenicillin N epimerase cefD - <i>Streptomyces clavuligerus</i>	54	6.1	52	6.0	17	48	36	342	Metabolism and Energy
	Pc06g00180	gj211581247	strong similarity to aldehyde dehydrogenase aldA - <i>Aspergillus niger</i>	54	5.9	52	6.0	16	49	43	273	Metabolism and Energy
248	Pc22g00890	gj211591151	strong similarity to carboxypeptidase y CPY - <i>Candida albicans</i>	62	4.9	50	4.2	12	51	30	377	Protein fate
249	Pc13g14920	gj211584514	strong similarity to hypothetical protein An09g01070 - <i>Aspergillus niger</i>	54	5.5	52	5.6	24	41	50	406	Unclassified
	Pc22g18320	gj211592754	strong similarity to diphosphomevalonate decarboxylase Erg19 - <i>Saccharomyces cerevisiae</i>	44	5.5	52	5.6	9	56	31	169	Metabolism and Energy
250	Pc12g11540	gj211582591	similarity to isopenicillin N epimerase cefD - <i>Streptomyces clavuligerus</i>	54	6.1	52	6.3	26	39	69	762	Metabolism and Energy
251	Pc21g17880	gj211590494	strong similarity to 4-aminobutyrate transaminase gatA - <i>Aspergillus nidulans</i>	55	8.8	51	8.1	23	40	51	595	Metabolism and Energy
252	Pc22g22810	gj211593192	strong similarity to sulphhydryl oxidase Sox from patent EP565172-A1 - <i>Aspergillus niger</i>	42	4.9	49	4.3	17	48	48	215	Metabolism and Energy
253	Pc22g01850	gj211591246	glucan 1,4-alpha-glucosidase BAC8255- <i>Penicillium chrysogenum</i> [Penicillium chrysogenum Wisconsin 54-1255]	52	5.0	51	4.5	24	41	54	503	Metabolism and Energy
254	Pc22g22810	gj211593192	strong similarity to sulphhydryl oxidase Sox from patent EP565172-A1 - <i>Aspergillus niger</i>	42	4.9	50	4.4	15	41	37	227	Metabolism and Energy
255	Pc22g19990	gj211592918	strong similarity to endonuclease Scl 75 kDa subunit Ens1 - <i>Saccharomyces cerevisiae</i>	73	5.6	50	4.5	8	57	11	90	Protein fate
256	Pc22g17560	gj211592684	glutamate dehydrogenase gdhA- <i>Penicillium chrysogenum</i>	50	6.0	49	7.1	28	27	71	778	Metabolism and Energy
257	Pc12g16020	gj211583019	strong similarity to cytoplasmic serine hydroxymethyltransferase Shm2 - <i>Saccharomyces cerevisiae</i>	52	7.2	51	7.8	15	50	28	159	Metabolism and Energy
258	Pc22g18320	gj211592754	strong similarity to diphosphomevalonate decarboxylase Erg19 - <i>Saccharomyces cerevisiae</i>	44	5.5	50	5.7	27	36	89	852	Metabolism and Energy
259	Pc22g12740	gj211592234	similarity to hypothetical protein tefF - <i>Aspergillus nidulans</i>	66	5.6	50	5.7	16	49	24	402	Unclassified
260	Pc18g02920	gj211586866	strong similarity to 2-dehydro-3-deoxyphosphoheptone aldolase - <i>Arabidopsis thaliana</i>	50	6.0	50	6.9	16	49	49	239	Metabolism and Energy

261	Pc20g04720	gj1211587749	strong similarity to precursor of dihydrolipoamide dehydrogenase Lpd1 - <i>Saccharomyces cerevisiae</i>	55	7.7	49	7.3	19	46	44	540	Metabolism and Energy
262	Pc22g09680	gj1211591995	similarity to hypothetical protein PA4204 - <i>Pseudomonas aeruginosa</i>	44	5.1	50	4.7	11	54	40	198	Unclassified
263	Pc22g17560	gj141017058	glutamate dehydrogenase gdhA - <i>Penicillium chrysogenum</i>	50	6.0	50	6.7	31	31	76	721	Metabolism and Energy
264	Pc12g16020	gj1211583019	strong similarity to cytoplasmic serine hydroxymethyltransferase Shm2 - <i>Saccharomyces cerevisiae</i>	52	7.2	50	8.0	27	35	53	476	Metabolism and Energy
265	Pc12g16020	gj1211583019	strong similarity to cytoplasmic serine hydroxymethyltransferase Shm2 - <i>Saccharomyces cerevisiae</i>	52	7.2	49	8.1	22	42	54	611	Metabolism and Energy
266	Pc13g05010	gj1211583556	strong similarity to cytoplasmic serine hydroxymethyltransferase Shm2 - <i>Saccharomyces cerevisiae</i>	58	8.7	50	7.5	22	41	43	429	Metabolism and Energy
267	Pc13g05010	gj1211583556	strong similarity to cytoplasmic serine hydroxymethyltransferase Shm2 - <i>Saccharomyces cerevisiae</i>	58	8.7	50	7.8	23	40	48	441	Metabolism and Energy
268	Pc22g17870	gj1211592713	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>	41	4.6	49	4.0	13	51	34	277	Unclassified
269	Pc12g04370	gj1211581925	strong similarity to phosphoserine transaminase Ser1 - <i>Saccharomyces cerevisiae</i>	48	5.5	49	5.9	21	43	49	631	Metabolism and Energy
270	Pc22g09580	gj1211591986	strong similarity to acid phosphatase aphA - <i>Aspergillus ficuum</i>	55	4.8	48	4.1	9	56	30	266	Metabolism and Energy
271	Pc14g01740	gj1211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47	5.3	49	4.9	15	50	51	543	Metabolism and Energy
272	Pc18g03600	gj1211586931	strong similarity to ornithine transaminase otaA - <i>Aspergillus nidulans</i>	49	5.9	49	6.6	18	45	59	558	Metabolism and Energy
273	Pc14g01740	gj1211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47	5.3	48	5.3	27	36	71	949	Metabolism and Energy
274	Pc18g01450	gj1211586723	strong similarity to 3-hydroxy-3-methylglutaryl-CoA synthase Erg13 - <i>Saccharomyces cerevisiae</i>	51	5.7	49	5.5	12	40	37	141	Metabolism and Energy
275	Pc12g03090	gj1211581814	strong similarity to soluble cytoplasmic fumarate reductase YEL047c - <i>Saccharomyces cerevisiae</i>	67	6.2	49	8.4	24	26	38	417	Metabolism and Energy
276	Pc20g03610	gj1211587647	strong similarity to precursor of mitochondrial isocitrate dehydrogenase icdA - <i>Aspergillus niger</i>	54	8.4	49	5.8	19	46	40	245	Metabolism and Energy
277	Pc12g03090	gj1211581814	strong similarity to soluble cytoplasmic fumarate reductase YEL047c - <i>Saccharomyces cerevisiae</i>	67	6.2	49	8.3	23	42	37	281	Metabolism and Energy
278	Pc21g18310	gj1211590536	similarity to hypothetical single-stranded TG1-3 binding protein tsg - <i>Schizosaccharomyces pombe</i>	40	7.7	49	5.7	10	55	36	221	Cell cycle and DNA processing
279	Pc20g03610	gj1211587647	strong similarity to precursor of mitochondrial isocitrate dehydrogenase icdA - <i>Aspergillus niger</i>	54	8.4	48	6.1	29	30	58	713	Metabolism and Energy
280	Pc14g01740	gj1211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47	5.3	48	5.1	17	47	55	533	Metabolism and Energy
281	Pc14g01740	gj1211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47	5.3	49	5.5	16	48	55	492	Metabolism and Energy
282	Pc20g03610	gj1211587647	strong similarity to precursor of mitochondrial isocitrate dehydrogenase icdA - <i>Aspergillus niger</i>	54	8.4	49	6.0	12	53	29	90	Metabolism and Energy
283	Pc21g18310	gj1211590536	similarity to hypothetical single-stranded TG1-3 binding protein tsg - <i>Schizosaccharomyces pombe</i>	40	7.7	48	6.8	9	56	33	256	Cell cycle and DNA processing
284	Pc21g06430	gj1211589399	strong similarity to actin like protein ad2p - <i>Schizosaccharomyces pombe</i>	48	6.2	49	7.1	24	40	61	678	Subcellular localization
285	Pc16g07470	gj1211585695	strong similarity to glycine decarboxylase subunit T Gcv1 - <i>Saccharomyces cerevisiae</i>	52	8.9	48	7.7	20	45	45	615	Metabolism and Energy

286	Pc13g09520	gij211583995	strong similarity to chitinase 1 precursor cts1 - <i>Coccidioides immitis</i>	44	4.5	48	3.9	8	26	32	325	Metabolism and Energy
287	Pc22g00890	gij211591151	strong similarity to carboxypeptidase Y CPY - <i>Candida albicans</i>	62	4.9	48	4.2	8	54	19	135	Protein fate
288	Pc22g16470	gij211592579	strong similarity to septin aspA - <i>Aspergillus nidulans</i>	43	5.0	48	4.6	26	37	73	520	Cell cycle and DNA processing
289	Pc22g05800	gij211591629	saccharopine reductase LysT - <i>Penicillium chrysogenum</i>	49	5.7	48	6.3	20	44	69	340	Metabolism and Energy
290	Pc22g23830	gij211593291	strong similarity to cytosolic aspartate transaminase Aat2 - <i>Saccharomyces cerevisiae</i>	47	6.2	48	7.2	17	48	33	252	Metabolism and Energy
291	Pc20g06040	gij211587868	strong similarity to proteasome 19S regulatory particle subunit Rpt1 - <i>Saccharomyces cerevisiae</i>	49	6.8	48	7.6	9	44	19	108	Regulation of metabolism and protein function
292	Pc13g05550	gij211583609	strong similarity to hypothetical protein nc01412.1 - <i>Neurospora crassa</i>	49	5.3	48	4.9	25	40	63	457	Unclassified
293	Pc22g23810	gij211593289	strong similarity to fumarate hydratase fumR - <i>Rhizopus oryzae</i>	58	7.7	48	6.2	14	30	36	433	Metabolism and Energy
294	Pc20g06040	gij211587868	strong similarity to proteasome 19S regulatory particle subunit Rpt1 - <i>Saccharomyces cerevisiae</i>	49	6.8	48	8.1	23	41	54	420	Regulation of metabolism and protein function
295	Pc20g03610	gij211587647	strong similarity to precursor of mitochondrial isocitrate dehydrogenase icdA - <i>Aspergillus niger</i>	54	8.4	48	5.7	9	56	25	130	Metabolism and Energy
296	Pc22g23810	gij211593289	strong similarity to fumarate hydratase fumR - <i>Rhizopus oryzae</i>	58	7.7	47	6.4	19	36	51	464	Metabolism and Energy
297	Pc13g09740	gij211584017	strong similarity to fructosyl amine oxygen oxidoreductase - <i>Aspergillus fumigatus</i>	49	7.6	48	8.0	20	45	50	423	Cell rescue, defense and virulence
298	Pc20g11850	gij211588406	strong similarity to elongation factor 1-gamma 1 Tef3 - <i>Saccharomyces cerevisiae</i>	46	5.8	48	5.5	17	42	53	321	Protein synthesis
299	Pc20g11850	gij211588406	strong similarity to elongation factor 1-gamma 1 Tef3 - <i>Saccharomyces cerevisiae</i>	46	5.8	47	5.8	25	38	70	535	Protein synthesis
300	Pc13g09660	gij211584009	strong similarity to 26S protease regulatory subunit tbaA - <i>Aspergillus niger</i>	47	5.2	47	5.1	23	41	54	413	Regulation of metabolism and protein function
301	Pc16g05080	gij211585478	strong similarity to adenosylhomocysteinase - <i>Homo sapiens</i>	49	5.6	47	5.7	14	51	30	334	Metabolism and Energy
	Pc20g11850	gij211588406	strong similarity to elongation factor 1-gamma 1 Tef3 - <i>Saccharomyces cerevisiae</i>	46	5.8	47	5.7	15	50	39	227	Protein synthesis
302	Pc16g05080	gij211585478	strong similarity to adenosylhomocysteinase - <i>Homo sapiens</i>	49	5.6	47	5.9	24	39	47	644	Metabolism and Energy
303	Pc12g10940	gij211582533	strong similarity to phosphogluconate dehydrogenase Gnd1 - <i>Saccharomyces cerevisiae</i>	56	5.9	47	6.3	25	38	55	747	Metabolism and Energy
304	Pc22g05150	gij211591567	similarity to precursor of phosphatidylinositol-specific phospholipase C - <i>Listeria monocytogenes</i>	54	6.5	47	7.5	14	21	25	216	Cellular communication /Signal transduction mechanism
305	Pc20g02910	gij211587580	strong similarity to aspartate-semialdehyde dehydrogenase Hom2 - <i>Saccharomyces cerevisiae</i>	40	6.4	47	7.5	10	16	39	362	Metabolism and Energy
306	Pc20g02910	gij211587580	strong similarity to aspartate-semialdehyde dehydrogenase Hom2 - <i>Saccharomyces cerevisiae</i>	40	6.4	46	7.8	23	40	68	808	Metabolism and Energy
307	Pc13g09660	gij211584009	strong similarity to 26S protease regulatory subunit tbaA - <i>Aspergillus niger</i>	47	5.2	47	5.0	20	43	54	392	Regulation of metabolism and protein function
308	Pc22g23810	gij211593289	strong similarity to fumarate hydratase fumR - <i>Rhizopus oryzae</i>	58	7.7	47	6.0	19	43	48	795	Metabolism and Energy
309	Pc16g07470	gij211585695	strong similarity to glycine decarboxylase subunit T Gov1 - <i>Saccharomyces cerevisiae</i>	52	8.9	47	7.6	16	49	34	466	Metabolism and Energy

310	Pc21g14230	gi 211590149	strong similarity to hypothetical nadh-dependent flavin oxidoreductase SPBC23G7.10c - <i>Schizosaccharomyces pombe</i>	48	6.0	46	7.1	21	43	54	586	Protein with binding function or cofactor requirement
312	Pc14g02010	gi 211584816	strong similarity to hypothetical protein contig_1_98_scaffold_6.tfa_1090cog - <i>Aspergillus nidulans</i>	44	6.2	46	7.4	26	38	70	630	Unclassified
313	Pc13g10770	gi 211584117	strong similarity to cAMP-dependent protein kinase regulatory subunit pkaR - <i>Aspergillus niger</i>	45	5.0	46	4.7	14	48	40	206	Regulation of metabolism and protein function
	Pc21g18150	gi 211590521	strong similarity to COP9 subunit 6 COPS6 - <i>Mus musculus</i>	44	5.1	46	4.7	5	60	19	171	Cellular communication /Signal transduction mechanism
314	Pc21g19210	gi 211590623	strong similarity to 442K curved DNA-binding protein SPAC23H4.09 - <i>Schizosaccharomyces pombe</i>	44	8.3	46	8.6	11	54	32	176	Protein with binding function or cofactor requirement
315	Pc15g01030	gi 211584950	strong similarity to 1,3-beta-glucanoyltransferase <i>gel1</i> - <i>Aspergillus fumigatus</i>	50	5.2	46	4.4	14	51	37	236	Metabolism and Energy
316	Pc22g11710	gi 211592134	strong similarity to alpha subunit E1 of the pyruvate dehydrogenase complex Pda1 - <i>Saccharomyces cerevisiae</i>	45	8.5	45	6.8	24	36	53	485	Metabolism and Energy
317	Pc21g19210	gi 211590623	strong similarity to 442K curved DNA-binding protein SPAC23H4.09 - <i>Schizosaccharomyces pombe</i>	44	8.3	46	8.7	21	44	56	459	Protein with binding function or cofactor requirement
318	Pc22g17180	gi 211592648	strong similarity to amidohydrolase like protein An02g00990 - <i>Aspergillus niger</i>	46	5.6	46	5.7	10	55	41	90	Protein fate
319	Pc22g11710	gi 211592134	strong similarity to alpha subunit E1 of the pyruvate dehydrogenase complex Pda1 - <i>Saccharomyces cerevisiae</i>	45	8.5	46	6.3	21	44	49	555	Metabolism and Energy
320	Pc22g08010	gi 211591834	weak similarity to heterogeneous nuclear ribonucleoprotein Hrp1 - <i>Saccharomyces cerevisiae</i>	50	9.2	45	4.1	4	24	8	90	Cellular transport, transport facilitation and transport routes
321	Pc15g01030	gi 211584950	strong similarity to 1,3-beta-glucanoyltransferase <i>gel1</i> - <i>Aspergillus fumigatus</i>	50	5.2	45	4.2	10	46	33	116	Metabolism and Energy
322	Pc22g08010	gi 211591834	weak similarity to heterogeneous nuclear ribonucleoprotein Hrp1 - <i>Saccharomyces cerevisiae</i>	50	9.2	46	4.9	6	59	15	97	Cellular transport, transport facilitation and transport routes
323	Pc22g11710	gi 211592134	strong similarity to alpha subunit E1 of the pyruvate dehydrogenase complex Pda1 - <i>Saccharomyces cerevisiae</i>	45	8.5	46	6.5	9	51	24	161	Metabolism and Energy
324	Pc18g01280	gi 211586706	strong similarity to ergosterol biosynthesis protein Kes1 - <i>Saccharomyces cerevisiae</i>	46	6.3	45	6.9	11	54	36	184	Metabolism and Energy
325	Pc20g13510	gi 211588565	strong similarity to methylcitrate synthase <i>mcsA</i> - <i>Aspergillus nidulans</i>	51	8.4	45	8.0	10	23	22	222	Metabolism and Energy
326	Pc06g02160	gi 211581432	strong similarity to retrotransposon Tio1 - <i>Nicotiana tabacum</i>	175	7.5	45	4.1	17	47	12	181	Transposable elements, viral and plasmid proteins
	Pc12g05400	gi 211582004	strong similarity to citrate synthase <i>citA</i> - <i>Aspergillus niger</i>	52	8.8	45	4.1	8	57	19	81	Metabolism and Energy
327	Pc13g08480	gi 211583893	strong similarity to muscle-specific interactor for beta(1) integrin melusin - <i>Mus musculus</i>	35	6.8	45	7.9	12	53	44	231	Unclassified
328	Pc12g05400	gi 211582004	strong similarity to citrate synthase <i>citA</i> - <i>Aspergillus niger</i>	52	8.8	45	8.4	23	42	56	634	Metabolism and Energy
329	Pc12g05400	gi 211582004	strong similarity to citrate synthase <i>citA</i> - <i>Aspergillus niger</i>	52	8.8	45	8.2	20	45	43	374	Metabolism and Energy
330	Pc13g08480	gi 211583893	strong similarity to muscle-specific interactor for beta(1) integrin melusin - <i>Mus musculus</i>	35	6.8	45	7.6	15	44	55	278	Unclassified
331	Pc12g05400	gi 211582004	strong similarity to citrate synthase <i>citA</i> - <i>Aspergillus niger</i>	52	8.8	45	8.1	12	53	28	365	Metabolism and Energy
332	Pc13g08480	gi 211583893	strong similarity to muscle-specific interactor for beta(1) integrin melusin - <i>Mus musculus</i>	35	6.8	45	7.5	19	46	62	338	Unclassified
333	Pc13g06650	gi 211583718	strong similarity to beta-succinyl CoA synthase precursor <i>scsB</i> - <i>Neocallimastix frontalis</i>	48	5.7	44	4.8	16	49	37	227	Metabolism and Energy

334	Pc13g06480	gj211583893	strong similarity to muscle-specific interactor for beta(1) integrin melusin - <i>Mus musculus</i>	35	6.8	45	7.7	9	32	32	93	Unclassified
335	Pc13g10890	gj211584129	similarity to hypothetical protein PA1213 - <i>Pseudomonas aeruginosa</i>	46	5.7	44	6.2	13	52	40	206	Unclassified
336	Pc21g05830	gj211589339	weak similarity to zeaxanthin epoxidase ABA2 - <i>Nicotiana plumbaginifolia</i>	45	5.5	44	5.5	13	52	29	85	Metabolism and Energy
337	Pc20g06210	gj211587885	strong similarity to Fe(II)-dependent sulfonate/alpha-ketoglutarate dioxygenase YLL057c - <i>Saccharomyces cerevisiae</i>	43	6.0	44	7.0	21	44	52	467	Metabolism and Energy
338	Pc22g10010	gj211592027	strong similarity to N-acetylglucosamine-6-phosphate deacetylase CaNAG2 - <i>Candida albicans</i>	46	5.8	43	6.6	23	40	72	707	Metabolism and Energy
339	Pc12g14620	gj211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	44	6.7	7	47	20	203	Protein with binding function or cofactor requirement
	Pc16g02020	gj211585198	strong similarity to hypothetical protein An08g00480 - <i>Aspergillus niger</i>	40	6.0	44	6.7	11	44	34	176	Unclassified
340	Pc13g10770	gj211584117	strong similarity to cAMP-dependent protein kinase regulatory subunit pkaR - <i>Aspergillus niger</i>	45	5.0	44	4.9	8	14	27	172	Regulation of metabolism and protein function
341	Pc22g10010	gj211592027	strong similarity to N-acetylglucosamine-6-phosphate deacetylase CaNAG2 - <i>Candida albicans</i>	46	5.8	44	6.3	21	44	73	464	Metabolism and Energy
342	Pc13g12030	gj211584240	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	48	7.8	44	7.5	20	42	39	611	Protein with binding function or cofactor requirement
343	Pc20g04810	gj211587757	strong similarity to estrogen receptor-binding cyclophilin cypD - <i>Bos primigenius taurus</i>	41	5.9	43	6.8	16	49	45	406	Protein with binding function or cofactor requirement
	Pc20g10120	gj211588241	strong similarity to actin-modulating protein severin - <i>Dictyostelium discoideum</i>	44	5.8	43	6.8	14	51	39	98	Cell cycle and DNA processing
344	Pc12g02970	gj211581802	strong similarity to hypothetical protein contig1492_0.1ta_4570cg - <i>Aspergillus fumigatus</i>	46	5.0	43	4.5	15	50	42	334	Unclassified
345	Pc12g08170	gj211582268	strong similarity to salt tolerance protein Met22 - <i>Saccharomyces cerevisiae</i>	37	5.0	44	4.7	21	44	58	426	Interaction with the cellular environment
346	Pc22g03070	gj211591367	strong similarity to glutamine synthase Gln1 - <i>Saccharomyces cerevisiae</i>	42	5.3	43	5.3	18	46	47	626	Metabolism and Energy
347	Pc22g20800	gj211592996	strong similarity to mannitol-1-phosphate 5-dehydrogenase m1D - <i>Streptococcus mutans</i>	43	5.5	43	5.5	26	39	78	828	Metabolism and Energy
348	Pc22g20800	gj211592996	strong similarity to mannitol-1-phosphate 5-dehydrogenase m1D - <i>Streptococcus mutans</i>	43	5.5	43	7.8	12	45	38	167	Metabolism and Energy
349	Pc16g05560	gj211585518	strong similarity to mitochondrial sulfide dehydrogenase (coenzyme Q2) SPBC2G5.06c - <i>Schizosaccharomyces pombe</i>	48	8.8	43	8.3	20	45	53	218	Metabolism and Energy
	Pc16g06210	gj211585580	strong similarity to hypothetical GTP-binding protein SPAC27E2.03c - <i>Schizosaccharomyces pombe</i>	44	7.0	43	8.3	8	57	31	215	Protein with binding function or cofactor requirement
350	Pc18g01240	gj211586702	strong similarity to saccharopine dehydrogenase LYS1 - <i>Candida albicans</i>	41	5.1	43	4.6	20	45	56	398	Metabolism and Energy
	Pc22g19990	gj211592918	strong similarity to endonuclease Scl 75 kDa subunit Ens1 - <i>Saccharomyces cerevisiae</i>	73	5.6	43	4.6	9	56	14	95	Protein fate
	Pc22g20800	gj211592996	strong similarity to mannitol-1-phosphate 5-dehydrogenase m1D - <i>Streptococcus mutans</i>	43	5.5	43	5.7	19	46	58	415	Metabolism and Energy
351	Pc16g04380	gj211585413	strong similarity to S-adenosylmethionine synthase eth-1 - <i>Neurospora crassa</i>	42	5.6	43	5.7	12	53	33	132	Metabolism and Energy
352	Pc12g14620	gj211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	41	7.2	12	53	38	445	Protein with binding function or cofactor requirement

353	Pc12g15010	gi 211582923	strong similarity to mitochondrial elongation factor like protein An08g04470 - <i>Aspergillus niger</i>	48	6.4	43	6.2	22	43	52	302	Protein synthesis
	Pc22g10040	gi 211592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	43	6.2	12	53	43	84	Metabolism and Energy
354	Pc22g19440	gi 211592864	strong similarity to cytosolic aspartate transaminase Aat2 - <i>Saccharomyces cerevisiae</i>	48	7.2	43	7.9	16	49	44	357	Metabolism and Energy
355	Pc12g14620	gi 211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	43	4.2	6	59	16	135	Protein with binding function or cofactor requirement
356	Pc12g14620	gi 211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	43	4.3	6	42	15	103	Protein with binding function or cofactor requirement
357	Pc12g02970	gi 211581802	strong similarity to hypothetical protein contig1492_0.tfa_4570cg - <i>Aspergillus fumigatus</i>	46	5.0	43	4.4	17	48	61	518	Unclassified
	Pc22g02580	gi 211591319	strong similarity to hypothetical protein 1556.170 - <i>Neurospora crassa</i>	37	5.0	43	4.4	7	57	32	119	Unclassified
358	Pc16g04380	gi 211585413	strong similarity to S-adenosylmethionine synthase eth-1 - <i>Neurospora crassa</i>	42	5.6	43	5.8	27	38	74	761	Metabolism and Energy
359	Pc12g05480	gi 211582012	similarity to core protein II of ubiquinol-cytochrome c reductase CAA42214.1 - <i>Bos primigenius taurus</i>	48	8.9	43	8.1	25	40	70	467	Metabolism and Energy
360	Pc18g01240	gi 211586702	strong similarity to saccharopine dehydrogenase LYS1 - <i>Candida albicans</i>	41	5.1	43	4.7	22	43	66	496	Metabolism and Energy
361	Pc16g06210	gi 211585580	strong similarity to hypothetical GTP-binding protein SPAC27E2.03c - <i>Schizosaccharomyces pombe</i>	44	7.0	43	8.3	24	41	65	745	Protein with binding function or cofactor requirement
362	Pc13g09680	gi 211584011	strong similarity to aspartyl proteinase candidapepsin - <i>Candida albicans</i>	44	5.1	42	4.0	11	54	32	415	Protein fate
363	Pc16g04380	gi 211585413	strong similarity to S-adenosylmethionine synthase eth-1 - <i>Neurospora crassa</i>	42	5.6	42	6.1	14	51	37	280	Metabolism and Energy
364	Pc12g14620	gi 211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	41	6.9	15	50	51	410	Protein with binding function or cofactor requirement
365	Pc12g04310	gi 211581920	strong similarity to acetate-inducible gene acIA - <i>Aspergillus nidulans</i>	50	6.3	42	7.3	25	40	58	422	Unclassified
366	Pc21g18000	gi 211590506	strong similarity to hypothetical DnaJ-like heat shock protein 17E5.120 - <i>Neurospora crassa</i>	39	9.2	42	9.2	8	23	27	162	Protein fate
367	Pc22g20290	gi 211592946	strong similarity to polygalacturonase pgal - <i>Aspergillus niger</i>	39	5.5	42	5.2	7	13	23	123	Metabolism and Energy
368	Pc18g04820	gi 211587046	weak similarity to single-stranded nucleic acid binding protein CBP - <i>Mus musculus</i>	40	5.6	42	5.9	17	48	57	290	Transcription
369	Pc22g03990	gi 211591455	strong similarity to hypothetical protein contig1492_0.tfa_4630cg - <i>Aspergillus fumigatus</i>	54	9.9	42	6.3	16	48	42	371	Unclassified
	Pc22g10040	gi 211592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	42	6.3	10	52	31	153	Metabolism and Energy
	Pc21g16520	gi 211590370	strong similarity to cytoplasmic ribosomal protein of the large subunit L4 - <i>Saccharomyces cerevisiae</i>	40	11.0	42	7.8	11	54	41	288	Protein synthesis
370	Pc06g01770	gi 211581395	strong similarity to adenylosuccinate synthase ade2p - <i>Schizosaccharomyces pombe</i>	47	6.4	42	7.8	17	48	37	264	Metabolism and Energy
	Pc12g14620	gi 211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	42	7.8	6	59	16	106	Protein with binding function or cofactor requirement
371	Pc22g14810	gi 211592419	strong similarity to hypothetical oxidoreductase CAB46711.1 - <i>Schizosaccharomyces pombe</i>	40	6.4	41	7.6	19	46	60	529	Unclassified
	Pc21g16520	gi 211590370	strong similarity to cytoplasmic ribosomal protein of the large subunit L4 - <i>Saccharomyces cerevisiae</i>	40	11.0	42	6.6	13	52	44	297	Protein synthesis
372	Pc12g14620	gi 211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	42	6.6	11	54	29	292	Protein with binding function or cofactor requirement

373	Pc15g00720	gi 211584920	strong similarity to 1,4-butanediol diacylate esterase BDA1 - <i>Brevibacterium linens</i>	43	5.1	41	4.6	17	46	64	684	Cell rescue, defense and virulence
374	Pc12g14620	gi 211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	41	6.7	13	52	44	406	Protein with binding function or cofactor requirement
375	Pc12g15600	gi 211582979	strong similarity to phosphatidylinositol-phosphatidylcholine transfer protein Sec14 - <i>Saccharomyces cerevisiae</i>	38	5.4	41	4.9	8	25	24	122	Cellular transport, transport facilitation and transport routes
376	Pc22g05790	gi 211591628	strong similarity to transcription activator Adr1 - <i>Saccharomyces cerevisiae</i>	36	5.4	41	5.6	23	39	64	507	Transcription
377	Pc22g10040	gi 211592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	41	5.8	18	46	65	479	Metabolism and Energy
378	Pc21g19480	gi 211590650	similarity to twintlin A Twf1 - <i>Saccharomyces cerevisiae</i>	37	5.3	41	4.7	13	52	45	357	Subcellular localization
379	Pc12g15600	gi 211582979	strong similarity to phosphatidylinositol-phosphatidylcholine transfer protein Sec14 - <i>Saccharomyces cerevisiae</i>	38	5.4	41	5.1	24	40	62	403	Cellular transport, transport facilitation and transport routes
380	Pc22g11240	gi 211592088	strong similarity to heat shock protein 70 hsp70 - <i>Ajellomyces capsulatus</i>	70	5.0	41	6.1	20	45	41	463	Protein fate
381	Pc21g18000	gi 211590506	strong similarity to hypothetical DnaJ-like heat shock protein 17E5.120 - <i>Neurospora crassa</i>	39	9.2	41	9.3	5	8	20	93	Protein fate
382	Pc12g14620	gi 211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	41	6.8	16	49	52	530	Protein with binding function or cofactor requirement
383	Pc14g01170	gi 211584732	strong similarity to NAD(+) -isocitrate dehydrogenase subunit 1 idh1 - <i>Ajellomyces capsulatus</i>	42	8.7	41	8.0	21	44	52	311	Metabolism and Energy
384	Pc12g05300	gi 211581996	strong similarity to cytosolic aspartate transaminase Aat2 - <i>Saccharomyces cerevisiae</i>	48	7.2	41	8.0	16	49	43	174	Metabolism and Energy
385	Pc22g10040	gi 211592030	strong similarity to 26S proteasome regulatory chain 12 rp12 - <i>Homo sapiens</i>	39	5.5	41	5.9	20	45	61	670	Regulation of metabolism and protein function
386	Pc22g19440	gi 211592864	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	41	6.5	10	55	36	185	Metabolism and Energy
387	Pc14g01170	gi 211584732	strong similarity to cytosolic aspartate transaminase Aat2 - <i>Saccharomyces cerevisiae</i>	48	7.2	41	8.0	24	39	58	705	Metabolism and Energy
388	Pc12g05380	gi 211582002	strong similarity to cytosolic aspartate transaminase Aat2 - <i>Saccharomyces cerevisiae</i>	48	7.2	41	8.1	21	44	55	434	Metabolism and Energy
389	Pc21g05590	gi 211584732	strong similarity to NAD(+) -isocitrate dehydrogenase subunit 1 idh1 - <i>Ajellomyces capsulatus</i>	42	8.7	41	8.1	19	46	52	253	Metabolism and Energy
390	Pc12g05380	gi 211582002	strong similarity to coproporphyrinogen oxidase III Hem13 - <i>Saccharomyces cerevisiae</i>	50	7.2	40	6.4	18	47	26	308	Metabolism and Energy
391	Pc21g05590	gi 211589316	strong similarity to D-amino-acid oxidase DAO - <i>Fusarium solani</i>	40	7.7	41	8.3	7	30	19	124	Metabolism and Energy
392	Pc21g19600	gi 211590662	strong similarity to hypothetical protein 1329_scaffold_5.fta_150cg - <i>Fusarium graminearum</i>	39	5.3	40	4.8	9	47	35	106	Unclassified
393	Pc21g20700	gi 211590768	weak similarity to hypothetical protein SPAC15E1.10 - <i>Schizosaccharomyces pombe</i>	37	5.2	40	4.9	20	45	78	577	Unclassified
394	Pc12g05910	gi 211582054	strong similarity to RNA-binding protein like protein An07g02990 - <i>Aspergillus niger</i>	37	8.4	40	5.3	15	50	51	108	Protein with binding function or cofactor requirement
395	Pc22g10040	gi 211592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	40	6.6	20	40	59	735	Metabolism and Energy
396	Pc12g01020	gi 211581621	strong similarity to threonine aldolase Gly1 - <i>Saccharomyces cerevisiae</i>	42	6.7	40	7.9	19	46	57	516	Metabolism and Energy
397	Pc12g16540	gi 211583071	strong similarity to cytosolic aspartate--RNA ligase Dps1 - <i>Saccharomyces cerevisiae</i>	62	5.8	40	6.0	12	53	28	421	Protein synthesis

397	Pc12g12040	gl 211582639	strong similarity to translation elongation factor eEF-2 - <i>Cricetulus griseus</i>	94	6.3	40	6.3	21	44	27	355	Protein synthesis
	Pc21g10370	gl 211589783	similarity to UDP-glucose 4-epimerase Gal10 - <i>Saccharomyces cerevisiae</i>	41	5.7	40	6.3	10	55	40	149	Metabolism and Energy
398	Pc22g10040	gl 211592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	40	6.8	21	42	67	530	Metabolism and Energy
399	Pc13g04680	gl 211583523	strong similarity to 7alpha-cephem-methoxylase subunit cmcJ - <i>Amycolatopsis lactamdurans</i>	34	5.6	40	5.6	7	32	14	123	Metabolism and Energy
400	Pc22g05330	gl 211591585	strong similarity to hypothetical protein contig_1_61_scaffold_4.tfa_1010cg - <i>Aspergillus nidulans</i>	36	6.0	40	7.6	5	60	23	107	Unclassified
401	Pc13g05760	gl 211583630	strong similarity to proteasome 19S regulatory particle subunit Rpt6 - <i>Saccharomyces cerevisiae</i>	44	8.2	40	8.6	18	42	50	352	Regulation of metabolism and protein function
402	Pc22g13130	gl 211592257	strong similarity to mitochondrial aspartate aminotransferase mAspAT - <i>Mus musculus</i>	47	9.1	40	8.6	24	38	61	731	Metabolism and Energy
403	Pc22g13130	gl 211592257	strong similarity to mitochondrial aspartate aminotransferase mAspAT - <i>Mus musculus</i>	47	9.1	40	9.1	18	33	49	465	Metabolism and Energy
404	Pc13g04680	gl 211583523	strong similarity to 7alpha-cephem-methoxylase subunit cmcJ - <i>Amycolatopsis lactamdurans</i>	34	5.6	39	5.7	19	46	70	461	Metabolism and Energy
405	Pc20g08410	gl 211588099	strong similarity to UDP-glucose 4-epimerase Gal10 - <i>Saccharomyces cerevisiae</i>	36	5.7	39	6.2	5	17	15	183	Metabolism and Energy
406	Pc22g13130	gl 211592257	strong similarity to mitochondrial aspartate aminotransferase mAspAT - <i>Mus musculus</i>	47	9.1	39	8.8	16	49	37	171	Metabolism and Energy
407	Pc22g13130	gl 211592257	strong similarity to mitochondrial aspartate aminotransferase mAspAT - <i>Mus musculus</i>	47	9.1	39	9.0	21	43	58	533	Metabolism and Energy
408	Pc21g07340	gl 211589486	strong similarity to sp1-GTPase binding protein sbp1p - <i>Schizosaccharomyces pombe</i>	28	4.8	39	4.1	5	36	38	149	Cell cycle and DNA processing
409	Pc12g12060	gl 211582641	strong similarity to branched-chain amino acid aminotransferase Bat2 - <i>Saccharomyces cerevisiae</i>	43	6.4	40	7.3	18	47	46	445	Metabolism and Energy
	Pc15g03260	gl 211585315	strong similarity to L-serine dehydratase Cha1 - <i>Saccharomyces cerevisiae</i>	40	6.4	40	7.3	17	48	64	206	Metabolism and Energy
410	Pc21g02430	gl 211589018	strong similarity to hypothetical protein Ecm4 - <i>Saccharomyces cerevisiae</i>	40	6.4	39	7.4	30	35	75	608	Unclassified
411	Pc14g01330	gl 211584748	strong similarity to Arp23 complex subunit sop2p - <i>Schizosaccharomyces pombe</i>	40	6.4	39	7.6	17	48	56	510	Biogenesis of cellular components
412	Pc21g10370	gl 211589783	strong similarity to UDP-glucose 4-epimerase Gal10 - <i>Saccharomyces cerevisiae</i>	41	5.7	39	6.4	14	51	53	260	Metabolism and Energy
	Pc21g11820	gl 211589924	weak similarity to U5 snRNP-specific 40 kDa protein - <i>Homo sapiens</i>	41	6.1	39	6.4	14	51	50	189	Transcription
413	Pc13g09680	gl 211584011	strong similarity to aspartyl proteinase candidapepsin - <i>Candida albicans</i>	44	5.1	38	4.0	15	50	45	490	Protein fate
414	Pc18g03540	gl 211586925	strong similarity to hypothetical thioredoxin SPAC577.08c - <i>Schizosaccharomyces pombe</i>	36	4.9	39	4.3	14	46	49	201	Cell rescue, defense and virulence
	Pc13g09680	gl 211584011	strong similarity to aspartyl proteinase candidapepsin - <i>Candida albicans</i>	44	5.1	39	4.3	9	51	29	112	Protein fate
415	Pc20g15430	gl 211588750	strong similarity to F-actin capping protein alpha-1 subunit CapZ - <i>Mus musculus</i>	30	5.0	39	4.7	9	56	32	347	Subcellular localization
416	Pc21g10370	gl 211589783	strong similarity to UDP-glucose 4-epimerase Gal10 - <i>Saccharomyces cerevisiae</i>	41	5.7	39	6.5	15	50	55	590	Metabolism and Energy
417	Pc22g04410	gl 211591496	strong similarity to Ngg1p-interacting factor Nif3 - <i>Saccharomyces cerevisiae</i>	39	5.4	39	5.1	15	50	56	447	Unclassified
	Pc18g01220	gl 211586700	strong similarity to fructose-bisphosphate aldolase Fba1 - <i>Saccharomyces cerevisiae</i>	39	5.4	39	5.1	7	58	21	121	Metabolism and Energy

418	Pc18g01220	gj 211586700	strong similarity to fructose-bisphosphate aldolase Fba1 - <i>Saccharomyces cerevisiae</i>	39	5.4	38	5.8	26	39	72	838	Metabolism and Energy
419	Pc16g01850	gj 211585182	strong similarity to septin aspD - <i>Aspergillus nidulans</i>	40	7.2	39	7.9	20	45	57	493	Cell cycle and DNA processing
420	Pc18g03540	gj 211586925	strong similarity to hypothetical thioredoxin SPAC577.08c - <i>Schizosaccharomyces pombe</i>	36	4.9	39	4.5	5	12	23	108	Cell rescue, defense and virulence
421	Pc22g19490	gj 211582869	strong similarity to trans-2-enoyl-ACP reductase like protein An16g05340 - <i>Aspergillus niger</i>	38	6.8	39	8.0	12	53	46	266	Metabolism and Energy
422	Pc16g01850	gj 211585182	strong similarity to septin aspD - <i>Aspergillus nidulans</i>	40	7.2	39	8.1	8	27	35	234	Cell cycle and DNA processing
423	Pc16g01850	gj 211585182	strong similarity to septin aspD - <i>Aspergillus nidulans</i>	40	7.2	39	8.2	26	39	74	613	Cell cycle and DNA processing
424	Pc13g04170	gj 211583473	similarity to cell polarity protein tea1p - <i>Schizosaccharomyces pombe</i>	36	5.2	39	4.9	23	42	83	521	Cell type differentiation
425	Pc21g04710	gj 211589228	strong similarity to tyr-inhibited DAHP synthase aroF - <i>Aspergillus nidulans</i>	40	6.1	39	6.7	20	45	58	589	Metabolism and Energy
426	Pc21g17460	gj 211590454	strong similarity to adenosine kinase like protein An17g01330 - <i>Aspergillus niger</i>	38	5.4	38	5.0	21	44	62	358	Metabolism and Energy
	Pc12g09820	gj 211582426	strong similarity to glyoxalase I Glo1- <i>Saccharomyces cerevisiae</i>	35	5.5	39	5.3	10	55	38	219	Cell rescue, defense and virulence
427	Pc21g021380	gj 211590824	isopenicillin N synthase ipiPbC - <i>Penicillium chrysogenum</i>	38	5.4	39	5.3	9	56	37	200	Metabolism and Energy
	Pc12g16540	gj 211583071	strong similarity to cytosolic aspartate-tRNA ligase Dps1 - <i>Saccharomyces cerevisiae</i>	62	5.8	39	5.3	10	55	23	97	Protein synthesis
428	Pc12g09820	gj 211582426	strong similarity to glyoxalase I Glo1 - <i>Saccharomyces cerevisiae</i>	35	5.5	38	5.5	14	51	48	373	Cell rescue, defense and virulence
	Pc18g01220	gj 211586700	strong similarity to fructose-bisphosphate aldolase Fba1 - <i>Saccharomyces cerevisiae</i>	39	5.4	38	5.5	13	52	39	212	Metabolism and Energy
429	Pc16g05740	gj 211585536	strong similarity to FMN2-dependent aliphatic sulfonate monooxygenase ssuD - <i>Escherichia coli</i>	40	5.6	39	6.0	22	43	75	461	Metabolism and Energy
	Pc18g01220	gj 211586700	strong similarity to fructose-bisphosphate aldolase Fba1 - <i>Saccharomyces cerevisiae</i>	39	5.4	39	6.0	13	52	36	188	Metabolism and Energy
430	Pc20g03970	gj 211587682	strong similarity to alpha subunit of the coatmer complex Ret1 - <i>Saccharomyces cerevisiae</i>	136	5.8	39	7.3	20	43	16	308	Cellular transport, transport facilitation and transport routes
	Pc21g05430	gj 211589300	strong similarity to cystathionine gamma-lyase - <i>Saccharomyces cerevisiae</i>	42	6.3	39	7.3	7	58	30	96	Metabolism and Energy
431	Pc21g03190	gj 211589094	strong similarity to hydroxypruvate dehydrogenase HPPA - <i>Methylobacterium extorquens</i>	37	6.5	38	7.7	9	35	40	119	Metabolism and Energy
	Pc16g03140	gj 211585305	strong similarity to thermostable alcohol dehydrogenase adhT - <i>Bacillus stearothermophilus</i>	37	6.6	38	7.7	5	41	14	86	Metabolism and Energy
432	Pc21g04710	gj 211589228	strong similarity to tyr-inhibited DAHP synthase aroF - <i>Aspergillus nidulans</i>	40	6.1	38	7.0	19	26	51	639	Metabolism and Energy
	Pc21g05430	gj 211589300	strong similarity to cystathionine gamma-lyase - <i>Saccharomyces cerevisiae</i>	42	6.3	38	7.5	9	56	36	182	Metabolism and Energy
433	Pc13g11380	gj 211584176	strong similarity to precursor of isocitrate dehydrogenase (NAD+) chain <i>ldh2</i> - <i>Saccharomyces cerevisiae</i>	42	8.7	38	7.5	14	51	36	98	Metabolism and Energy
434	Pc21g05430	gj 211589300	strong similarity to cystathionine gamma-lyase - <i>Saccharomyces cerevisiae</i>	42	6.3	38	7.6	12	38	40	381	Metabolism and Energy

435	Pc13g11380	gl 211584176	strong similarity to precursor of isocitrate dehydrogenase (NAD ⁺) chain <i>lch2</i> - <i>Saccharomyces cerevisiae</i>	42	8.7	38	7.8	25	39	51	659	Metabolism and Energy
436	Pc16g08460	gl 211585789	strong similarity to sorbitol dehydrogenase <i>gulB</i> - <i>Bacillus subtilis</i>	37	5.9	38	7.2	17	48	53	421	Metabolism and Energy
437	Pc16g08460	gl 211585789	strong similarity to sorbitol dehydrogenase <i>gulB</i> - <i>Bacillus subtilis</i>	37	5.9	38	6.9	13	52	35	281	Metabolism and Energy
438	Pc21g14890	gl 211590213	cysteine synthase BAC82550- <i>Penicillium chrysogenum</i>	40	8.6	38	8.2	16	38	61	406	Metabolism and Energy
439	Pc22g20490	gl 211592966	strong similarity to cinnamyl-alcohol dehydrogenase CAD1 - <i>Eucalyptus gunnii</i>	37	6.6	38	7.9	20	45	46	320	Metabolism and Energy
440	Pc13g08990	gl 211583944	strong similarity to homoserine dehydrogenase YJR139c - <i>Saccharomyces cerevisiae</i>	39	5.0	38	4.5	16	46	50	455	Metabolism and Energy
442	Pc12g12040	gl 211582639	strong similarity to translation elongation factor eEF-2 - <i>Cricetulus griseus</i>	94	6.3	37	5.9	10	25	12	249	Protein synthesis
443	Pc12g12040	gl 211582639	strong similarity to translation elongation factor eEF-2 - <i>Cricetulus griseus</i>	94	6.3	37	6.0	21	44	25	412	Protein synthesis
444	Pc22g06170	gl 211591665	strong similarity to hypothetical oxidoreductase SPAC977.14c - <i>Schizosaccharomyces pombe</i>	39	6.0	37	6.8	13	52	37	317	Unclassified
445	Pc21g11540	gl 211589896	strong similarity to xytil dehydrogenase <i>xth</i> - <i>Galactocandida mastotermis</i>	38	6.5	37	7.5	16	33	52	222	Metabolism and Energy
446	Pc06g01800	gl 211581398	strong similarity to quinone oxidoreductase P1 - <i>Arabidopsis thaliana</i>	37	6.5	37	7.7	14	51	56	312	Cell rescue, defense and virulence
447	Pc21g04710	gl 211589228	strong similarity to tyr-inhibited DAHP synthase <i>aroF</i> - <i>Aspergillus nidulans</i>	40	6.1	37	7.7	5	60	17	76	Metabolism and Energy
448	Pc22g02940	gl 211591355	thioredoxin reductase TrxB- <i>Penicillium chrysogenum</i>	36	5.1	37	4.7	6	59	28	134	Cell rescue, defense and virulence
449	Pc21g18040	gl 211590510	similarity to conserved domain protein - <i>Pseudomonas syringae</i>	37	5.3	37	5.2	13	52	46	213	Unclassified
450	Pc20g05830	gl 211587847	strong similarity to enoyl reductase of the lovastatin biosynthesis <i>lovC</i> - <i>Aspergillus terreus</i>	37	6.2	37	6.6	8	24	26	129	Metabolism and Energy
451	Pc20g11270	gl 211588351	strong similarity to hypothetical protein 1114_scaffold_1.fta_260wg - <i>Fusarium graminearum</i>	36	5.9	37	7.0	8	52	28	225	Unclassified
452	Pc13g10260	gl 211584068	strong similarity to hypothetical protein contig_1_84_scaffold_5.fta_850cg - <i>Aspergillus nidulans</i>	38	9.5	37	4.5	4	13	10	99	Unclassified
453	Pc22g02940	gl 211591355	thioredoxin reductase TrxB- <i>Penicillium chrysogenum</i>	36	5.1	37	4.8	11	22	39	310	Cell rescue, defense and virulence
454	Pc20g05270	gl 211587798	strong similarity to aflatoxin B1 aldehyde reductase AFAR - <i>Rattus norvegicus</i>	39	5.5	37	4.9	15	50	53	183	Cell rescue, defense and virulence
455	Pc12g11030	gl 211582542	similarity to hypothetical phosphoglycerate mutase SPAC5H10.03 - <i>Schizosaccharomyces pombe</i>	31	5.1	37	4.9	8	57	42	73	Metabolism and Energy
456	Pc18g03000	gl 211586873	strong similarity to hypothetical protein YMR099c - <i>Saccharomyces cerevisiae</i>	35	5.0	37	4.4	15	49	78	560	Unclassified
457	Pc12g14200	gl 211582844	similarity to enoyl reductase of the lovastatin biosynthesis <i>lovC</i> - <i>Aspergillus terreus</i>	37	3.0	37	6.9	9	56	46	210	Metabolism and Energy
458	Pc20g15580	gl 211588765	strong similarity to NADPH-dependent aldehyde reductase - <i>Sporobolomyces salmonicolar</i>	37	6.1	37	6.9	6	59	20	104	Metabolism and Energy
459	Pc16g08460	gl 211585789	strong similarity to sorbitol dehydrogenase <i>gulB</i> - <i>Bacillus subtilis</i>	37	5.9	37	7.3	12	53	36	281	Metabolism and Energy
460	Pc12g14840	gl 211582907	strong similarity to sterol transmethylease ERG6 - <i>Candida albicans</i>	42	6.1	37	7.3	16	49	46	239	Metabolism and Energy

458	Pc18g02290	gj 211586806	strong similarity to 2-nitropropane dioxygenase precursor ncd2 - <i>Neurospora crassa</i>	37	6.2	37	6.9	19	46	64	402	Metabolism and Energy
459	Pc20g15580	gj 211588765	strong similarity to NADPH-dependent aldehyde reductase - <i>Sporobolomyces salmonicolor</i>	37	6.1	37	7.2	17	48	57	466	Metabolism and Energy
460	Pc22g02940	gj 211591355	thioredoxin reductase TrxB- <i>Penicillium chrysogenum</i>	36	5.1	37	4.8	12	52	50	377	Cell rescue, defense and virulence
461	Pc13g06360	gj 211583690	strong similarity to salt tolerance protein Met22 - <i>Saccharomyces cerevisiae</i>	38	5.1	37	4.8	12	53	50	92	Interaction with the cellular environment
461	Pc20g05830	gj 211587847	strong similarity to enoyl reductase of the lovastatin biosynthesis lovC - <i>Aspergillus terreus</i>	37	6.2	37	7.1	15	29	59	449	Metabolism and Energy
462	Pc21g18620	gj 211590565	strong similarity to hypothetical protein mg06182.1 - <i>Magnaporthe grisea</i>	38	5.9	37	5.6	16	49	50	298	Unclassified
463	Pc22g15930	gj 211592526	strong similarity to pyridoxine synthesis component pyroA - <i>Aspergillus nidulans</i>	33	5.7	36	5.5	15	50	42	262	Metabolism and Energy
463	Pc21g13770	gj 211590105	strong similarity to thiosulfate sulfurtransferase TST - <i>Homo sapiens</i>	36	5.5	36	5.5	14	44	41	204	Cell rescue, defense and virulence
464	Pc20g00720	gj 211587369	strong similarity to hypothetical chorismate mutase/prephenate dehydratase pheA - <i>Aquifex aeolicus</i>	36	5.6	36	6.1	17	37	60	167	Metabolism and Energy
465	Pc18g00110	gj 211586591	weak similarity to isoflavone reductase IFR - <i>Medicago sativa</i>	36	5.8	36	6.3	19	27	59	336	Metabolism and Energy
466	Pc13g07960	gj 211583842	strong similarity to alcohol dehydrogenase ADH like protein An04g02690 - <i>Aspergillus niger</i>	37	6.5	36	7.9	19	46	68	514	Metabolism and Energy
468	An13g01080 Pc21g08820; putative sequencing error	gj 145247997 gj 211589632	hypothetical protein An13g01080 [<i>Aspergillus niger</i>] strong similarity to ATP phosphoribosyltransferase his1p - <i>Schizosaccharomyces pombe</i> [putative sequencing error]	33	5.7	36	5.8	8	57	42	192	Unclassified
469	Pc22g19990	gj 211592918	strong similarity to endonuclease Scl 75 kDa subunit Ens1 - <i>Saccharomyces cerevisiae</i>	73	5.6	36	6.6	12	33	20	144	Protein fate
470	Pc13g07960	gj 211583842	strong similarity to alcohol dehydrogenase ADH like protein An04g02690 - <i>Aspergillus niger</i>	37	6.5	36	7.7	15	22	48	298	Metabolism and Energy
471	Pc12g14200	gj 211582844	similarity to enoyl reductase of the lovastatin biosynthesis lovC - <i>Aspergillus terreus</i>	37	6.0	35	7.0	17	48	63	478	Metabolism and Energy
472	Pc13g07960	gj 211583842	strong similarity to alcohol dehydrogenase ADH like protein An04g02690 - <i>Aspergillus niger</i>	37	6.5	36	7.7	15	27	48	321	Metabolism and Energy
473	Pc22g23250	gj 211593233	strong similarity to acetylglutamate kinase/N-acetyl-gamma-glutamyl-phosphate reductase precursor arg-6 - <i>Neurospora crassa</i>	98	8.0	36	8.1	19	46	23	575	Metabolism and Energy
474	Pc12g14200	gj 211582844	similarity to enoyl reductase of the lovastatin biosynthesis lovC - <i>Aspergillus terreus</i>	37	6.0	36	6.7	7	23	30	161	Metabolism and Energy
475	Pc12g00420	gj 211581565	strong similarity to proteasome 19S regulatory particle multiubiquitin chain binding subunit like protein An15g03020 - <i>Aspergillus niger</i>	30	5.0	36	4.6	7	19	42	239	Regulation of metabolism and protein function
476	Pc21g01220	gj 211588904	strong similarity to alcohol dehydrogenase (NADP+) ALR - <i>Sus scrofa</i>	35	6.0	36	6.8	8	52	24	166	Metabolism and Energy
476	Pc12g14200	gj 211582844	similarity to enoyl reductase of the lovastatin biosynthesis lovC - <i>Aspergillus terreus</i>	37	6.0	36	6.8	10	55	22	99	Metabolism and Energy
478	Pc12g00420	gj 211581565	strong similarity to proteasome 19S regulatory particle multiubiquitin chain binding subunit like protein An15g03020 - <i>Aspergillus niger</i>	30	5.0	36	4.5	10	55	46	267	Regulation of metabolism and protein function
479	Pc13g11570	gj 211584194	strong similarity to cytoplasmic ribosomal protein of the large subunit L1 - <i>Saccharomyces cerevisiae</i>	35	8.4	36	4.8	9	16	32	151	Protein synthesis
480	Pc21g14560	gj 211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase gpdA - <i>Aspergillus niger</i>	36	6.2	35	7.1	21	44	75	555	Metabolism and Energy
481	Pc20g13270	gj 211588542	strong similarity to nascent polypeptide-associated complex alpha chain alpha-NAC - <i>Mus musculus</i>	22	4.7	35	3.9	6	25	31	194	Protein fate

483	Pc22g20070	gj 211592926	strong similarity to protein vip1p - <i>Schizosaccharomyces pombe</i>	28	5.8	35	6.2	14	40	41	182	Cellular communication /Signal transduction mechanism
484	Pc21g14560	gj 211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase <i>gpdA</i> - <i>Aspergillus niger</i>	36	6.2	35	7.5	23	42	79	618	Metabolism and Energy
485	Pc20g01610	gj 211587455	strong similarity to mitochondrial malate dehydrogenase Mdh1 - <i>Saccharomyces cerevisiae</i>	36	8.4	35	7.2	19	45	59	683	Metabolism and Energy
486	Pc22g03140	gj 211591374	strong similarity to Gbeta like protein <i>cpcB</i> - <i>Aspergillus nidulans</i>	35	6.3	35	7.4	23	41	77	671	Protein with binding function or cofactor requirement
487	Pc12g06870	gj 211582144	strong similarity to succinyl coenzyme A synthase alpha subunit SVRTSA - <i>Rattus norvegicus</i>	35	8.9	35	7.9	16	49	45	423	Metabolism and Energy
488	Pc21g14560	gj 211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase <i>gpdA</i> - <i>Aspergillus niger</i>	36	6.2	35	8.5	22	43	78	378	Metabolism and Energy
489	Pc22g04850	gj 211591539	strong similarity to D-arabinose dehydrogenase Ara1 - <i>Saccharomyces cerevisiae</i>	37	5.5	35	5.6	24	40	73	618	Metabolism and Energy
490	Pc21g02210	gj 211588997	strong similarity to hypothetical protein contig31_part_ii.fta_2190wg - <i>Aspergillus fumigatus</i>	35	5.6	35	5.8	9	56	40	158	Unclassified
491	Pc13g06860	gj 211583739	similarity to phenazine biosynthesis oxidoreductase phzF - <i>Pseudomonas fluorescens</i>	34	5.6	35	5.8	4	61	16	154	Metabolism and Energy
491	Pc20g03500	gj 211587636	strong similarity to hypothetical protein SPBC31F10.03 - <i>Schizosaccharomyces pombe</i>	29	5.2	35	5.0	10	55	33	139	Unclassified
492	Pc21g02210	gj 211588997	strong similarity to hypothetical protein contig31_part_ii.fta_2190wg - <i>Aspergillus fumigatus</i>	35	5.6	35	6.0	15	50	63	371	Unclassified
493	Pc21g14560	gj 211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase <i>gpdA</i> - <i>Aspergillus niger</i>	36	6.2	35	7.7	14	26	44	227	Metabolism and Energy
494	Pc13g11570	gj 211584194	strong similarity to cytoplasmic ribosomal protein of the large subunit L1 - <i>Saccharomyces cerevisiae</i>	35	8.4	35	8.9	17	47	53	428	Protein synthesis
495	Pc22g19890	gj 211592908	strong similarity to eukaryotic translation initiation factor 3 subunit p42 - <i>Homo sapiens</i>	32	8.2	35	8.7	20	40	49	311	Protein synthesis
496	Pc21g14980	gj 211590221	strong similarity to methionine adenosyltransferase regulatory beta subunit like protein An02g10660 - <i>Aspergillus niger</i>	35	6.1	35	6.7	10	48	36	126	Regulation of metabolism and protein function
497	Pc21g14500	gj 211590175	weak similarity to D-arabinose dehydrogenase Ara1 - <i>Saccharomyces cerevisiae</i>	35	6.7	35	7.9	18	47	50	295	Metabolism and Energy
497	Pc21g14560	gj 211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase <i>gpdA</i> - <i>Aspergillus niger</i>	36	6.2	35	7.9	18	47	64	219	Metabolism and Energy
499	Pc22g15930	gj 211592526	strong similarity to pyridoxine synthesis component pyroA - <i>Aspergillus nidulans</i>	33	5.7	34	5.7	14	23	36	326	Metabolism and Energy
500	Pc20g02780	gj 211587567	strong similarity to hypothetical protein PA3829 - <i>Pseudomonas aeruginosa</i>	34	5.4	34	5.1	14	50	54	475	Unclassified
500	Pc12g04750	gj 211581963	strong similarity to malate dehydrogenase precursor MDH - <i>Mus musculus</i>	34	5.4	34	5.1	12	53	42	97	Metabolism and Energy
501	Pc20g08420	gj 211588100	strong similarity to nicotinate-nucleotide pyrophosphorylase (carboxylating) QPRTase - <i>Homo sapiens</i>	33	6.1	35	7.0	15	50	56	268	Metabolism and Energy
501	Pc12g08930	gj 211582340	strong similarity to hypothetical protein contig1492_0.fta_1890wg - <i>Aspergillus fumigatus</i>	34	6.1	35	7.0	14	51	52	247	Unclassified
502	Pc22g22650	gj 211593176	strong similarity to androgen-inducible aldehyde reductase alar - <i>Rattus norvegicus</i>	37	5.8	34	6.1	22	33	71	530	Metabolism and Energy
503	Pc13g08810	gj 211583926	strong similarity to elongation factor 1beta EF-1 - <i>Oryctolagus cuniculus</i>	25	4.4	34	3.9	8	57	59	539	Protein synthesis
504	Pc12g04750	gj 211581963	strong similarity to malate dehydrogenase precursor MDH - <i>Mus musculus</i>	34	5.4	34	5.5	26	37	92	820	Metabolism and Energy

505	Pc12g04650	gj 211581953	strong similarity to purine-nucleoside phosphorylase - <i>Bos taurus</i>	33	5.9	34	6.2	20	43	61	570	Metabolism and Energy
506	Pc16g11070	gj 211586036	strong similarity to mitochondrial heat shock protein Hsp60 - <i>Saccharomyces cerevisiae</i>	62	5.6	34	6.3	16	49	30	197	Protein fate
507	Pc21g16950	gj 211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	36	5.5	34	4.9	18	46	58	509	Metabolism and Energy
508	Pc20g01610	gj 211587455	strong similarity to mitochondrial malate dehydrogenase Mdh1 - <i>Saccharomyces cerevisiae</i>	36	8.4	34	7.3	11	24	38	241	Metabolism and Energy
509	Pc12g14620	gj 211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	34	6.9	8	57	24	165	Protein with binding function or cofactor requirement
	Pc16g01190	gj 211585160	strong similarity to actin-binding protein like protein An03g06960 - <i>Aspergillus niger</i>	84	4.9	34	6.9	11	54	21	113	Subcellular localization
510	Pc12g08930	gj 211582340	strong similarity to hypothetical protein contig1492_0.1fa_1890cg - <i>Aspergillus fumigatus</i>	34	6.1	34	7.0	19	40	70	574	Unclassified
511	Pc21g16950	gj 211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	36	5.5	33	4.9	12	53	43	113	Metabolism and Energy
512	Pc20g01610	gj 211587455	strong similarity to mitochondrial malate dehydrogenase Mdh1 - <i>Saccharomyces cerevisiae</i>	36	8.4	33	7.2	19	44	57	855	Metabolism and Energy
513	Pc21g16950	gj 211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	36	5.5	34	5.4	19	46	64	412	Metabolism and Energy
514	Pc21g16950	gj 211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	36	5.5	33	5.2	22	42	65	560	Metabolism and Energy
515	Pc22g21450	gj 211593058	strong similarity to Hsp70-interacting protein Chip - <i>Drosophila melanogaster</i>	31	5.9	34	6.6	11	34	36	127	Protein with binding function or cofactor requirement
516	Pc20g01610	gj 211587455	strong similarity to mitochondrial malate dehydrogenase Mdh1 - <i>Saccharomyces cerevisiae</i>	36	8.4	34	6.7	9	42	34	115	Metabolism and Energy
517	Pc12g12040	gj 211582639	strong similarity to translation elongation factor eEF-2 - <i>Cricetulus griseus</i>	94	6.3	34	6.4	19	46	21	332	Protein synthesis
518	Pc21g02070	gj 211588983	strong similarity to exosome component like protein An15g06320 - <i>Aspergillus niger</i>	34	6.5	34	7.8	11	54	41	82	Transcription
519	Pc22g10040	gj 211592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	33	8.1	14	35	46	451	Metabolism and Energy
520	Pc20g01610	gj 211587455	strong similarity to mitochondrial malate dehydrogenase Mdh1 - <i>Saccharomyces cerevisiae</i>	36	8.4	33	6.8	13	29	45	223	Metabolism and Energy
521	Pc20g01610	gj 211587455	strong similarity to mitochondrial malate dehydrogenase Mdh1 - <i>Saccharomyces cerevisiae</i>	36	8.4	33	7.9	14	51	48	221	Metabolism and Energy
522	Pc21g14600	gj 211590185	strong similarity to mRNA cleavage factor 1 25 kDa subunit like protein An16g01870 - <i>Aspergillus niger</i>	32	5.7	33	5.8	9	51	40	224	Transcription
523	Pc20g01610	gj 211587455	strong similarity to mitochondrial malate dehydrogenase Mdh1 - <i>Saccharomyces cerevisiae</i>	36	8.4	33	7.5	15	33	52	457	Metabolism and Energy
524	Pc22g06960	gj 211591738	strong similarity to ribose-phosphate pyrophosphokinase Prs2 - <i>Saccharomyces cerevisiae</i>	35	6.5	33	7.7	11	53	47	180	Metabolism and Energy
525	Pc22g21440	gj 211593057	strong similarity to ribose-5-phosphate isomerase p1B - <i>Escherichia coli</i>	17	6.7	33	6.1	12	53	76	489	Metabolism and Energy
526	Pc20g07400	gj 211587998	strong similarity to phosphoribosylaminoimidazolecarboxamide synthase Ade1 - <i>Saccharomyces cerevisiae</i>	33	5.5	33	5.6	12	53	49	158	Metabolism and Energy
	Pc16g09710	gj 211585905	strong similarity to proliferating cell nuclear antigen pcn1p - <i>Schizosaccharomyces pombe</i>	34	4.9	33	4.0	9	56	40	273	Cell cycle and DNA processing
527	Pc22g25320	gj 211593435	strong similarity to actin-capping protein beta chain - <i>Gallus gallus</i>	30	4.5	33	4.0	14	51	67	218	Subcellular localization

528	Pc15g00670	gj 211584915	weak similarity to hypothetical UbE1/COO5 family methyltransferase - <i>Caulobacter crescentus</i>	33	5.0	32	4.6	18	37	81	549	Metabolism and Energy
529	Pc18g02740	gj 211586948	strong similarity to regucalcin also known as senescence marker protein-30 like protein An04g03420 - <i>Aspergillus niger</i>	35	5.0	33	4.5	19	45	53	506	Unclassified
530	Pc20g07230	gj 211587983	strong similarity to inorganic pyrophosphatase lpp1 - <i>Saccharomyces cerevisiae</i>	33	5.1	32	4.8	14	50	60	507	Metabolism and Energy
531	Pc15g00670	gj 211584915	weak similarity to hypothetical UbE1/COO5 family methyltransferase - <i>Caulobacter crescentus</i>	33	5.0	33	4.3	9	18	35	140	Metabolism and Energy
531	Pc20g07230	gj 211587983	strong similarity to inorganic pyrophosphatase lpp1 - <i>Saccharomyces cerevisiae</i>	33	5.1	33	4.3	4	23	12	100	Metabolism and Energy
532	Pc20g07230	gj 211587983	strong similarity to inorganic pyrophosphatase lpp1 - <i>Saccharomyces cerevisiae</i>	33	5.1	33	4.9	14	51	57	335	Metabolism and Energy
533	Pc14g01140	gj 211584729	strong similarity to proteasome 20S subunit Pre10 - <i>Saccharomyces cerevisiae</i>	29	5.1	33	6.9	15	50	53	369	Protein fate
534	Pc22g20340	gj 211592951	strong similarity to aldol/keto reductase Gcy1 - <i>Saccharomyces cerevisiae</i>	34	6.5	33	7.6	23	42	72	671	Metabolism and Energy
535	Pc15g00670	gj 211584915	weak similarity to hypothetical UbE1/COO5 family methyltransferase - <i>Caulobacter crescentus</i>	33	5.0	32	3.9	15	45	66	179	Metabolism and Energy
536	Pc20g11240	gj 211588348	strong similarity to mediator of 40S ribosomal subunit assembly like protein An16g07470 - <i>Aspergillus niger</i>	40	9.5	33	8.6	18	41	43	335	Protein synthesis
537	Pc22g21440	gj 211593057	strong similarity to ribose-5-phosphate isomerase rpiB - <i>Escherichia coli</i>	17	6.7	32	6.1	13	52	79	290	Metabolism and Energy
538	Pc22g01470	gj 211591208	strong similarity to nitrilase Ntr1 - <i>Mus musculus</i>	32	6.4	32	7.3	18	46	67	539	Metabolism and Energy
539	Pc16g09250	gj 211585866	strong similarity to cytochrome-b5 reductase Mcr1 - <i>Saccharomyces cerevisiae</i>	36	8.9	32	8.4	20	45	60	543	Metabolism and Energy
540	Pc13g12400	gj 211584276	strong similarity to spermidine synthase Spe3 - <i>Saccharomyces cerevisiae</i>	33	5.5	32	5.5	8	57	27	216	Metabolism and Energy
541	Pc16g05650	gj 211585527	strong similarity to aldol/keto reductase Gcy1 - <i>Saccharomyces cerevisiae</i>	33	6.2	32	7.2	20	45	66	502	Metabolism and Energy
542	Pc21g07030	gj 211589456	strong similarity to vacuolar ATPase subunit D Yma-8 - <i>Neurospora crassa</i>	30	7.8	32	8.3	9	31	26	159	Metabolism and Energy
543	Pc18g05100	gj 211587071	strong similarity to carboxyphosphoenolpyruvate phosphonmutase bcpA - <i>Streptomyces hygroscopicus</i>	33	5.6	32	5.7	21	44	71	473	Metabolism and Energy
544	Pc12g16540	gj 211583071	strong similarity to cytosolic aspartate--RNA ligase Dps1 - <i>Saccharomyces cerevisiae</i>	62	5.8	32	6.0	11	54	26	163	Protein synthesis
545	Pc22g16150	gj 211592548	strong similarity to prostaglandin f(2alpha) synthase TdpGFS - <i>Trypanosoma brucei</i>	31	6.1	32	7.0	22	43	79	404	Metabolism and Energy
546	Pc22g16150	gj 211592548	strong similarity to prostaglandin f(2alpha) synthase TdpGFS - <i>Trypanosoma brucei</i>	31	6.1	32	4.1	11	54	54	120	Metabolism and Energy
547	Pc22g15910	gj 211592524	strong similarity to cytoplasmic form of aminopeptidase like protein An03g04230 - <i>Aspergillus niger</i>	68	5.5	32	6.9	7	58	14	153	Protein fate
548	Pc22g25320	gj 211593435	strong similarity to actin-capping protein beta chain - <i>Gallus gallus</i>	30	4.5	32	4.0	11	54	45	82	Subcellular localization
549	Pc20g06330	gj 211587897	strong similarity to NADPH-dependent beta-ketoacyl reductase RhlG - <i>Pseudomonas aeruginosa</i>	32	6.1	32	6.7	13	52	46	175	Metabolism and Energy
550	Pc15g00670	gj 211584915	weak similarity to hypothetical UbE1/COO5 family methyltransferase - <i>Caulobacter crescentus</i>	33	5.0	31	4.9	16	46	71	270	Metabolism and Energy
551	Pc12g05820	gj 211582045	strong similarity to esterase D ESD - <i>Homo sapiens</i>	31	5.9	32	6.6	15	50	64	386	Metabolism and Energy

552	Pc14g01140	gjl211584729	strong similarity to proteasome 20S subunit Pre10 - <i>Saccharomyces cerevisiae</i>	29	5.1	31	4.8	9	56	37	215	Protein fate
553	Pc13g12400	gjl211584276	strong similarity to spermidine synthase Spe3 - <i>Saccharomyces cerevisiae</i>	33	5.5	32	5.5	4	20	11	148	Metabolism and Energy
554	Pc12g05820	gjl211582045	strong similarity to esterase D ESD - <i>Homo sapiens</i>	31	5.9	32	6.2	9	31	30	200	Metabolism and Energy
555	Pc21g14560	gjl211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase <i>gpdA</i> - <i>Aspergillus niger</i>	36	6.2	31	7.9	9	56	35	92	Metabolism and Energy
556	Pc13g15120	gjl211584528	strong similarity to prostaglandin (12alpha) synthase TBP-GFS - <i>Trypanosoma brucei</i>	31	5.9	31	6.9	10	55	40	151	Metabolism and Energy
	Pc21g14560	gjl211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase <i>gpdA</i> - <i>Aspergillus niger</i>	36	6.2	31	6.9	12	53	40	93	Metabolism and Energy
557	Pc15g00670	gjl211584915	weak similarity to hypothetical UbiE/COO5 family methyltransferase - <i>Caulobacter crescentus</i>	33	5.0	31	4.7	14	45	82	320	Metabolism and Energy
558	Pc22g19990	gjl211592918	strong similarity to endonuclease Scl 75 kDa subunit Ens1 - <i>Saccharomyces cerevisiae</i>	73	5.6	31	3.7	10	55	19	119	Protein fate
	Pc21g12600	gjl211589998	strong similarity to hypothetical protein 1465_scaffold_9.fta_470wg - <i>Fusarium graminearum</i>	28	5.5	31	5.3	18	47	75	604	Unclassified
559	Pc13g05890	gjl211583643	strong similarity to hypothetical protein YJR133w - <i>Saccharomyces cerevisiae</i>	26	5.5	31	5.3	12	53	53	108	Unclassified
560	Pc21g12600	gjl211589998	strong similarity to hypothetical protein 1465_scaffold_9.fta_470wg - <i>Fusarium graminearum</i>	28	5.5	31	5.7	20	45	67	649	Unclassified
561	Pc14g01270	gjl211584742	strong similarity to hypothetical 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase BH2000 - <i>Bacillus halodurans</i>	31	6.3	31	7.2	22	43	66	440	Metabolism and Energy
563	Pc20g06970	gjl211587957	weak similarity to mitochondrial pyruvate dehydrogenase complex protein Pdx1 - <i>Saccharomyces cerevisiae</i>	30	9.5	31	9.0	20	45	69	478	Metabolism and Energy
564	Pc12g14630	gjl211582886	strong similarity to hypothetical protein contig1477_1.fta_1140cg - <i>Aspergillus fumigatus</i>	34	5.5	31	5.6	19	46	64	547	Unclassified
	Pc21g12600	gjl211589998	strong similarity to hypothetical protein 1465_scaffold_9.fta_470wg - <i>Fusarium graminearum</i>	28	5.5	31	5.6	12	53	46	142	Unclassified
565	Pc13g01530	gjl211583218	strong similarity to hypothetical protein contig589.fta_450wg - <i>Aspergillus fumigatus</i>	32	7.0	31	8.3	8	37	29	139	Unclassified
566	Pc16g04380	gjl211585413	strong similarity to S-adenosylmethionine synthase <i>eth-1</i> - <i>Neurospora crassa</i>	42	5.6	31	7.6	19	46	42	688	Metabolism and Energy
567	Pc13g12400	gjl211584276	strong similarity to spermidine synthase Spe3 - <i>Saccharomyces cerevisiae</i>	33	5.5	31	5.5	13	52	41	357	Metabolism and Energy
568	Pc21g20440	gjl211590742	strong similarity to ribose 5-phosphate isomerase RPI - <i>Mus musculus</i>	29	5.8	31	6.0	12	53	46	277	Metabolism and Energy
569	Pc18g00620	gjl211586642	strong similarity to small Ras-like GTPase Vps21 - <i>Saccharomyces cerevisiae</i>	27	4.7	31	4.3	14	39	57	323	Cellular transport, transport facilitation and transport routes
570	Pc21g03920	gjl211589152	strong similarity to hypothetical short chain dehydrogenase SPCC736.13 - <i>Schizosaccharomyces pombe</i>	34	8.5	30	8.8	24	31	75	532	Metabolism and Energy
571	Pc16g05780	gjl211585539	similarity to inhibitor of endosome-lysosome fusion DotA - <i>Legionella pneumophila</i>	24	6.1	30	7.0	13	52	64	225	Biogenesis of cellular components
	Pc22g10010	gjl211592027	strong similarity to N-acetylglucosamine-6-phosphate deacetylase CanAG2 - <i>Candida albicans</i>	46	5.8	30	7.0	12	53	39	88	Metabolism and Energy
572	Pc15g00670	gjl211584915	weak similarity to hypothetical UbiE/COO5 family methyltransferase - <i>Caulobacter crescentus</i>	33	5.0	30	4.6	11	25	55	163	Metabolism and Energy
573	Pc21g16970	gjl211590408	vacuolar serine proteinase AAG4693- <i>Penicillium chrysogenum</i>	53	6.1	30	7.2	11	45	18	180	Protein fate

574	Pc21g16970	gj211590408	vacuolar serine proteinase AAG44693- <i>Penicillium chrysogenum</i>	53	6.1	30	7.8	15	48	27	463	Protein fate
575	Pc22g08740	gj211591903	strong similarity to ubiquitin thioesterase L3 - <i>Homo sapiens</i>	27	4.8	30	4.1	10	48	47	89	Protein fate
576	Pc16g05780	gj211585539	similarity to inhibitor of endosome-lysosome fusion DotA - <i>Legionella pneumophila</i>	24	6.1	30	7.5	11	50	53	214	Biogenesis of cellular components
	Pc16g04380	gj211585413	strong similarity to S-adenosylmethionine synthase eth-1 - <i>Neurospora crassa</i>	42	5.6	30	7.5	8	57	22	147	Metabolism and Energy
	Pc21g16970	gj7963902	vacuolar serine proteinase AAG44693- <i>Penicillium chrysogenum</i> . <i>Avicennia marina</i>	53	6.1	30	8.0	16	49	31	216	Protein fate
578	Pc12g14620	gj211582885	strong similarity to flavohemoglobin Flp - <i>Alcaligenes eutrophus</i>	46	6.0	30	8.0	7	58	27	80	Protein with binding function or cofactor requirement
579	Pc13g05520	gj211583606	strong similarity to hypothetical protein confg_1_100_scaffold_7.fta_30wg - <i>Aspergillus nidulans</i>	44	10.1	30	5.1	8	57	15	109	Unclassified
580	Pc21g12310	gj211589973	hypothetical protein <i>Penicillium chrysogenum</i> Wisconsin 54-1255	30	5.6	30	5.8	13	25	49	245	Unclassified
581	Pc22g23970	gj211593304	strong similarity to hypothetical phosphoglycerate mutase YKR043c - <i>Saccharomyces cerevisiae</i>	27	5.6	30	6.0	8	41	26	178	Metabolism and Energy
582	Pc21g16970	gj211590408	vacuolar serine proteinase AAG44693- <i>Penicillium chrysogenum</i>	53	6.1	30	7.6	14	51	24	267	Protein fate
	Pc16g04380	gj211585413	strong similarity to S-adenosylmethionine synthase eth-1 - <i>Neurospora crassa</i>	42	5.6	30	7.6	11	54	26	189	Metabolism and Energy
583	Pc22g20960	gj211593010	strong similarity to urate oxidase uaz - <i>Aspergillus flavus</i>	34	7.2	30	8.2	12	40	45	203	Metabolism and Energy
584	Pc21g13600	gj211590088	strong similarity to translation initiation factor eIF-4E - <i>Schizosaccharomyces pombe</i>	27	5.7	30	6.2	12	38	37	380	Protein synthesis
585	Pc20g02910	gj211587580	strong similarity to aspartate-semialdehyde dehydrogenase Hom2 - <i>Saccharomyces cerevisiae</i>	40	6.4	30	6.4	9	56	31	144	Metabolism and Energy
586	Pc13g05520	gj211583606	strong similarity to hypothetical protein confg_1_100_scaffold_7.fta_30wg - <i>Aspergillus nidulans</i>	44	10.1	30	5.3	17	48	27	283	Unclassified
587	Pc13g05520	gj211583606	strong similarity to hypothetical protein confg_1_100_scaffold_7.fta_30wg - <i>Aspergillus nidulans</i>	44	10.1	30	5.7	11	36	20	122	Unclassified
589	Pc12g08070	gj211582258	weak similarity to hypothetical protein Ta1372 - <i>Thermoplasma acidophilum</i>	27	5.0	30	7.0	13	52	56	297	Unclassified
590	Pc18g02880	gj211586862	strong similarity to NAD-dependent D-arabinitol dehydrogenase ard - <i>Candida tropicalis</i>	31	5.2	29	4.8	10	55	41	243	Metabolism and Energy
	Pc13g02200	gj211583281	similarity to hypothetical protein - <i>Mycobacterium avium</i>	27	5.2	29	4.8	5	60	29	98	Unclassified
591	Pc13g06610	gj211583714	strong similarity to hypothetical protein YBL036c - <i>Saccharomyces cerevisiae</i>	29	6.5	29	7.5	16	40	55	374	Unclassified
592	Pc22g24360	gj211593341	strong similarity to vacuolar ATPase subunit E Vma-4 - <i>Neurospora crassa</i>	26	6.1	29	6.6	14	51	54	460	Metabolism and Energy
593	Pc18g03560	gj211586927	strong similarity to cytochrome-c peroxidase precursor Ccp1 - <i>Saccharomyces cerevisiae</i>	40	8.3	29	5.9	14	51	46	237	Cell rescue, defense and virulence
	Pc21g11760	gj211589918	weak similarity to hypothetical protein RV0923c - <i>Mycobacterium tuberculosis</i>	26	5.7	29	5.9	9	56	42	168	Unclassified
	Pc18g00870	gj211586666	strong similarity to hypothetical protein YLL056c - <i>Saccharomyces cerevisiae</i>	32	5.9	29	6.8	11	54	40	166	Unclassified
594	Pc20g09400	gj211588171	strong similarity to dipeptidyl-peptidase V DPP V - <i>Aspergillus fumigatus</i>	79	5.6	29	6.8	8	57	21	127	Protein fate

595	Pc13g09810	gi 211584024	strong similarity to hypothetical protein contig336.tfa_680cg - <i>Aspergillus fumigatus</i>	40	8.4	29	7.4	12	53	38	139	Unclassified
597	Pc18g00870	gi 211586666	strong similarity to hypothetical protein YL056c - <i>Saccharomyces cerevisiae</i>	32	5.9	29	7.0	17	48	66	389	Unclassified
598	Pc21g10370	gi 211589783	strong similarity to UDP-glucose 4-epimerase Gal10 - <i>Saccharomyces cerevisiae</i>	41	5.7	29	6.4	9	56	31	207	Metabolism and Energy
599	Pc21g05900	gi 211589346	strong similarity to hypothetical short chain dehydrogenase SPAC521.03 - <i>Schizosaccharomyces pombe</i>	29	7.8	29	8.3	8	27	40	162	Metabolism and Energy
600	Pc22g17950	gi 211592721	strong similarity to hypothetical protein contig40.tfa_680wg - <i>Aspergillus fumigatus</i>	36	9.5	29	9.1	18	47	51	517	Unclassified
601	Pc22g17950	gi 211592721	strong similarity to hypothetical protein contig40.tfa_680wg - <i>Aspergillus fumigatus</i>	36	9.5	29	8.9	17	48	65	402	Unclassified
602	Pc22g22640	gi 211593175	strong similarity to hypothetical protein An04g02910 - <i>Aspergillus niger</i>	30	6.1	29	7.2	4	61	21	84	Unclassified
603	Pc21g11050	gi 211589848	strong similarity to U2 snRNA-specific protein A - <i>Homo sapiens</i>	28	6.5	29	7.6	15	50	65	317	Transcription
604	Pc21g12310	gi 211589973	hypothetical protein, <i>Penicillium chrysogenum</i> Wisconsin 54-1255	30	5.6	29	5.6	18	47	65	268	Unclassified
605	Pc21g03140	gi 211589089	strong similarity to cell cycle regulator p21 protein wos2p - <i>Schizosaccharomyces pombe</i>	22	4.5	28	3.8	7	58	35	163	Cell cycle and DNA processing
606	Pc21g10370	gi 211589783	strong similarity to UDP-glucose 4-epimerase Gal10 - <i>Saccharomyces cerevisiae</i>	41	5.7	29	6.0	5	60	16	136	Metabolism and Energy
608	Pc22g02600	gi 211591321	strong similarity to hypothetical membrane protein YNL010w - <i>Saccharomyces cerevisiae</i>	29	4.8	28	4.1	6	58	28	138	Unclassified
609	Pc22g15380	gi 211592472	weak similarity to hypothetical glutathione S-transferase BAB68404.1 - <i>Gibberella fujikuroi</i>	30	5.8	28	6.2	22	42	67	485	Cell rescue, defense and virulence
610	Pc16g04380	gi 211585413	strong similarity to S-adenosylmethionine synthase eth-1 - <i>Neurospora crassa</i>	42	5.6	29	8.0	9	56	22	278	Metabolism and Energy
611	Pc22g23000	gi 211593211	strong similarity to lysophospholipase extended like protein An12g07760 - <i>Aspergillus niger</i>	27	5.3	28	5.6	14	51	50	279	Metabolism and Energy
612	--	gi 212534950	proteasome component Pre6, putative [Penicillium marneffei ATCC 18224]	30	8.3	28	7.6	9	56	30	332	Protein fate
613	Pc13g03800	gi 211583437	strong similarity to precursor of mitochondrial nuclease Nuc1 - <i>Saccharomyces cerevisiae</i>	36	9.2	28	8.3	12	53	43	238	Cell cycle and DNA processing
615	Pc22g21430	gi 211593056	strong similarity to hypothetical protein contig31_part_1.tfa_2550cg - <i>Aspergillus fumigatus</i>	30	5.2	28	4.8	12	53	43	295	Unclassified
616	Pc22g01530	gi 211591214	strong similarity to hypothetical protein YAL049c - <i>Saccharomyces cerevisiae</i>	27	5.6	28	5.0	9	49	33	217	Unclassified
617	Pc21g12310	gi 211589973	hypothetical protein, <i>Penicillium chrysogenum</i> Wisconsin 54-1255	30	5.6	28	5.8	17	44	62	364	Unclassified
618	Pc12g11810	gi 211582617	strong similarity to hypothetical suppressor of deletion of TFIS Ssm1 - <i>Saccharomyces cerevisiae</i>	22	5.3	28	7.1	5	52	20	190	Cell rescue, defense and virulence
619	Pc12g01420	gi 211581659	strong similarity to riboflavin biosynthesis protein Rib7 - <i>Saccharomyces cerevisiae</i>	30	7.0	28	7.9	17	47	61	357	Metabolism and Energy
620	Pc21g18110	gi 211590517	strong similarity to electron transfer flavoprotein beta chain ETF-beta - <i>Paracoccus denitrificans</i>	28	9.0	28	9.2	10	55	46	105	Metabolism and Energy
621	Pc20g03920	gi 211587677	strong similarity to proteasome 19S regulatory particle subunit Rpn12 - <i>Saccharomyces cerevisiae</i>	31	5.0	28	4.4	19	43	79	451	Regulation of metabolism and protein function
622	Pc22g25470	gi 211593449	strong similarity to Sol1 - <i>Saccharomyces cerevisiae</i>	29	5.5	28	5.3	9	56	38	139	Unclassified

623	Pc22g21430	gj 211593056	strong similarity to hypothetical protein contig31_part_1.ifa_2550cg - <i>Aspergillus fumigatus</i>	30	5.2	28	4.7	9	56	31	219	Unclassified
624	Pc21g17430	gj 211590451	strong similarity to cytoplasmic ribosomal protein of the large subunit L8 e P12b - <i>Saccharomyces cerevisiae</i>	27	11.0	28	6.6	9	56	42	168	Protein synthesis
625	Pc13g02360	gj 211586813	weak similarity to hypothetical protein CG17807 - <i>Drosophila melanogaster</i>	29	5.9	28	6.6	9	56	40	82	Unclassified
625	Pc21g07040	gj 211589457	strong similarity to small nuclear ribonucleoprotein U2B snRNP U2B - <i>Homo sapiens</i>	27	6.4	28	7.1	15	39	66	625	Transcription
626	Pc22g25470	gj 211593449	strong similarity to Sol1 - <i>Saccharomyces cerevisiae</i>	29	5.5	28	5.7	13	52	56	380	Unclassified
627	Pc22g10040	gj 211592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	28	7.7	17	44	51	596	Metabolism and Energy
628	Pc13g13550	gj 211586277	weak similarity to hypothetical protein - <i>Streptomyces avermitilis</i>	25	5.5	28	6.2	11	34	56	394	Unclassified
629	Pc20g01960	gj 211587490	strong similarity to formyltetrahydrofolate deformylase purU - <i>Corynebacterium</i> sp."	32	6.7	28	7.8	11	51	53	649	Metabolism and Energy
630	Pc13g06830	gj 211583736	strong similarity to 3-hydroxybutyryl-CoA dehydrogenase like protein An14g00430 - <i>Aspergillus niger</i>	34	8.4	28	6.3	12	53	44	164	Metabolism and Energy
631	Pc12g16540	gj 211583071	strong similarity to cytosolic aspartate-tRNA ligase Dps1 - <i>Saccharomyces cerevisiae</i>	62	5.8	27	8.5	9	56	19	526	Protein synthesis
632	Pc20g01470	gj 211587441	strong similarity to proteasome 20S subunit Pre8 - <i>Saccharomyces cerevisiae</i>	30	5.4	27	5.4	14	51	50	282	Protein fate
633	Pc22g20280	gj 211592945	weak similarity to hypothetical protein SCD82.04c - <i>Streptomyces coelicolor</i>	29	6.2	27	7.3	12	53	61	228	Unclassified
634	Pc20g11580	gj 211588379	strong similarity to proteasome 20S subunit C2 - <i>Rattus norvegicus</i>	28	5.4	27	4.1	12	43	52	109	Protein fate
635	Pc20g11580	gj 211588379	strong similarity to proteasome 20S subunit C2 - <i>Rattus norvegicus</i>	28	5.4	27	5.5	14	31	55	504	Protein fate
636	Pc15g00320	gj 211584881	strong similarity to proteasome 20S subunit Pup2 - <i>Saccharomyces cerevisiae</i>	27	4.8	27	4.2	12	53	65	191	Protein fate
637	Pc12g00830	gj 211581603	strong similarity to sorbitol utilization protein sou2 - <i>Candida albicans</i>	29	5.3	27	5.2	17	45	78	422	Metabolism and Energy
639	Pc12g08450	gj 211582294	strong similarity to proteasome 20S subunit Pre9 - <i>Saccharomyces cerevisiae</i>	29	5.8	27	6.1	17	48	79	386	Protein fate
640	Pc21g06950	gj 211589448	strong similarity to hypothetical protein YML079w - <i>Saccharomyces cerevisiae</i>	24	5.1	27	4.7	8	57	30	236	Unclassified
640	Pc12g00550	gj 211581577	strong similarity to ribulose-5-phosphate-epimerase Rpe1 - <i>Saccharomyces cerevisiae</i>	28	5.0	27	4.7	7	58	34	131	Metabolism and Energy
641	Pc12g00830	gj 211581603	strong similarity to sorbitol utilization protein sou2 - <i>Candida albicans</i>	29	5.3	27	4.9	13	52	61	211	Metabolism and Energy
642	Pc15g00320	gj 211584881	strong similarity to proteasome 20S subunit Pup2 - <i>Saccharomyces cerevisiae</i>	27	4.8	27	4.3	7	58	45	144	Protein fate
643	Pc21g06950	gj 211589448	strong similarity to hypothetical protein YML079w - <i>Saccharomyces cerevisiae</i>	24	5.1	26	4.6	11	54	45	443	Unclassified
644	Pc13g09540	gj 211583997	strong similarity to proteasome 20S subunit Sol1 - <i>Saccharomyces cerevisiae</i>	28	5.8	27	6.3	21	44	76	562	Protein fate
645	Pc13g08270	gj 211583872	adenylylsulfate kinase AA81521- <i>Penicillium chrysogenum</i>	24	6.0	27	6.6	19	46	89	535	Metabolism and Energy
646	Pc22g01530	gj 211591214	strong similarity to hypothetical protein YAL049c - <i>Saccharomyces cerevisiae</i>	27	5.6	26	5.8	13	52	44	348	Unclassified

647	Pc21g21370	gl 211590823	acyl-coenzyme A:isopenicillin N acyltransferase (acyltransferase) AAT / PenDE - <i>Penicillium chrysogenum</i>	40	6.2	26	6.8	10	55	33	203	Metabolism and Energy
648	Pc22g01530	gl 211591214	strong similarity to hypothetical protein YAL049c - <i>Saccharomyces cerevisiae</i>	27	5.6	26	5.3	7	58	31	243	Unclassified
	Pc16g13050	gl 211586227	strong similarity to hypothetical protein 1148_scaffold_2.1fa_850cg - <i>Fusarium graminearum</i>	30	5.5	26	5.3	14	51	41	126	Unclassified
649	Pc21g08590	gl 211589609	strong similarity to hydroxyacylglutathione hydrolase RSP29 - <i>Rattus norvegicus</i>	31	7.2	26	7.7	21	44	81	531	Metabolism and Energy
650	Pc22g21270	gl 211593041	strong similarity to mitochondrial enoyl-CoA hydratase ECHS1 - <i>Rattus norvegicus</i>	32	8.8	26	8.1	16	49	49	254	Metabolism and Energy
651	Pc16g11920	gl 211586115	strong similarity to sepiapterin reductase like protein An01g01080 - <i>Aspergillus niger</i>	27	6.6	26	8.0	13	42	55	330	Metabolism and Energy
652	Pc20g11690	gl 211588390	strong similarity to imidazoleglycophosphate dehydratase igh - <i>Trichoderma harzianum</i>	26	6.4	26	8.0	10	42	59	236	Metabolism and Energy
653	Pc21g23590	gl 211591037	strong similarity to cyanamide hydratase - <i>Myrothecium verrucaria</i>	28	5.5	26	5.1	5	53	24	146	Cell rescue, defense and virulence
654	Pc21g23590	gl 211591037	strong similarity to cyanamide hydratase - <i>Myrothecium verrucaria</i>	28	5.5	26	5.4	13	52	44	369	Cell rescue, defense and virulence
655	Pc16g11920	gl 211586115	strong similarity to sepiapterin reductase like protein An01g01080 - <i>Aspergillus niger</i>	27	6.6	26	7.8	14	51	50	292	Metabolism and Energy
	Pc22g24530	gl 211593358	similarity to hypothetical protein MDB19 - <i>Arabidopsis thaliana</i>	28	6.5	26	7.8	11	54	44	161	Unclassified
656	Pc13g08810	gl 211583926	strong similarity to elongation factor 1 beta EF-1 - <i>Oryctolagus cuniculus</i>	25	4.4	26	3.9	4	61	34	113	Protein synthesis
657	Pc12g00220	gl 211581546	strong similarity to galactoside acetyltransferase lacA - <i>Escherichia coli</i>	26	5.5	26	5.2	11	54	54	189	Metabolism and Energy
	Pc21g10100	gl 211589756	weak similarity to glutathione S-transferase omega - <i>Sus scrofa</i>	26	5.9	26	5.7	12	53	60	416	Cell rescue, defense and virulence
658	Pc22g16280	gl 211592561	similarity to thyroidal receptor for N-acetylglucosamine TGR-CL10C - <i>Homo sapiens</i>	26	5.7	26	5.7	11	54	70	163	Unclassified
659	Pc18g03110	gl 211586884	weak similarity to hypothetical protein CAC04238.1 - <i>Streptomyces coelicolor</i>	27	5.6	26	5.7	5	60	48	139	Unclassified
	Pc22g16280	gl 211592561	similarity to thyroidal receptor for N-acetylglucosamine TGR-CL10C - <i>Homo sapiens</i>	26	5.7	26	5.6	10	55	54	273	Unclassified
660	Pc16g04730	gl 211585447	phosphoglycerate kinase pgkA - <i>Penicillium chrysogenum</i>	44	6.0	26	8.5	13	52	36	279	Metabolism and Energy
661	Pc22g01260	gl 211591187	strong similarity to small G-protein Gsp1 - <i>Candida albicans</i>	24	6.5	25	7.3	17	48	80	573	Cellular communication /Signal transduction mechanism
662	Pc22g01260	gl 211591187	strong similarity to small G-protein Gsp1 - <i>Candida albicans</i>	24	6.5	25	6.5	14	51	75	321	Cellular communication /Signal transduction mechanism
663	Pc22g01260	gl 211591187	strong similarity to small G-protein Gsp1 - <i>Candida albicans</i>	24	6.5	25	6.7	15	49	76	387	Cellular communication /Signal transduction mechanism
664	Pc12g14390	gl 211582863	strong similarity to hypothetical glutathione S-transferase CAA19067.1 - <i>Schizosaccharomyces pombe</i>	27	5.6	25	6.0	14	51	66	510	Cell rescue, defense and virulence
665	Pc22g01260	gl 211591187	strong similarity to small G-protein Gsp1 - <i>Candida albicans</i>	24	6.5	25	7.8	10	55	49	180	Cellular communication /Signal transduction mechanism
666	Pc22g01260	gl 211591187	strong similarity to small G-protein Gsp1 - <i>Candida albicans</i>	24	6.5	25	7.4	10	55	40	232	Cellular communication /Signal transduction mechanism
667	Pc15g00490	gl 211584898	strong similarity to orotidine-5-monophosphate pyrophosphorylase - <i>Ajeilomyces capsulatus</i>	26	6.0	25	6.2	18	47	67	649	Metabolism and Energy

668	Pc16g02160	gj211585211	strong similarity to cytoplasmic ribosomal protein of the large subunit L9b - <i>Saccharomyces cerevisiae</i>	22	9.6	25	9.6	14	51	69	299	Protein synthesis
669	Pc21g23500	gj211591028	similarity to IgE-dependent histamine-releasing factor - <i>Homo sapiens</i>	21	4.8	25	4.2	7	55	58	139	Cellular communication /Signal transduction mechanism
670	Pc22g23120	gj211593222	strong similarity to hypothetical glutathione S-transferase like protein - <i>Bradyrhizobium japonicum</i>	27	6.0	25	7.0	15	49	61	183	Cell rescue, defense and virulence
671	Pc18g00790	gj211586658	strong similarity to glutathione S-transferase like protein An02g06560 - <i>Aspergillus niger</i>	29	6.6	24	7.7	20	43	64	585	Cell rescue, defense and virulence
672	Pc16g02160	gj211585211	strong similarity to cytoplasmic ribosomal protein of the large subunit L9b - <i>Saccharomyces cerevisiae</i>	22	9.6	25	9.9	9	51	60	214	Protein synthesis
673	Pc20g08550	gj211588113	strong similarity to protein-L-isospartate(D-aspartate) O-methyltransferase PCMT1 - <i>Mus musculus</i>	26	5.5	25	5.6	4	51	20	74	Metabolism and Energy
674	Pc22g23120	gj211593222	strong similarity to hypothetical glutathione S-transferase like protein - <i>Bradyrhizobium japonicum</i>	27	6.0	25	6.8	14	51	58	169	Cell rescue, defense and virulence
675	Pc21g23710	gj211591049	strong similarity to gamma subunit of translation elongation factor eEF-1 - <i>Homo sapiens</i>	25	5.1	25	4.9	7	53	26	181	Protein synthesis
676	Pc21g03400	gj211589115	strong similarity to triose-phosphate-isomerase <i>tpiA</i> from patent WO8704464-A - <i>Aspergillus niger</i>	27	5.3	24	5.1	21	44	91	789	Metabolism and Energy
677	Pc21g08140	gj211589565	strong similarity to hypothetical protein contig_1_107_scaffold_7.tfa_1090cg - <i>Aspergillus nidulans</i>	27	6.0	25	6.6	13	52	66	358	Unclassified
678	Pc18g00790	gj211586658	strong similarity to glutathione S-transferase like protein An02g06560 - <i>Aspergillus niger</i>	29	6.6	25	7.6	9	44	30	104	Cell rescue, defense and virulence
679	Pc14g01230	gj211584738	strong similarity to proteasome 20S subunit Pre7 - <i>Saccharomyces cerevisiae</i>	28	5.5	24	5.2	11	42	52	239	Protein fate
680	Pc21g01810	gj211588958	strong similarity to uracil phosphoribosyltransferase <i>upp</i> - <i>Bacillus caldolyticus</i>	25	5.5	24	5.3	6	59	41	84	Metabolism and Energy
681	Pc21g03090	gj211589084	strong similarity to meso-2,3-butanediol dehydrogenase like protein An14g05430 - <i>Aspergillus niger</i>	26	7.9	24	8.5	12	53	73	418	Metabolism and Energy
682	---	gj154285684	woronin body major protein [<i>Ajlomyces capsulatus</i> NAM1] glutathione S-transferase <i>penicillium chrysogenum</i> , strong similarity to hypothetical protein contig_1_172_scaffold_16.tfa_550cg - <i>Aspergillus nidulans</i>	36	6.7	24	8.4	8	57	33	283	Cell rescue, defense and virulence
683	Pc18g01320	gj211586710	hypothetical protein contig_1_172_scaffold_16.tfa_550cg - <i>Aspergillus nidulans</i>	27	5.7	24	5.7	15	49	58	644	Cell rescue, defense and virulence
684	Pc22g06690	gj211591714	weak similarity to versicolorm reductase <i>vera</i> - <i>Aspergillus nidulans</i>	26	5.8	24	6.3	16	49	77	577	Metabolism and Energy
685	Pc21g08140	gj211589565	strong similarity to hypothetical protein contig_1_107_scaffold_7.tfa_1090cg - <i>Aspergillus nidulans</i>	27	6.0	24	6.6	6	25	33	190	Unclassified
686	Pc14g01260	gj211584741	strong similarity to proteasome 20S subunit Pre4 - <i>Saccharomyces cerevisiae</i>	29	6.8	24	7.8	12	53	48	437	Protein fate
687	Pc18g02780	gj211586852	strong similarity to hypothetical protein contig1488_2.tfa_1170cg - <i>Aspergillus fumigatus</i>	25	5.2	24	4.8	17	48	73	361	Unclassified
688	Pc16g04730	gj211585447	phosphoglycerate kinase <i>pgkA</i> - <i>Penicillium chrysogenum</i>	44	6.0	24	8.0	10	55	26	251	Metabolism and Energy
689	Pc16g08960	gj211585838	strong similarity to cytoplasmic ribosomal protein of the large subunit S8 - <i>Rattus norvegicus</i>	23	11.1	24	6.1	5	51	36	104	Protein synthesis
690	Pc13g14980	gj211584520	weak similarity to developmental protein C-factor <i>csgA</i> - <i>Myxococcus xanthus</i>	23	9.1	24	7.4	11	47	70	317	Development
691	Pc13g06320	gj211583686	strong similarity to hypothetical protein PA5433 - <i>Pseudomonas aeruginosa</i>	27	8.6	24	8.9	12	48	54	217	Unclassified
692	Pc21g06220	gj211589378	strong similarity to rho GDP dissociation inhibitor Rdi1 - <i>Saccharomyces cerevisiae</i>	22	5.2	24	4.7	10	55	57	258	Protein with binding function or cofactor requirement

693	Pc18g00790	gj 211586658	strong similarity to glutathione S-transferase like protein An02g06560 - <i>Aspergillus niger</i>	29	6.6	24	7.2	15	50	57	350	Cell rescue, defense and virulence
694	Pc22g24980	gj 211593403	strong similarity to hypothetical protein binA - <i>Aspergillus nidulans</i>	21	5.1	24	4.6	5	29	30	132	Unclassified
695	Pc12g10270	gj 211582469	strong similarity to proteasome 20S subunit Pup1 - <i>Saccharomyces cerevisiae</i>	30	6.2	24	8.1	8	57	24	82	Protein fate
696	Pc21g04870	gj 211589244	strong similarity to uridine-monophosphate kinase Ura6 - <i>Saccharomyces cerevisiae</i>	23	5.3	24	5.0	7	50	43	219	Metabolism and Energy
697	Pc20g03290	gj 211587616	strong similarity to hypothetical protein contig1495_2.ifa.640cg - <i>Aspergillus fumigatus</i>	25	5.4	24	5.5	12	53	44	215	Unclassified
698	Pc21g15310	gj 211590252	weak similarity to hypothetical intracellular protease/amidase related enzyme of the ThiJ family CAC2826 - <i>Clostridium acetobutylicum</i>	25	5.6	24	5.4	11	50	42	365	Metabolism and Energy
	Pc22g04730	gj 211591528	strong similarity to hypothetical intracellular protease/amidase related enzyme of the ThiJ family CAC2826 - <i>Clostridium acetobutylicum</i>	24	5.6	24	5.4	5	60	43	103	Metabolism and Energy
699	Pc20g11630	gj 211588384	gamma-actin act- <i>Penicillium chrysogenum</i>	42	5.5	24	5.2	6	59	21	237	Subcellular localization
700	Pc21g15310	gj 211590252	weak similarity to hypothetical intracellular protease/amidase related enzyme of the ThiJ family CAC2826 - <i>Clostridium acetobutylicum</i>	25	5.6	23	5.3	10	36	29	359	Metabolism and Energy
	Pc22g04730	gj 211591528	strong similarity to hypothetical intracellular protease/amidase related enzyme of the ThiJ family CAC2826 - <i>Clostridium acetobutylicum</i>	24	5.6	23	5.3	5	47	43	103	Metabolism and Energy
701	Pc21g15310	gj 211590252	weak similarity to hypothetical intracellular protease/amidase related enzyme of the ThiJ family CAC2826 - <i>Clostridium acetobutylicum</i>	25	5.6	23	5.7	14	51	58	332	Metabolism and Energy
702	Pc20g10030	gj 211588232	similarity to hypothetical protein contig31_part_ii.ifa.3600cg - <i>Aspergillus fumigatus</i>	27	5.4	23	7.5	14	51	70	707	Unclassified
703	Pc22g04730	gj 211591528	strong similarity to hypothetical intracellular protease/amidase related enzyme of the ThiJ family CAC2826 - <i>Clostridium acetobutylicum</i>	24	5.6	23	5.8	7	58	51	286	Metabolism and Energy
704	Pc12g02320	gj 211581742	weak similarity to phosphothricin acetyltransferase pat - <i>Streptomyces coelicolor</i>	27	7.8	23	6.6	7	48	39	87	Cell rescue, defense and virulence
707	Pc12g10470	gj 211582487	strong similarity to L-proline-analogue resistance gene Mpr1 - <i>Saccharomyces cerevisiae</i>	24	6.8	23	8.2	6	59	36	163	Cell rescue, defense and virulence
708	Pc22g23270	gj 211593235	similarity to hypothetical dihydrofolate reductase CAB16576.1 - <i>Schizosaccharomyces pombe</i>	24	5.9	23	6.8	7	58	32	86	Metabolism and Energy
709	Pc13g15810	gj 211584596	strong similarity to ketol-acid reductoisomerase iv-2 - <i>Neurospora crassa</i>	44	8.8	23	7.2	11	37	28	144	Metabolism and Energy
710	Pc12g10470	gj 211582487	strong similarity to L-proline-analogue resistance gene Mpr1 - <i>Saccharomyces cerevisiae</i>	24	6.8	23	8.0	12	30	55	309	Cell rescue, defense and virulence
711	Pc20g11630	gj 211588384	gamma-actin act- <i>Penicillium chrysogenum</i>	42	5.5	23	7.7	8	57	33	233	Subcellular localization
712	Pc22g25560	gj 211593458	strong similarity to dUTP pyrophosphatase DUT1 - <i>Candida albicans</i>	20	6.3	23	6.7	10	44	56	203	Metabolism and Energy
713	Pc14g01130	gj 211584728	strong similarity to 6,7-dimethyl-8-ribitylumazine synthase Rib4 - <i>Saccharomyces cerevisiae</i>	22	5.7	23	6.2	8	38	61	89	Metabolism and Energy
714	Pc21g14440	gj 211590169	strong similarity to Atp2/3 complex 21kDa subunit ARC21 - <i>Homo sapiens</i>	21	6.1	23	7.3	8	45	52	223	Subcellular localization
715	Pc16g14220	gj 211586343	manganese superoxide dismutase AAC36553- <i>Penicillium chrysogenum</i>	23	6.8	22	7.6	9	56	60	265	Cell rescue, defense and virulence
	Pc12g12010	gj 211582636	weak similarity to hypothetical mlf5689 - <i>Mesorhizobium loti</i>	25	5.1	22	4.8	5	60	35	100	Unclassified
716	Pc16g08430	gj 211585787	strong similarity to translation initiation factor Eif-5a.2 - <i>Saccharomyces cerevisiae</i>	18	5.1	22	4.8	5	60	32	99	Protein synthesis

717	Pc20g04720	gj211587749	strong similarity to precursor of dihydropyrimidine dehydrogenase Lpd1 - <i>Saccharomyces cerevisiae</i>	55	7.7	23	5.4	9	32	22	122	Metabolism and Energy
718	Pc16g14220	gj211586343	manganese superoxide dismutase AAC36583- <i>Penicillium chrysogenum</i>	23	6.8	22	7.9	11	54	75	490	Cell rescue, defense and virulence
719	Pc16g14220	gj211586343	manganese superoxide dismutase AAC36583- <i>Penicillium chrysogenum</i>	23	6.8	22	8.1	5	60	34	143	Cell rescue, defense and virulence
720	Pc14g01930	gj211584808	strong similarity to GTP-binding protein rho1p - <i>Schizosaccharomyces pombe</i>	22	6.3	22	7.4	7	35	54	221	Protein with binding function or cofactor requirement
721	Pc21g05750	gj211589332	strong similarity to fatty acid regulation protein like protein An14g07060 - <i>Aspergillus niger</i>	24	5.3	22	5.0	13	52	66	325	Metabolism and Energy
722	Pc22g03110	gj211591371	strong similarity to hypothetical protein An01g08830 - <i>Aspergillus niger</i>	24	7.9	22	5.5	6	35	34	129	Unclassified
723	Pc12g07410	gj211582195	strong similarity to chaperone involved in mitochondrial protein import Mge1 - <i>Saccharomyces cerevisiae</i>	27	9.2	22	6.0	9	56	40	189	Protein fate
724	Pc16g11840	gj211586107	strong similarity to phosphothreonine acetyltransferase like protein An04g09830 - <i>Aspergillus niger</i>	30	7.0	22	6.2	12	53	52	93	Cell rescue, defense and virulence
725	Pc16g14220	gj211586343	manganese superoxide dismutase AAC36583- <i>Penicillium chrysogenum</i>	23	6.8	22	7.7	11	54	58	350	Cell rescue, defense and virulence
726	Pc21g05750	gj211589332	strong similarity to fatty acid regulation protein like protein An14g07060 - <i>Aspergillus niger</i>	24	5.3	22	5.3	9	33	53	397	Metabolism and Energy
727	Pc12g13370	gj211582764	strong similarity to hypothetical protein SPAC9E9.15 - <i>Schizosaccharomyces pombe</i>	25	5.6	22	5.2	8	42	57	79	Unclassified
728	Pc21g11750	gj211589917	strong similarity to adenine phosphoribosyltransferase Apt1 - <i>Saccharomyces cerevisiae</i>	22	5.4	22	4.7	7	58	45	220	Metabolism and Energy
729	Pc13g05400	gj211583594	strong similarity to leukotriene-A4 hydrolase - <i>Mus musculus</i>	76	5.9	22	8.3	13	47	20	169	Metabolism and Energy
730	Pc13g15850	gj211584600	strong similarity to heat shock protein hsp30 - <i>Aspergillus nidulans</i>	18	5.9	22	6.6	8	49	55	129	Protein fate
731	Pc20g1330	gj211587427	strong similarity to 5-formyltetrahydrofolate cyclo-ligase MTHFS - <i>Homo sapiens</i>	23	8.8	22	9.2	7	36	34	116	Metabolism and Energy
732	Pc21g06220	gj211589378	strong similarity to rho GDP dissociation inhibitor Rdi1 - <i>Saccharomyces cerevisiae</i>	22	5.2	22	5.5	9	52	52	166	Protein with binding function or cofactor requirement
733	Pc16g01840	gj211585181	strong similarity to guanylate kinase Guk1- <i>Saccharomyces cerevisiae</i>	22	6.2	22	6.7	19	46	84	538	Metabolism and Energy
734	Pc24g02750	gj211904527	hypothetical protein BAC82546- <i>Penicillium chrysogenum</i>	23	5.9	22	7.0	5	36	32	225	Unclassified
735	Pc22g18890	gj211592809	strong similarity to proteasome 20S subunit Pre1 - <i>Saccharomyces cerevisiae</i>	23	6.3	21	7.3	5	60	33	188	Protein fate
736	Pc21g09090	gj211589659	strong similarity to proteasome 20S subunit Pre3 - <i>Saccharomyces cerevisiae</i>	21	5.4	21	5.7	10	46	68	364	Protein fate
737	Pc22g13950	gj211592336	strong similarity to vacuolar aminopeptidase Ysc1 - <i>Saccharomyces cerevisiae</i>	56	5.7	21	8.7	15	50	27	331	Protein fate
738	Pc13g15290	gj211584544	strong similarity to protein het-c2 - <i>Podospira anserina</i>	22	5.6	21	6.0	5	43	43	83	Cell fate
739	Pc22g13950	gj211592336	strong similarity to vacuolar aminopeptidase Ysc1 - <i>Saccharomyces cerevisiae</i>	56	5.7	21	8.8	11	43	23	341	Protein fate
741	Pc13g04100	gj211583466	strong similarity to peptidyl-prolyl cis/trans isomerase pinA - <i>Aspergillus nidulans</i>	19	5.9	21	6.1	9	56	56	217	Protein fate
742	Pc20g07270	gj211587987	weak similarity to hypothetical cystathione beta-synthase cysB - <i>Dictyostelium discoideum</i>	20	5.6	21	5.4	7	39	26	116	Metabolism and Energy

744	Pc20g06940	gi 211587954	strong similarity to aminopeptidase like protein An02g11940 - <i>Aspergillus niger</i>	53	5.9	21	10.0	11	54	26	98	Protein fate
745	Pc22g18820	gi 211592802	strong similarity to proteasome 20S subunit Pup3 - <i>Saccharomyces cerevisiae</i>	23	4.7	21	4.0	7	49	44	208	Protein fate
747	Pc20g00590	gi 211587356	strong similarity to hypothetical protein rcu01441.1 - <i>Neurospora crassa</i>	21	5.4	21	5.5	10	49	59	183	Unclassified
750	Pc16g06200	gi 211585579	strong similarity to hypothetical protein YER007c-a - <i>Saccharomyces cerevisiae</i>	18	6.7	21	8.5	8	57	57	127	Unclassified
751	Pc20g09400	gi 211588171	strong similarity to dipeptidyl-peptidase V DPP V - <i>Aspergillus fumigatus</i>	79	5.6	21	8.6	18	47	23	410	Protein fate
752	Pc22g13950	gi 211592336	strong similarity to vacuolar aminopeptidase Ysci - <i>Saccharomyces cerevisiae</i>	56	5.7	21	8.7	12	53	25	263	Protein fate
753	Pc13g02920	gi 211583350	strong similarity to host infection protein cap20 - <i>Colletotrichum gloeosporioides</i>	21	7.0	20	8.4	8	47	52	112	Cell rescue, defense and virulence
754	Pc22g25220	gi 211593426	strong similarity to 1,4-benzoquinone reductase qr - <i>Phanerochaete chrysosporium</i>	21	5.7	20	6.4	8	57	68	632	Cell rescue, defense and virulence
755	Pc22g04680	gi 211591523	strong similarity to superoxide dismutase like protein An04g04870 - <i>Aspergillus niger</i>	25	8.7	20	7.7	11	54	78	311	Cell rescue, defense and virulence
756	Pc18g00580	gi 211586638	weak similarity to arp2/3 complex 16kD subunit arc16 - <i>Homo sapiens</i>	20	5.2	20	4.6	9	56	56	201	Biogenesis of cellular components
757	Pc13g04070	gi 211583463	strong similarity to CAP binding protein CBP20-X - <i>Xenopus laevis</i>	21	5.3	20	5.2	7	58	30	88	Protein with binding function or cofactor requirement
759	Pc21g03140	gi 211589089	strong similarity to cell cycle regulator p21 protein wos2p - <i>Schizosaccharomyces pombe</i>	22	4.5	20	3.9	8	57	42	205	Cell cycle and DNA processing
760	Pc18g01380	gi 211586716	strong similarity to hypothetical protein contig. 1_51_scaffold_3.tfa_410cg - <i>Aspergillus nidulans</i>	28	8.4	20	5.0	7	42	37	142	Unclassified
764	Pc22g19060	gi 211592826	strong similarity to cyclophilin cypB - <i>Aspergillus nidulans</i>	29	6.5	20	7.5	12	53	44	346	Protein fate
765	Pc13g02920	gi 211583350	strong similarity to host infection protein cap20 - <i>Colletotrichum gloeosporioides</i>	21	7.0	19	8.1	7	58	61	145	Cell rescue, defense and virulence
766	Pc12g03390	gi 211581843	strong similarity to 19 kD subunit of DNA-directed RNA polymerase II rpb7p - <i>Schizosaccharomyces pombe</i>	19	5.7	19	6.3	8	45	52	111	Transcription
767	Pc20g02710	gi 211587560	weak similarity to DOPA-dioxygenase dodA - <i>Amanita muscaria</i>	20	5.7	19	5.9	10	55	54	302	Metabolism and Energy
768	Pc21g12490	gi 211589989	hypothetical protein <i>Penicillium chrysogenum</i>	22	6.2	19	5.4	7	53	43	107	Unclassified
769	Pc12g10000	gi 211582444	similarity to hypothetical protein CC3654 - <i>Caulobacter crescentus</i>	19	5.0	19	4.4	12	41	95	559	Unclassified
770	Pc13g13470	gi 211584381	strong similarity to tubulin beta chain beta-tubulin like protein An08g03190 - <i>Aspergillus niger</i>	50	4.8	19	4.0	11	54	42	78	Subcellular localization
771	Pc20g03510	gi 211587637	strong similarity to hypothetical protein contig. 1_51_scaffold_3.tfa_1450cg - <i>Aspergillus nidulans</i>	16	5.3	19	7.0	10	55	80	569	Unclassified
772	Pc20g03510	gi 211587637	strong similarity to hypothetical protein contig. 1_51_scaffold_3.tfa_1450cg - <i>Aspergillus nidulans</i>	16	5.3	18	7.9	7	58	49	311	Unclassified
773	Pc22g23760	gi 211593284	strong similarity to type 2 peroxiredoxin like protein An12g08570 - <i>Aspergillus niger</i>	19	4.9	18	3.7	9	56	64	260	Cell rescue, defense and virulence
774	Pc18g05190	gi 211587079	strong similarity to cyclophilin-like peptidyl prolyl cis-trans isomerase cypA - <i>Aspergillus niger</i>	19	8.6	18	8.8	12	53	79	371	Protein fate
775	Pc18g05190	gi 211587079	strong similarity to cyclophilin-like peptidyl prolyl cis-trans isomerase cypA - <i>Aspergillus niger</i>	19	8.6	18	9.2	7	27	40	155	Protein fate

776	Pc18g05190	gj211587079	strong similarity to cyclophilin-like peptidyl prolyl cis-trans isomerase cypA - <i>Aspergillus niger</i>	19	8.6	18	9.0	6	20	36	159	Protein fate
777	Pc18g05190	gj211587079	strong similarity to cyclophilin-like peptidyl prolyl cis-trans isomerase cypA - <i>Aspergillus niger</i>	19	8.6	18	8.4	7	40	36	202	Protein fate
778	Pc22g10250	gj211592050	strong similarity to estrogen receptor-binding cyclophilin cypD - <i>Bos primigenius taurus</i>	19	6.4	18	7.0	12	52	80	297	Unclassified
779	Pc13g06880	gj211583741	strong similarity to hypothetical protein contig49_fta_1800cg - <i>Aspergillus fumigatus</i>	18	5.9	18	5.5	12	38	70	340	Unclassified
780	Pc22g23760	gj211593284	strong similarity to type 2 peroxiredoxin like protein An12g08570 - <i>Aspergillus niger</i>	19	4.9	18	3.9	4	28	27	148	Cell rescue, defense and virulence
781	Pc22g23760	gj211593284	strong similarity to type 2 peroxiredoxin like protein An12g08570 - <i>Aspergillus niger</i>	19	4.9	18	4.9	12	51	71	399	Cell rescue, defense and virulence
782	Pc15g01900	gj211585027	strong similarity to alpha-1,6-glucosidase glcA - <i>Aspergillus parasiticus</i>	87	5.4	18	6.3	17	48	21	247	Metabolism and Energy
783	Pc22g23760	gj211593284	strong similarity to type 2 peroxiredoxin like protein An12g08570 - <i>Aspergillus niger</i>	19	4.9	18	4.2	10	42	70	398	Cell rescue, defense and virulence
784	Pc22g23760	gj211593284	strong similarity to type 2 peroxiredoxin like protein An12g08570 - <i>Aspergillus niger</i>	19	4.9	18	4.0	15	50	71	532	Cell rescue, defense and virulence
785	Pc22g23760	gj211593284	strong similarity to type 2 peroxiredoxin like protein An12g08570 - <i>Aspergillus niger</i>	19	4.9	17	10.0	7	58	43	104	Cell rescue, defense and virulence
787	Pc12g04760	gj211581964	similarity to cytoplasmic ribosomal protein of the large subunit L28 - <i>Rattus norvegicus</i>	17	10.3	17	9.6	12	53	65	417	Protein synthesis
788	Pc14g01740	gj211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47	5.3	17	9.3	10	55	28	352	Metabolism and Energy
789	Pc22g24640	gj211593369	strong similarity to peroxisomal membrane protein PMP20 - <i>Candida boidinii</i>	18	5.4	17	4.9	7	58	65	206	Subcellular localization
790	Pc18g01180	gj211586697	strong similarity to ubiquitin conjugating enzyme Ubc4 - <i>Saccharomyces cerevisiae</i>	17	5.3	17	5.0	10	48	68	162	Protein fate
791	Pc22g23060	gj211593216	weak similarity to hypothetical protein SPAC25B8.18 - <i>Schizosaccharomyces pombe</i>	17	5.6	17	5.4	10	55	81	336	Unclassified
792	Pc24g02700	gj211904522	similarity to hypothetical protein An12g08810 - <i>Aspergillus niger</i>	15	6.6	17	7.9	9	56	75	108	Unclassified
795	Pc22g23430	gj211593251	strong similarity to hypothetical protein contig_1_159_scaffold_13_fta_90cg - <i>Aspergillus nidulans</i>	18	6.2	17	7.1	8	57	44	252	Unclassified
796	Pc08g00080	gj211581481	weak similarity to scytalone dehydratase - <i>Pyricularia oryzae</i>	17	6.1	17	6.8	10	55	78	218	Metabolism and Energy
797	Pc16g03290	gj211585318	weak similarity to cytoplasmic dynein light chain dlc1p - <i>Schizosaccharomyces pombe</i>	16	4.7	17	3.9	9	53	70	203	Cellular transport, transport facilitation and transport routes
798	Pc22g09580	gj211591986	strong similarity to acid phosphatase aphA - <i>Aspergillus ficuum</i>	55	4.8	16	5.6	6	45	12	165	Metabolism and Energy
798	Pc22g25220	gj211593426	strong similarity to 1,4-benzoquinone reductase qr - <i>Phanerochaete chrysosporium</i>	21	5.7	16	5.6	6	59	50	168	Cell rescue, defense and virulence
798	Pc22g25560	gj211593458	strong similarity to dUTP pyrophosphatase DUT1 - <i>Candida albicans</i>	20	6.3	16	5.6	11	54	56	105	Metabolism and Energy
799	Pc16g13060	gj211586228	strong similarity to cyclophilin cypB - <i>Aspergillus nidulans</i>	18	6.9	16	7.8	10	55	72	359	Protein fate
801	Pc18g02330	gj211586810	strong similarity to cytoplasmic ribosomal protein of the small subunit S12 - <i>Sus scrofa</i>	16	4.9	16	4.1	7	51	49	171	Protein synthesis
802	Pc18g02330	gj211586810	strong similarity to cytoplasmic ribosomal protein of the small subunit S12 - <i>Sus scrofa</i>	16	4.9	16	4.0	5	48	61	112	Protein synthesis

803	Pc20g13690	gj211588582	strong similarity to hypothetical protein An02g03710 - <i>Aspergillus niger</i>	17	5.8	16	6.1	6	56	32	309	Unclassified
804	Pc12g10650 truncated	gj1144952798	strong similarity to hypothetical protein ng06840.1 - <i>Magnaporthe grisea</i> . <i>P. chrysogenum</i> 16 kDa allergen	16	5.2	16	4.1	4	61	21	79	Unclassified
805	Pc20g02850	gj211587574	strong similarity to component of the retrieval machinery Grt19 - <i>Saccharomyces cerevisiae</i>	17	7.9	16	8.4	15	50	80	545	Protein with binding function or cofactor requirement
807	Pc12g02950	gj211581800	hypothetical protein <i>Penicillium chrysogenum</i>	20	5.1	16	3.9	3	26	32	120	Unclassified
809	Pc22g18480	gj211592770	similarity to coflin Cof1 - <i>Saccharomyces cerevisiae</i>	16	4.9	15	5.2	5	23	38	164	Subcellular localization
810	Pc22g05220	gj211591574	strong similarity to ubiquitin conjugating enzyme like protein Mms2 - <i>Saccharomyces cerevisiae</i>	16	6.1	15	6.6	7	20	51	195	Protein fate
811	Pc21g10090	gj211589755	similarity to coflin Cof1 - <i>Saccharomyces cerevisiae</i>	17	5.4	15	5.0	10	39	79	398	Subcellular localization
812	Pc21g10090	gj211589755	similarity to coflin Cof1 - <i>Saccharomyces cerevisiae</i>	17	5.4	15	7.3	2	29	22	105	Subcellular localization
	Pc22g21440	gj211593057	strong similarity to ribose-5-phosphate isomerase <i>rpiB</i> - <i>Escherichia coli</i>	17	6.7	15	7.3	5	26	37	84	Metabolism and Energy
813	Pc21g15880	gj211590308	strong similarity to nucleoside-diphosphate kinase NDK-1 - <i>Neurospora crassa</i>	17	7.8	15	8.4	15	47	92	525	Metabolism and Energy
814	Pc21g10090	gj211589755	similarity to coflin Cof1 - <i>Saccharomyces cerevisiae</i>	17	5.4	15	3.4	9	56	51	351	Subcellular localization
815	Pc22g05220	gj211591574	strong similarity to ubiquitin conjugating enzyme like protein Mms2 - <i>Saccharomyces cerevisiae</i>	16	6.1	15	6.2	12	53	66	338	Protein fate
816	Pc22g16880	gj211592618	strong similarity to steroid membrane binding protein like protein An04g00560 - <i>Aspergillus niger</i>	14	5.1	15	4.6	11	36	87	365	Protein with binding function or cofactor requirement
817	Pc24g01570	gj211904428	hypothetical protein <i>Penicillium chrysogenum</i>	16	5.4	15	5.3	9	56	50	303	Unclassified
818	Pc20g07930	gj211588051	strong similarity to ubiquitin conjugating enzyme Ubc4 - <i>Saccharomyces cerevisiae</i>	17	6.4	15	6.7	9	56	55	251	Protein fate
820	Pc12g00650	gj211581586	similarity to hypothetical protein CC3092 - <i>Caulobacter crescentus</i>	15	6.1	15	7.4	14	51	99	405	Unclassified
822	Pc22g21460	gj211593059	strong similarity to hypothetical protein config31_part_i.tfa_2570wg - <i>Aspergillus fumigatus</i>	16	5.2	14	5.2	10	55	54	208	Unclassified
823	Pc06g01290	gj211581349	strong similarity to glycine-rich RNA-binding protein like protein An02g12270 - <i>Aspergillus niger</i>	14	5.4	14	3.4	8	57	52	310	Protein with binding function or cofactor requirement
824	Pc18g05040	gj211587065	strong similarity to peptidyl-prolyl isomerase FKBP-21 - <i>Neurospora crassa</i>	14	5.3	14	4.8	10	55	66	295	Protein fate
826	Pc18g01750	gj211586752	similarity to cytoplasmic ribosomal protein of the large subunit L22 - <i>Xenopus laevis</i>	14	9.3	14	9.5	5	60	46	263	Protein synthesis
827	Pc06g01290	gj211581349	strong similarity to glycine-rich RNA-binding protein like protein An02g12270 - <i>Aspergillus niger</i>	14	5.4	14	4.7	8	57	53	166	Protein with binding function or cofactor requirement
828	Pc13g03940	gj211583450	strong similarity to hypothetical protein An09g03330 - <i>Aspergillus niger</i>	15	5.3	14	5.1	8	57	66	146	Unclassified
829	Pc16g01040	gj211585145	strong similarity to cytidine deaminase - <i>Homo sapiens</i>	18	6.8	14	7.3	8	56	60	386	Metabolism and Energy
830	Pc12g03750	gj211581874	similarity to hypothetical protein An12g08810 - <i>Aspergillus niger</i>	15	5.9	14	5.5	12	53	69	308	Unclassified
831	Pc21g11730	gj211588915	strong similarity to hypothetical protein PH0854 - <i>Pyrococcus horikoshii</i>	13	5.6	13	5.1	7	58	84	229	Unclassified

832	Pc17g01220	gi 211586572	hypothetical protein, <i>Penicillium chrysogenum</i> Wisconsin 54-1255	13	5.0	13	5.6	2	63	17	135	Unclassified
833	Pc13g06110	gi 211583665	strong similarity to peptidyl-prolyl isomerase like protein An14g00850 - <i>Aspergillus niger</i>	13	6.1	13	7.3	5	60	58	244	Protein fate
834	Pc12g13990	gi 211582824	strong similarity to profilin cdc3p - <i>Schizosaccharomyces pombe</i>	14	4.9	12	4.0	2	63	32	74	Protein with binding function or cofactor requirement
835	Pc12g13990	gi 211582824	strong similarity to profilin cdc3p - <i>Schizosaccharomyces pombe</i>	14	4.9	12	4.2	5	60	61	198	Protein with binding function or cofactor requirement
836	Pc18g05330	gi 211587093	strong similarity to hypothetical protein contig248.tfa_180wg - <i>Aspergillus fumigatus</i>	50	8.8	12	8.1	8	57	16	158	Unclassified
837	Pc13g10440	gi 211584085	strong similarity to nuclear transport factor NT2 - <i>Saccharomyces cerevisiae</i>	14	4.5	12	3.7	3	62	26	155	Cellular transport, transport facilitation and transport routes
839	Pc06g01300	gi 211581350	strong similarity to thioredoxin - <i>Aspergillus nidulans</i>	12	4.9	11	4.4	7	39	68	139	Cell rescue, defense and virulence
842	Pc20g10130	gi 211588242	similarity to RNA-binding protein 30 - <i>Nicotiana plumbaginifolia</i>	10	5.3	11	4.7	4	61	86	148	Protein with binding function or cofactor requirement
845	Pc21g16930	gi 211590404	strong similarity to 100 kDa coactivator snd1 - <i>Homo sapiens</i>	98	5.7	114	6.3	23	42	33	321	Transcription
846	Pc22g09380	gi 211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanase gel3 - <i>Aspergillus fumigatus</i>	58	4.9	112	5.1	13	52	28	247	Biogenesis of cellular components
847	Pc12g05640	gi 211582027	strong similarity to heat shock protein sspB - <i>Aspergillus niger</i>	80	4.9	112	5.1	19	46	29	103	Protein fate
847	Pc21g02440	gi 211589019	strong similarity to ubiquitin activating protein Uba1 - <i>Saccharomyces cerevisiae</i>	115	5.0	122	4.8	22	43	38	177	Protein fate
848	Pc16g06530	gi 211585612	strong similarity to tRNA ligase Trt1 - <i>Saccharomyces cerevisiae</i>	94	6.7	81	6.7	18	47	25	90	Transcription
850	Pc22g06680	gi 211591713	acetate-CoA ligase <i>facA</i> - <i>Penicillium chrysogenum</i>	75	5.9	77	6.6	17	48	26	179	Metabolism and Energy
856	Pc20g07710	gi 211588029	sulfate adenylyltransferase <i>aps</i> - <i>Penicillium chrysogenum</i>	64	6.4	74	8.1	38	27	65	833	Metabolism and Energy
857	Pc16g07440	gi 211585692	strong similarity to cytoplasmic proline-tRNA ligase like protein An08g02860 - <i>Aspergillus niger</i>	70	6.5	77	8.1	17	43	36	250	Protein synthesis
858	Pc22g14170	gi 211592358	strong similarity to tRNA Met processing protein Gcd14 - <i>Saccharomyces cerevisiae</i>	57	8.2	65	8.5	13	52	35	155	Transcription
859	Pc22g03860	gi 211591424	strong similarity to isocitrate lyase <i>acuD</i> - <i>Aspergillus nidulans</i>	60	6.5	65	8.2	16	39	41	261	Metabolism and Energy
860	Pc20g15010	gi 211588710	strong similarity to carnitine O-acetyltransferase <i>cat2</i> - <i>Candida tropicalis</i>	73	8.5	67	8.1	21	44	33	223	Metabolism and Energy
861	Pc21g02050	gi 211588981	strong similarity to multifunctional glutamine-proline-tRNA ligase Aats-glupro - <i>Drosophila melanogaster</i>	70	6.2	74	7.2	13	52	29	75	Protein synthesis
862	Pc18g00980	gi 211586677	strong similarity to hypothetical trunk lateral cell specific gene HTLC1 - <i>Halocynthia roretzi</i>	57	5.6	59	6.4	11	48	23	139	Cell rescue, defense and virulence
863	Pc18g00980	gi 211586677	strong similarity to hypothetical trunk lateral cell specific gene HTLC1 - <i>Halocynthia roretzi</i>	57	5.6	57	6.4	21	44	48	357	Cell rescue, defense and virulence
864	Pc09g00050	gi 211581488	similarity to hypothetical protein YER080w - <i>Saccharomyces cerevisiae</i>	62	5.5	71	5.7	12	53	33	137	Unclassified
865	Pc21g23240	gi 211591002	strong similarity to alpha-isopropylmalate synthase Leu4 - <i>Saccharomyces cerevisiae</i>	71	5.4	76	5.5	14	49	22	113	Metabolism and Energy
866	Pc21g18000	gi 211590506	strong similarity to hypothetical DnaJ-like heat shock protein 17E5.120 - <i>Neurospora crassa</i>	39	9.2	41	8.7	16	49	59	377	Protein fate

867	Pc22g04940	gij211591548	strong similarity to 2-ketaldonate reductase like protein An01g07030 - <i>Aspergillus niger</i>	37	5.8	38	6.2	7	58	34	121	Metabolism and Energy
868	Pc22g22650	gij211593176	strong similarity to androgen-inducible aldehyde reductase air - <i>Rattus norvegicus</i>	37	5.8	33	6.2	27	35	77	708	Metabolism and Energy
869	Pc12g04650	gij211581953	strong similarity to purine-nucleoside phosphorylase - <i>Bos taurus</i>	33	5.9	34	6.2	19	43	64	793	Metabolism and Energy
870	Pc21g14600	gij211590185	strong similarity to mRNA cleavage factor 125 kDa subunit like protein An16g01870 - <i>Aspergillus niger</i>	32	5.7	33	5.9	12	53	56	464	Transcription
875	Pc12g09360	gij211582382	strong similarity to phosphoenolpyruvate carboxykinase KIPck1 - <i>Kluyveromyces fragilis</i>	67	5.8	66	5.6	27	36	59	487	Metabolism and Energy
876	Pc13g05480	gij211583602	strong similarity to fimbrin - <i>Gibberella pulicaris</i>	72	5.4	72	5.4	16	49	33	247	Subcellular localization
877	Pc16g09030	gij211585845	strong similarity to polyadenylate-binding protein Pabp - <i>Saccharomyces cerevisiae</i>	80	5.5	69	5.4	20	45	30	298	Protein synthesis
878	Pc13g09300	gij211583974	strong similarity to pyruvate decarboxylase pdcA - <i>Aspergillus oryzae</i>	63	5.7	61	5.8	30	35	60	822	Metabolism and Energy
879	Pc22g09390	gij211591968	strong similarity to mannitol dehydrogenase mtlD - <i>Pseudomonas fluorescens</i>	59	5.6	59	5.9	15	50	33	77	Metabolism and Energy
880	Pc18g01390	gij211586717	strong similarity to phosphoglucomutase pgmB - <i>Aspergillus nidulans</i>	60	5.7	63	5.7	15	50	37	100	Metabolism and Energy
881	Pc13g09300	gij211583974	strong similarity to pyruvate decarboxylase pdcA - <i>Aspergillus oryzae</i>	63	5.7	61	5.6	25	40	54	491	Metabolism and Energy
882	Pc22g22460	gij211593157	strong similarity to TCP1 complex beta chain TCP1 Beta - <i>Saccharomyces cerevisiae</i>	57	5.7	59	6.2	21	44	49	326	Protein fate
	Pc18g00980	gij211586677	strong similarity to hypothetical trunk lateral cell specific gene HtTLC1 - <i>Halocynthia roretzi</i>	57	5.6	59	6.2	17	48	34	180	Cell rescue, defense and virulence
883	Pc22g12740	gij211592234	similarity to hypothetical protein rfeF - <i>Aspergillus nidulans</i>	66	5.6	60	6.1	17	48	35	346	Transcription
884	Pc13g07730	gij211583822	strong similarity to threonine deaminase ILV - <i>Arxula adenivorans</i>	62	5.9	62	6.6	17	48	43	220	Metabolism and Energy
	Pc12g13060	gij211582733	strong similarity to coronin Crn1 - <i>Saccharomyces cerevisiae</i>	65	5.5	62	6.6	15	50	34	151	Subcellular localization
885	Pc16g04730	gij211585447	phosphoglycerate kinase pgkA - <i>Penicillium chrysogenum</i>	44	6.0	47	6.6	24	41	65	669	Metabolism and Energy
886	Pc12g05420	gij211582006	strong similarity to O-acetylhomoserine (thiol)-lyase cysD - <i>Aspergillus nidulans</i>	47	5.9	47	6.8	20	45	58	636	Metabolism and Energy
887	Pc16g04730	gij211585447	phosphoglycerate kinase pgkA - <i>Penicillium chrysogenum</i>	44	6.0	46	6.6	10	55	33	119	Metabolism and Energy
888	Pc22g11710	gij211592134	strong similarity to alpha subunit E1 of the pyruvate dehydrogenase complex Pda1 - <i>Saccharomyces cerevisiae</i>	45	8.5	46	6.7	22	33	58	698	Metabolism and Energy
889	Pc12g16040	gij211583021	strong similarity to phosphoglycerate mutase pgm - <i>Bacillus subtilis</i>	57	5.2	57	4.8	25	38	51	591	Metabolism and Energy
890	Pc20g08320	gij211588090	strong similarity to hypothetical protein FLJ10830 - <i>Homo sapiens</i>	53	5.0	54	4.7	16	42	48	217	Unclassified
891	Pc15g00670	gij211584915	weak similarity to hypothetical UniE/COQ5 family methyltransferase - <i>Caulobacter crescentus</i>	33	5.0	32	5.2	11	54	55	120	Metabolism and Energy
	Pc21g12600	gij211589998	strong similarity to hypothetical protein 1465_scaffold_9_ita_470wg - <i>Fusarium graminearum</i>	28	5.5	32	5.2	11	54	33	90	Unclassified
893	Pc21g14440	gij211590169	strong similarity to Arp2/3 complex 2 kDa subunit ARC21 - <i>Homo sapiens</i>	21	6.1	23	6.9	11	54	56	218	Subcellular localization

894	Pc12g12040	gj211582639	strong similarity to translation elongation factor eEF-2 - <i>Cricetulus griseus</i>	94	6.3	33	6.5	8	39	12	110	Protein synthesis
895	Pc21g18110	gj211590517	strong similarity to electron transfer flavoprotein beta chain ETF-beta - <i>Paracoccus denitrificans</i>	28	9.0	28	9.3	9	56	49	207	Metabolism and Energy
899	Pc13g03800	gj211583437	strong similarity to precursor of mitochondrial nuclease Nuc1 - <i>Saccharomyces cerevisiae</i>	36	9.2	28	8.7	11	54	35	217	Cell cycle and DNA processing
910	Pc21g15880	gj211590308	strong similarity to nucleoside-diphosphate kinase NDK-1 - <i>Neurospora crassa</i>	17	7.8	15	7.8	7	58	47	143	Metabolism and Energy
911	Pc22g01600	gj211591221	strong similarity to histidine triad protein Hnt1 - <i>Saccharomyces cerevisiae</i>	15	6.7	13	7.7	7	45	77	194	Cell fate
913	Pc12g00650	gj211581586	similarity to hypothetical protein CC3092 - <i>Caulobacter crescentus</i>	15	6.1	15	7.0	9	37	80	322	Unclassified
919	Pc22g25560	gj211593458	strong similarity to dUTP pyrophosphatase DUT1 - <i>Candida albicans</i>	20	6.3	16	5.9	7	26	48	155	Metabolism and Energy
929	Pc12g14390	gj211582863	strong similarity to hypothetical glutathione S-transferase CAA19067.1 - <i>Schizosaccharomyces pombe</i>	27	5.6	26	5.9	6	59	42	97	Cell rescue, defense and virulence
938	Pc22g15930	gj211592526	strong similarity to pyridoxine synthesis component pyroA - <i>Aspergillus nidulans</i>	33	5.7	35	5.3	9	25	37	209	Metabolism and Energy
	Pc22g04850	gj211591539	strong similarity to D-arabinose dehydrogenase Ara1 - <i>Saccharomyces cerevisiae</i>	37	5.5	35	5.3	5	30	23	102	Metabolism and Energy
947	Pc12g14620	gj211582885	strong similarity to flavohemoglobin Fhp- <i>Alcaligenes eutrophus</i>	46	6.0	44	7.3	10	55	32	199	Protein with binding function or cofactor requirement
	Pc12g04310	gj211581920	strong similarity to acetate-inducible gene acA - <i>Aspergillus nidulans</i>	50	6.3	44	7.3	14	51	31	160	Unclassified
948	Pc21g05430	gj211589300	strong similarity to cystathionine gamma-lyase - <i>Saccharomyces cerevisiae</i>	42	6.3	38	7.4	9	22	42	436	Metabolism and Energy
949	Pc22g10040	gj211592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	37	7.4	12	53	40	196	Metabolism and Energy
950	Pc22g10040	gj211592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	41	7.4	15	50	46	241	Metabolism and Energy
	Pc22g22180	gj211593130	strong similarity to phe-inhibited DAHP synthase aroG - <i>Aspergillus nidulans</i>	41	6.3	41	7.4	12	53	30	85	Metabolism and Energy
	Pc12g04310	gj211581920	strong similarity to acetate-inducible gene acA - <i>Aspergillus nidulans</i>	50	6.3	43	7.5	16	42	37	133	Unclassified
951	Pc12g14620	gj211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	43	7.5	4	53	20	105	Protein with binding function or cofactor requirement
	Pc22g20230	gj211592940	strong similarity to hypothetical conserved protein SPOC61.03 - <i>Schizosaccharomyces pombe</i>	40	6.3	43	7.5	10	48	37	100	Unclassified
952	Pc12g14620	gj211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	41	7.5	15	50	51	405	Protein with binding function or cofactor requirement
	Pc22g14810	gj211592419	strong similarity to hypothetical oxidoreductase CAB46711.1 - <i>Schizosaccharomyces pombe</i>	40	6.4	41	7.5	13	52	41	132	Unclassified
	Pc06g01770	gj211581395	strong similarity to adenylosuccinate synthase ade2p - <i>Schizosaccharomyces pombe</i>	47	6.4	41	7.5	13	52	30	82	Metabolism and Energy
953	Pc06g01800	gj211581398	strong similarity to quinone oxidoreductase P1 - <i>Arabidopsis thaliana</i>	37	6.5	39	7.7	14	29	59	225	Cell rescue, defense and virulence
958	Pc20g08200	gj211588078	strong similarity to hypothetical protein involved in ergosterol biosynthesis SPBC646.08c - <i>Schizosaccharomyces pombe</i> [truncated ORF]	45	6.2	49	7.4	16	36	52	333	Metabolism and Energy
959	Pc21g08400	gj211589590	strong similarity to 3-hydroxyisobutyryl-coenzyme A hydrolase like protein An14g06290 - <i>Aspergillus niger</i>	53	7.8	49	8.0	12	19	32	138	Metabolism and Energy

894	Pc12g12040	gl 211582639	strong similarity to translation elongation factor eEF-2 - <i>Circetulus griseus</i>	94	6.3	33	6.5	8	39	12	110	Protein synthesis
895	Pc21g18110	gl 211590517	strong similarity to electron transfer flavoprotein beta chain ETF-beta - <i>Paracoccus denitrificans</i>	28	9.0	28	9.3	9	56	49	207	Metabolism and Energy
899	Pc13g03800	gl 211583437	strong similarity to precursor of mitochondrial nuclease Nuc1 - <i>Saccharomyces cerevisiae</i>	36	9.2	28	8.7	11	54	35	217	Cell cycle and DNA processing
910	Pc21g15880	gl 211590308	strong similarity to nucleoside-diphosphate kinase NDK-1 - <i>Neurospora crassa</i>	17	7.8	15	7.8	7	58	47	143	Metabolism and Energy
911	Pc22g01600	gl 211591221	strong similarity to histidine triad protein Hnt1 - <i>Saccharomyces cerevisiae</i>	15	6.7	13	7.7	7	45	77	194	Cell fate
913	Pc12g00650	gl 211581586	similarity to hypothetical protein CC3092 - <i>Caulobacter crescentus</i>	15	6.1	15	7.0	9	37	80	322	Unclassified
919	Pc22g25560	gl 211593458	strong similarity to dUTP pyrophosphatase DUT1 - <i>Candida albicans</i>	20	6.3	16	5.9	7	26	48	155	Metabolism and Energy
929	Pc12g14390	gl 211582863	strong similarity to hypothetical glutathione S-transferase CAA19067.1 - <i>Schizosaccharomyces pombe</i>	27	5.6	26	5.9	6	59	42	97	Cell rescue, defense and virulence
938	Pc22g15930	gl 211592526	strong similarity to pyridoxine synthesis component pyroA - <i>Aspergillus nidulans</i>	33	5.7	35	5.3	9	25	37	209	Metabolism and Energy
	Pc22g04850	gl 211591539	strong similarity to D-arabinose dehydrogenase Ara1 - <i>Saccharomyces cerevisiae</i>	37	5.5	35	5.3	5	30	23	102	Metabolism and Energy
947	Pc12g14620	gl 211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	44	7.3	10	55	32	199	Protein with binding function or cofactor requirement
	Pc12g04310	gl 211581920	strong similarity to acetate-inducible gene acA - <i>Aspergillus nidulans</i>	50	6.3	44	7.3	14	51	31	160	Unclassified
948	Pc21g05430	gl 211589300	strong similarity to cystathionine gamma-lyase - <i>Saccharomyces cerevisiae</i>	42	6.3	38	7.4	9	22	42	436	Metabolism and Energy
949	Pc22g10040	gl 211592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	37	7.4	12	53	40	196	Metabolism and Energy
950	Pc22g10040	gl 211592030	strong similarity to glucosamine-6-phosphate deaminase like protein An16g09070 - <i>Aspergillus niger</i>	40	6.1	41	7.4	15	50	46	241	Metabolism and Energy
	Pc22g22180	gl 211593130	strong similarity to phe-inhibited DAHP synthase aroG - <i>Aspergillus nidulans</i>	41	6.3	41	7.4	12	53	30	85	Metabolism and Energy
	Pc12g04310	gl 211581920	strong similarity to acetate-inducible gene acA - <i>Aspergillus nidulans</i>	50	6.3	43	7.5	16	42	37	133	Unclassified
951	Pc12g14620	gl 211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	43	7.5	4	53	20	105	Protein with binding function or cofactor requirement
	Pc22g20230	gl 211592940	strong similarity to hypothetical conserved protein SPCC61.03 - <i>Schizosaccharomyces pombe</i>	40	6.3	43	7.5	10	48	37	100	Unclassified
	Pc12g14620	gl 211582885	strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>	46	6.0	41	7.5	15	50	51	405	Protein with binding function or cofactor requirement
952	Pc22g14810	gl 211592419	strong similarity to hypothetical oxidoreductase CAB46711.1 - <i>Schizosaccharomyces pombe</i>	40	6.4	41	7.5	13	52	41	132	Unclassified
	Pc06g01770	gl 211581395	strong similarity to adenylosuccinate synthase ade2p - <i>Schizosaccharomyces pombe</i>	47	6.4	41	7.5	13	52	30	82	Metabolism and Energy
953	Pc06g01800	gl 211581398	strong similarity to quinone oxidoreductase P1 - <i>Arabidopsis thaliana</i>	37	6.5	39	7.7	14	29	59	225	Cell rescue, defense and virulence
958	Pc20g08200	gl 211588078	strong similarity to hypothetical protein involved in ergosterol biosynthesis SPBC646.08c - <i>Schizosaccharomyces pombe</i> [truncated ORF]	45	6.2	49	7.4	16	36	52	333	Metabolism and Energy
959	Pc21g08400	gl 211589590	strong similarity to 3-hydroxyisobutyryl-coenzyme A hydrolase like protein An14g06290 - <i>Aspergillus niger</i>	53	7.8	49	8.0	12	19	32	138	Metabolism and Energy
960	Pc12g05970	gl 211582060	strong similarity to cell division control protein CDC3 - <i>Candida albicans</i>	47	6.3	61	7.0	11	27	37	144	Cell cycle and DNA processing
975	Pc13g10120	gl 211584054	strong similarity to acetolactate synthase precursor ALS - <i>Schizosaccharomyces pombe</i>	77	6.7	72	6.7	13	39	25	84	Metabolism and Energy
976	Pc18g02490	gl 211586826	strong similarity to cytoplasmic ribosomal protein of the small subunit S19 - <i>Saccharomyces cerevisiae</i>	17	8.7	16	8.4	13	52	66	327	Protein synthesis

Supplementary Table 2. Isoforms identified in the cytosolic proteome reference map of *P. chrysogenum* Wisconsin 54-1255. Proteins are ordered according to their ORF name.

Spot	ORF name	Accession N.	Isoforms	Spot	ORF name	Accession N.	Isoforms	Spot	ORF name	Accession N.	Isoforms
233 240 241 244 245 247	Pc06g00180	gil211581247	6	172 884	Pc12g13060	gil211582733	2	433 435	Pc13g11380	gil211584176	2
823 827	Pc06g01290	gil211581349	2	834 835	Pc12g13990	gil211582824	2	479 494	Pc13g11570	gil211584194	2
370 952	Pc06g01770	gil211581395	2	456 471 474 476	Pc12g14200	gil211582844	4	540 553 567	Pc13g12400	gil211584276	3
446 953	Pc06g01800	gil211581398	2	664 929	Pc12g14390	gil211582863	2	89 90 91 92	Pc13g12450	gil211584281	4
475 478	Pc12g00420	gil211581565	2	339 352 355 356 364 370 372 374 382 509 578 947 951 952	Pc12g14620	gil211582885	14	221 770	Pc13g13470	gil211584381	2
820 913	Pc12g00650	gil211581586	2	375 379	Pc12g15600	gil211582979	2	533 552	Pc14g01140	gil211584729	2
637 641	Pc12g00830	gil211581603	2	257 264 265	Pc12g16020	gil211583019	3	383 387	Pc14g01170	gil211584732	2
344 357	Pc12g02970	gil211581802	2	153 889	Pc12g16040	gil211583021	2	271 273 280 281 788	Pc14g01740	gil211584789	5
275 277	Pc12g03090	gil211581814	2	396 427 544 631	Pc12g16540	gil211583071	4	636 642	Pc15g00320	gil211584881	2
166 168 173	Pc12g03130	gil211581818	3	753 765	Pc13g02920	gil211583350	2	231 236	Pc15g00640	gil211584913	2
207 219 224	Pc12g03370	gil211581841	3	613 899	Pc13g03800	gil211583437	2	528 531 535	Pc15g00670	gil211584915	7
365 947 951	Pc12g04310	gil211581920	3	399 404	Pc13g04680	gil211583523	2	550 557 572 891	Pc15g01030	gil211584950	3
113 119 131 183	Pc12g04590	gil211581947	4	266 267	Pc13g05010	gil211583556	2	8 509	Pc16g01190	gil211585160	2
505 869	Pc12g04650	gil211581953	2	208 213	Pc13g05320	gil211583586	2	419 422 423	Pc16g01850	gil211585182	3
500 504	Pc12g04750	gil211581963	2	579 586 587	Pc13g05520	gil211583606	3	668 672	Pc16g02160	gil211585211	2
326 328 329 331	Pc12g05400	gil211582004	4	163 884	Pc13g07730	gil211583822	2	41 43	Pc16g03220	gil211585311	2
69 846	Pc12g05640	gil211582027	2	466 470 472	Pc13g07960	gil211583842	3	351 358 363 566 576 582 610	Pc16g04380	gil211585413	7
551 554	Pc12g05820	gil211582045	2	327 330 332 334	Pc13g08480	gil211583893	4	80 87 93	Pc16g04640	gil211585438	3
211 228 960	Pc12g05970	gil211582060	3	503 656	Pc13g08810	gil211583926	2	660 688 885 887	Pc16g04730	gil211585447	4
12 14	Pc12g06650	gil211582124	2	878 881	Pc13g09300	gil211583974	2	301 302	Pc16g05080	gil211585478	2
501 510	Pc12g08930	gil211582340	2	300 307	Pc13g09660	gil211584009	2	576 571	Pc16g05780	gil211585539	2
73 76	Pc12g09250	gil211582371	2	362 413 414	Pc13g09680	gil211584011	3	349 361	Pc16g06210	gil211585580	2
137 875	Pc12g09360	gil211582382	2	230 246 340 313	Pc13g10770	gil211584117	4	81 857	Pc16g07440	gil211585692	2
427 428	Pc12g09820	gil211582426	2					285 309	Pc16g07470	gil211585695	2
707 710	Pc12g10470	gil211582487	2								
247 250	Pc12g11540	gil211582591	2								
397 442 443 517 894	Pc12g12040	gil211582639	5								

Spot	ORF name	Accession N.	Isoforms	Spot	ORF name	Accession N.	Isoforms	Spot	ORF name	Accession N.	Isoforms
436	Pc16g08460	gil211585789	3	774	Pc18g05190	gil211587079	4	425	Pc21g04710	gil211589228	3
437				775				432			
457				776				446			
27	Pc16g09030	gil211585845	6	777				430	Pc21g05430	gil211589300	4
28				177	Pc18g06000	gil211587160	3	433			
38				179				434			
101				183				948			
107				485	Pc20g01610	gil211587455	7	721	Pc21g05750	gil211589332	2
877				508				726			
135	Pc16g11070	gil211586036	5	512				692	Pc21g06220	gil211589378	2
165				516				732			
167				520				640	Pc21g06950	gil211589448	2
175				521				643			
506				523				677	Pc21g08140	gil211589565	2
29	Pc16g11860	gil211586109	12	144	Pc20g01630	gil211587457	2	685			
36				152				230	Pc21g10070	gil211589753	2
39				305	Pc20g02910	gil211587580	3	239			
40				306				811	Pc21g10090	gil211589755	3
45				585				812			
46				184	Pc20g03330	gil211587620	4	814			
48				186				397	Pc21g10370	gil211589783	5
51				188				412			
54				213				416			
57				771	Pc20g03510	gil211587637	2	598			
60				772				606			
61				276	Pc20g03610	gil211587647	4	98	Pc21g11280	gil211589871	2
651	Pc16g11920	gil211586115	2	279				134			
655				295				169	Pc21g11950	gil211589937	2
110	Pc16g12680	gil211586190	2	282	Pc20g04720	gil211587749	2	175			
145				261				580	Pc21g12310	gil211589973	3
234	Pc16g13280	gil211586250	3	717	Pc20g05830	gil211587847	2	604			
238				461				617			
715	Pc16g14220	gil211586343	4	291	Pc20g06040	gil211587868	2	559	Pc21g12600	gil211589998	4
718				294				560			
719				225	Pc20g06940	gil211587954	3	564			
725				237				891			
671	Pc18g00790	gil211586658	3	744				201	Pc21g12790	gil211590017	6
678				530	Pc20g07230	gil211587983	3	202			
693				531				205			
594	Pc18g00870	gil211586666	2	532				206			
597				96	Pc20g07710	gil211588029	3	214			
178	Pc18g00980	gil211586677	8	97				215			
184				856				714	Pc21g14440	gil211590169	2
197				594	Pc20g09400	gil211588171	2	893			
198				751				480	Pc21g14560	gil211590181	7
199				115	Pc20g10360	gil211588263	2	484			
862				121				488			
863				634	Pc20g11580	gil211588379	2	493			
882				635				497			
417	Pc18g01220	gil211586700	4	699	Pc20g11630	gil211588384	2	555			
418				711				556			
428				298	Pc20g11850	gil211588406	3	522	Pc21g14600	gil211590185	2
429				301				870			
360	Pc18g01240	gil211586702	2	456	Pc20g15580	gil211588765	2	698	Pc21g15310	gil211590252	3
350				459				700			
157	Pc18g01390	gil211586717	2	94	Pc21g02050	gil211588981	2	701	Pc21g15880	gil211590308	2
880				861				910			
801	Pc18g02330	gil211586810	2	490	Pc21g02210	gil211588997	2	370	Pc21g16520	gil211590370	2
802				492				372			
22	Pc18g02580	gil211586833	4	186	Pc21g02360	gil211589011	3	18	Pc21g16930	gil211590404	2
42				187				845			
47				189				507	Pc21g16950	gil211590406	4
190	Pc18g02760	gil211586850	2	19	Pc21g02440	gil211589019	2	511			
193				847				513			
420	Pc18g03540	gil211586925	2	605	Pc21g03140	gil211589089	2	514			
414				759				573	Pc21g16970	gil211590408	4
								574			
								578			
								582			

Spot	ORF name	Accession N.	Isoforms	Spot	ORF name	Accession N.	Isoforms	Spot	ORF name	Accession N.	Isoforms
235 251	Pc21g17880	gil211590494	2	182 195 879	Pc22g09390	gil211591968	3	600 601	Pc22g17950	gil211592721	2
366 381 866	Pc21g18000	gil211590506	3	270 798	Pc22g09580	gil211591986	2	249 258	Pc22g18320	gil211592754	2
620 895	Pc21g18110	gil211590517	2	34 56	Pc22g09990	gil211592025	2	49 52 53	Pc22g18630	gil211592785	3
278 283	Pc21g18310	gil211590536	2	338 341 571	Pc22g10010	gil211592027	3	125 126 127 132	Pc22g19100	gil211592830	4
314 317	Pc21g19210	gil211590623	2	353 369 377 385 394 398	Pc22g10040	gil211592030	10	354 383 386 387	Pc22g19440	gil211592864	4
20 21 23	Pc21g19270	gil211590629	3	519 627 949 950				85 88 124 204 255 350 469 558	Pc22g19990	gil211592918	8
653 654	Pc21g23590	gil211591037	2	88 99 100	Pc22g10220	gil211592047	3	347 348 351	Pc22g20800	gil211592996	3
194 248 287	Pc22g00890	gil211591151	3	65 77 84 380	Pc22g11240	gil211592088	4	185 191	Pc22g21240	gil211593038	2
661 662 663 665 666	Pc22g01260	gil211591187	5	316 319 323 888	Pc22g11710	gil211592134	4	615 623	Pc22g21430	gil211593056	2
616 646 648	Pc22g01530	gil211591214	3	259 883	Pc22g12740	gil211592234	2	525 537 812	Pc22g21440	gil211593057	3
55 58 59 62	Pc22g02000	gil211591261	4	402 403 406 407	Pc22g13130	gil211592257	4	1 602	Pc22g22640	gil211593175	2
447 453 460	Pc22g02940	gil211591355	3	116 118 136	Pc22g13590	gil211592302	3	502 868	Pc22g22650	gil211593176	2
154 162 859	Pc22g03660	gil211591424	3	737 739 752	Pc22g13950	gil211592336	3	146 147	Pc22g22710	gil211593182	2
698 700 703	Pc22g04730	gil211591528	3	371 952	Pc22g14810	gil211592419	2	252 254	Pc22g22810	gil211593192	2
10 13 17	Pc22g04770	gil211591532	3	71 72	Pc22g15510	gil211592485	2	670 674	Pc22g23120	gil211593222	2
236 243	Pc22g04840	gil211591538	2	82 86 547	Pc22g15910	gil211592524	3	773 780 781 783 784 785	Pc22g23760	gil211593284	6
489 938	Pc22g04850	gil211591539	2	463 499 938	Pc22g15930	gil211592526	3	293 296 308	Pc22g23810	gil211593289	3
810 815	Pc22g05220	gil211591574	2	545 546	Pc22g16150	gil211592548	2	754 798	Pc22g25220	gil211593426	2
2 3	Pc22g05690	gil211591618	2	658 659	Pc22g16280	gil211592561	2	527 548	Pc22g25320	gil211593435	2
170 171	Pc22g05940	gil211591643	2	221 227	Pc22g17420	gil211592672	2	622 626	Pc22g25470	gil211593449	2
74 850	Pc22g06680	gil211591713	2	256 263	Pc22g17560	gil211592684	2	712 798 919	Pc22g25560	gil211593458	3
320 322	Pc22g08010	gil211591834	2	114 150 268	Pc22g17870	gil211592713	3				
117 120 129 130	Pc22g08080	gil211591841	4	125 126 128	Pc22g17940	gil211592720	3				
222 227	Pc22g08480	gil211591877	2								
64 66 67 68 70 112 846	Pc22g09380	gil211591967	7								

Supplementary Table 3. Proteins identified in the extracellular proteome reference map of *P. chrysogenum* Wisconsin 5

Spot	ORF name	Accession Number	Description	Theoretical		Estimated		Non-Matched	Score	Protein coverage (%)	Function
				Mass (kDa)	pI	Mass (kDa)	pI				
1	Pc18g02900	gi 211586864	lysophospholipase phospholipase B plb1 - <i>Penicillium chrysogenum</i>	66.2	4.5	105.0	4.6	9	341	21	Cell rescue, defense and virulence
2	Pc12g15470	gi 211582966	strong similarity to hypothetical protein contig1492_0.fta_1880cg - <i>A. fumigatus</i>	70.7	5.2	96.7	4.9	27	594	50	Utilization of nutrients
3	Pc12g15470	gi 211582966	strong similarity to hypothetical protein contig1492_0.fta_1880cg - <i>A. fumigatus</i>	70.7	5.2	97.0	5.0	24	709	42	Utilization of nutrients
4	Pc12g15470	gi 211582966	strong similarity to hypothetical protein contig1492_0.fta_1880cg - <i>A. fumigatus</i>	70.7	5.2	95.3	5.0	28	698	54	Utilization of nutrients
5	Pc22g06490	gi 211591694	strong similarity to alkaline phosphatase - <i>Neurospora crassa</i>	73.8	4.8	90.5	4.8	14	405	29	Utilization of nutrients
6	Pc18g02580	gi 211586833	strong similarity to acid phosphatase AFPPhoA - <i>A. ficuum</i>	50.2	5.0	87.8	5.1	9	473	18	Utilization of nutrients
7	Pc22g06490	gi 211591694	strong similarity to alkaline phosphatase - <i>Neurospora crassa</i>	73.8	4.8	87.8	4.8	27	863	48	Utilization of nutrients
8	Pc22g06490	gi 211591694	strong similarity to alkaline phosphatase - <i>Neurospora crassa</i>	73.8	4.8	87.8	4.9	27	858	51	Utilization of nutrients
9	Pc22g06490	gi 211591694	strong similarity to alkaline phosphatase - <i>Neurospora crassa</i>	73.8	4.8	89.4	4.8	23	473	41	Utilization of nutrients
10	Pc18g02580	gi 211586833	strong similarity to acid phosphatase AFPPhoA - <i>A. ficuum</i>	50.2	5.0	87.8	5.0	9	437	18	Utilization of nutrients
11	Pc18g02580	gi 211586833	strong similarity to acid phosphatase AFPPhoA - <i>A. ficuum</i>	50.2	5.0	88.1	5.0	7	274	16	Utilization of nutrients
12	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	88.1	4.2	21	360	33	Cell rescue, defense and virulence
13	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	87.5	4.3	22	337	34	Cell rescue, defense and virulence
	Pc22g02000	gi 211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	87.5	4.3	21	179	30	General metabolism
14	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	87.8	4.3	23	491	37	Cell rescue, defense and virulence
15	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	88.1	4.3	23	490	36	Cell rescue, defense and virulence
16	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	86.7	4.4	16	207	25	Cell rescue, defense and virulence
	Pc22g02000	gi 211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	86.7	4.4	13	124	23	General metabolism
17	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	86.5	4.4	18	277	28	Cell rescue, defense and virulence
	Pc22g02000	gi 211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	86.5	4.4	17	175	28	General metabolism
18	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	87.0	4.5	16	369	26	Cell rescue, defense and virulence
19	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	81.2	5.5	27	699	44	Cell rescue, defense and virulence
20	Pc16g11860	gi 211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	87.3	6.0	27	600	46	Cell rescue, defense and virulence

21	Pc16g11860	gj211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	86.2	4.5	22	42	342	35	Cell rescue, defense and virulence
	Pc16g11860	gj211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	86.2	4.6	20	44	171	32	Cell rescue, defense and virulence
22	Pc22g19230	gj211592843	strong similarity to endo-beta-1,4-glucanase A egIA - <i>A. nidulans</i>	51.9	4.7	86.2	4.6	8	55	127	20	Plant cell wall degradation
	Pc21g06670	gj211589422	strong similarity to carboxypeptidase S1 - <i>Penicillium janthinellum</i>	63.2	4.6	86.2	4.6	9	56	123	22	Proteases
	Pc21g23210	gj211590999	strong similarity to cell wall protein Cn11 - <i>Saccharomyces cerevisiae</i>	36.1	4.7	86.2	4.6	10	55	90	33	Cell wall morphogenesis
23	Pc16g11860	gj211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	86.5	4.5	22	43	315	35	Cell rescue, defense and virulence
	Pc22g19230	gj211592843	strong similarity to endo-beta-1,4-glucanase A egIA - <i>A. nidulans</i>	51.9	4.7	86.5	4.5	8	57	153	22	Plant cell wall degradation
24	Pc16g11860	gj211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	86.7	5.7	33	27	681	46	Cell rescue, defense and virulence
25	Pc22g02000	gj211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	83.9	6.1	33	24	814	52	General metabolism
26	Pc16g11860	gj211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	84.4	5.5	30	30	687	43	Cell rescue, defense and virulence
27	Pc16g11860	gj211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	83.6	5.6	29	32	722	45	Cell rescue, defense and virulence
28	Pc22g02000	gj211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	86.5	6.2	27	31	648	42	General metabolism
29	Pc16g11860	gj211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	86.2	5.7	34	27	753	47	Cell rescue, defense and virulence
30	Pc22g02000	gj211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	86.0	6.0	33	28	911	50	General metabolism
31	Pc16g11860	gj211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	83.9	5.6	27	37	515	42	Cell rescue, defense and virulence
32	Pc22g09380	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanase transferase gel3 - <i>A. fumigatus</i>	57.6	4.9	78.7	4.9	14	50	498	25	Cell wall morphogenesis
33	Pc22g09380	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanase transferase gel3 - <i>A. fumigatus</i>	57.6	4.9	78.5	5.0	15	50	370	28	Cell wall morphogenesis
34	Pc18g02580	gj211586833	strong similarity to acid phosphatase AFPhoA - <i>A. ficuum</i>	50.2	5.0	79.9	5.1	6	58	448	16	Utilization of nutrients
35	Pc22g09380	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanase transferase gel3 - <i>A. fumigatus</i>	57.6	4.9	79.4	4.9	13	52	429	33	Cell wall morphogenesis
36	Pc20g11630	gj211588384	gamma-actin act. <i>Penicillium chrysogenum</i>	42.0	5.5	79.9	5.1	8	56	246	25	Cytoskeleton
37	Pc22g09380	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanase transferase gel3 - <i>A. fumigatus</i>	57.6	4.9	77.8	4.9	17	47	501	33	Cell wall morphogenesis
38	Pc18g02580	gj211586833	strong similarity to acid phosphatase AFPhoA - <i>A. ficuum</i>	50.2	5.0	78.7	5.2	8	57	267	16	Utilization of nutrients
39	Pc21g09860	gj211589733	strong similarity to hypothetical amidase - <i>Schizosaccharomyces pombe</i>	62.9	4.7	77.1	4.8	20	32	369	39	General metabolism
40	Pc22g19230	gj211592843	strong similarity to endo-beta-1,4-glucanase A egIA - <i>A. nidulans</i>	51.9	4.7	75.9	4.5	14	48	467	40	Plant cell wall degradation
	Pc21g23210	gj211590999	strong similarity to cell wall protein Cn11 - <i>Saccharomyces cerevisiae</i>	36.1	4.7	75.9	4.5	10	55	100	34	Cell wall morphogenesis

41	Pc20g09560	gj211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakiense</i>	66.5	5.2	78.0	5.5	12	53	133	24	Cell rescue, defense and virulence
42	Pc22g19230	gj211592843	strong similarity to endo-beta-1,4-glucanase A egIA - <i>A. nidulans</i>	51.9	4.7	75.7	4.5	13	49	411	39	Plant cell wall degradation
43	Pc18g02580	gj211586833	strong similarity to acid phosphatase AFPhA - <i>A. ficuum</i>	50.2	5.0	75.2	5.1	6	58	349	16	Utilization of nutrients
44	Pc22g01750	gj211591236	strong similarity to hypothetical amidase - <i>Schizosaccharomyces pombe</i>	54.0	5.9	75.7	5.7	14	51	434	32	General metabolism
45	Pc22g15910	gj211592524	strong similarity to cytoplasmic form of aminopeptidase like protein An03g04230 - <i>A. niger</i>	68.0	5.5	75.9	5.8	27	38	505	65	Proteases
46	Pc22g01750	gj211591236	strong similarity to hypothetical amidase - <i>Schizosaccharomyces pombe</i>	54.0	5.9	75.0	5.6	19	45	648	39	General metabolism
47	Pc18g04640	gj211585438	similarity to L-galactonolactone oxidase Alo - <i>Saccharomyces cerevisiae</i>	54.8	5.9	74.1	6.0	13	31	462	32	Cell rescue, defense and virulence
48	Pc22g09380	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - <i>A. fumigatus</i>	57.6	4.9	72.4	4.9	15	49	510	30	Cell wall morphogenesis
49	Pc22g16510	gj211592582	strong similarity to isoamyl alcohol oxidase mreA - <i>A. oryzae</i>	61.9	4.8	69.0	4.8	20	42	629	41	General metabolism
50	Pc22g09380	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - <i>A. fumigatus</i>	57.6	4.9	70.6	4.9	11	37	194	24	Cell wall morphogenesis
51	Pc20g10360	gj211588263	beta-N-acetylhexosaminidase hex - <i>Penicillium chrysogenum</i> (68 kDa allergen)	66.8	5.3	69.8	5.3	13	52	250	24	Cell wall morphogenesis
52	Pc20g10360	gj211588263	beta-N-acetylhex osaminidase hex - <i>Penicillium chrysogenum</i> (68 kDa allergen)	66.8	5.3	69.6	5.2	17	48	252	32	Cell wall morphogenesis
53	Pc12g13400	gj211582767	strong similarity to cephalosporin esterase - <i>Rhodospiridium toruloides</i>	60.2	5.2	69.8	5.5	18	44	477	46	Cell rescue, defense and virulence
54	Pc22g09380	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - <i>A. fumigatus</i>	57.6	4.9	69.4	4.9	16	49	451	29	Cell wall morphogenesis
55	Pc20g10360	gj211588263	beta-N-acetylhex osaminidase hex - <i>Penicillium chrysogenum</i> (68 kDa allergen)	66.8	5.3	68.6	5.2	22	38	661	45	Cell wall morphogenesis
56	Pc20g10240	gj211588252	strong similarity to serine-type carboxypeptidase precursor cpdS - <i>A. phoenicis</i>	57.9	5.2	69.0	5.0	7	58	199	14	Proteases
	Pc20g10360	gj211588263	beta-N-acetylhex osaminidase hex - <i>Penicillium chrysogenum</i> (68 kDa allergen)	66.8	5.3	69.0	5.0	12	52	153	28	Cell wall morphogenesis
57	Pc22g09380	gj211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - <i>A. fumigatus</i>	57.6	4.9	66.8	4.9	13	50	481	25	Cell wall morphogenesis
58	Pc22g16510	gj211592582	strong similarity to isoamyl alcohol oxidase mreA - <i>A. oryzae</i>	61.9	4.8	65.6	4.8	17	47	558	43	General metabolism
59	Pc22g02800	gj211591341	strong similarity to calcium-binding protein precursor <i>cnx1p</i> - <i>Schizosaccharomyces pombe</i>	62.0	4.8	65.3	4.7	10	55	176	20	Control of cellular functions
	Pc22g16510	gj211592582	strong similarity to isoamyl alcohol oxidase mreA - <i>A. oryzae</i>	61.9	4.8	65.3	4.7	11	54	117	24	General metabolism
60	Pc22g09580	gj211591986	strong similarity to acid phosphatase aphA - <i>A. ficuum</i>	55.1	4.8	65.1	4.9	11	54	361	39	Utilization of nutrients
61	Pc21g22000	gj211590882	strong similarity to 1,3-beta-glucanoyltransferase gel1 - <i>A. fumigatus</i>	51.6	4.7	63.4	4.8	15	50	416	34	Cell wall morphogenesis
62	Pc22g02000	gj211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	85.9	6.0	64.5	5.5	14	51	209	20	General metabolism

63	Pc22g02800	gl 211591341	strong similarity to calcium-binding protein precursor <i>cnx1p</i> - <i>Schizosaccharomyces pombe</i>	62.0	4.8	63.8	4.8	18	47	267	33	Control of cellular functions
64	Pc22g022710	gl 211593182	strong similarity to dihydroxy-acid dehydratase <i>liv3</i> - <i>Saccharomyces cerevisiae</i>	65.3	6.2	64.0	5.7	20	42	484	40	General metabolism
65	Pc22g022710	gl 211593182	strong similarity to dihydroxy-acid dehydratase <i>liv3</i> - <i>Saccharomyces cerevisiae</i>	65.3	6.2	63.6	5.6	9	56	202	13	General metabolism
66	Pc18g01390	gl 211586717	strong similarity to phosphoglucumutase <i>pgmB</i> - <i>A. nidulans</i>	60.5	5.7	63.6	5.8	21	44	216	42	General metabolism
67	Pc18g01390	gl 211586717	strong similarity to phosphoglucumutase <i>pgmB</i> - <i>A. nidulans</i>	60.5	5.7	62.7	5.8	29	34	557	57	General metabolism
68	Pc22g09390	gl 211591968	strong similarity to mannitol dehydrogenase <i>mID</i> - <i>Pseudomonas fluorescens</i>	59.5	5.6	62.1	5.8	14	51	211	32	Utilization of nutrients
69	Pc14g00170	gl 211584633	strong similarity to hypothetical protein contig1490_3.tfa_630cg - <i>A. fumigatus</i>	47.1	4.8	62.3	4.9	8	57	208	29	Cell rescue, defense and virulence
70	Pc22g09390	gl 211591968	strong similarity to mannitol dehydrogenase <i>mID</i> - <i>Pseudomonas fluorescens</i>	59.5	5.6	61.6	5.9	24	41	436	48	Utilization of nutrients
	Pc18g01390	gl 211586717	strong similarity to phosphoglucumutase <i>pgmB</i> - <i>A. nidulans</i>	60.5	5.7	61.6	5.9	24	41	349	52	General metabolism
71	Pc18g01390	gl 211586717	strong similarity to phosphoglucumutase <i>pgmB</i> - <i>A. nidulans</i>	60.5	5.7	62.1	5.8	17	27	175	36	General metabolism
72	Pc22g17870	gl 211592713	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>	41.2	4.6	59.3	4.6	14	48	516	38	Cell wall morphogenesis
73	Pc22g17870	gl 211592713	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>	41.2	4.6	59.7	4.7	15	45	580	38	Cell wall morphogenesis
74	Pc22g12670	gl 211592227	strong similarity to hypothetical protein contig_1_67_scaffold_4.tfa_440wg - <i>A. nidulans</i>	77.1	4.3	59.3	4.2	13	52	477	28	Utilization of nutrients
	Pc21g23210	gl 211590999	strong similarity to cell wall protein <i>Cwh1</i> - <i>Saccharomyces cerevisiae</i>	36.1	4.7	59.3	4.2	10	55	116	37	Cell wall morphogenesis
75	Pc12g14860	gl 211582909	extracellular acid phosphatase <i>PhoA</i> - <i>Penicillium chrysogenum</i>	45.9	5.2	57.8	5.2	14	51	791	50	Utilization of nutrients
76	Pc12g14860	gl 211582909	extracellular acid phosphatase <i>PhoA</i> - <i>Penicillium chrysogenum</i>	45.9	5.2	56.5	5.3	13	25	716	47	Utilization of nutrients
77	Pc14g00170	gl 211584633	strong similarity to hypothetical protein contig1490_3.tfa_630cg - <i>A. fumigatus</i>	47.1	4.8	59.0	4.8	15	50	448	47	Cell rescue, defense and virulence
78	Pc14g00170	gl 211584633	strong similarity to hypothetical protein contig1490_3.tfa_630cg - <i>A. fumigatus</i>	47.1	4.8	57.6	4.8	15	50	641	52	Cell rescue, defense and virulence
79	Pc12g14860	gl 211582909	extracellular acid phosphatase <i>PhoA</i> - <i>Penicillium chrysogenum</i>	45.9	5.2	60.0	5.4	14	51	549	50	Utilization of nutrients
80	Pc14g00170	gl 211584633	strong similarity to hypothetical protein contig1490_3.tfa_630cg - <i>A. fumigatus</i>	47.1	4.8	58.8	4.9	15	50	525	53	Cell rescue, defense and virulence
81	Pc15g01880	gl 211585025	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	58.8	5.5	17	48	505	45	Cell rescue, defense and virulence
82	Pc15g01880	gl 211585025	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	57.0	5.1	20	45	462	53	Cell rescue, defense and virulence
83	Pc15g01880	gl 211585025	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	56.1	5.2	18	47	482	49	Cell rescue, defense and virulence
84	Pc16g04220	gl 211585400	strong similarity to trehalose metabolism factor <i>Pmu1</i> - <i>Saccharomyces cerevisiae</i>	39.8	6.1	57.0	6.4	12	52	493	30	Utilization of nutrients
85	Pc18g00980	gl 211586677	strong similarity to hypothetical trunk lateral cell specific gene <i>HrTLC1</i> - <i>Halocynthia roretzi</i>	57.2	5.6	57.5	6.0	26	38	573	50	Cell rescue, defense and virulence

86	Pc12g14860	gij211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	56.5	5.6	12	41	241	43	Utilization of nutrients
87	Pc18g00980	gij211586677	strong similarity to hypothetical trunk lateral cell specific gene HrtLC1 - <i>Halocynthia roretzi</i>	57.2	5.6	57.0	5.9	21	43	443	48	Cell rescue, defense and virulence
88	Pc14g00370	gij211584653	strong similarity to rhamnogalacturonase B precursor rthgB - <i>A. aculeatus</i>	57.1	6.2	57.0	6.4	15	46	532	36	Plant cell wall degradation
89	Pc21g12590	gij211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	54.7	5.2	53.6	5.0	17	48	528	35	Utilization of nutrients
90	Pc18g00980	gij211586677	strong similarity to hypothetical trunk lateral cell specific gene HrtLC1 - <i>Halocynthia roretzi</i>	57.2	5.6	56.8	5.8	15	50	232	37	Cell rescue, defense and virulence
91	Pc22g19730	gij211592892	strong similarity to glucose-6-phosphate isomerase Pg1 - <i>Saccharomyces cerevisiae</i>	61.3	5.8	56.6	6.3	20	42	337	56	Utilization of nutrients
92	Pc20g03330	gij211587620	strong similarity to glucose-6-phosphate 1-dehydrogenase gsdA - <i>A. niger</i>	58.6	6.0	56.0	6.3	21	44	301	52	General metabolism
93	Pc21g14970	gij211590220	strong similarity to protein D-lactate dehydrogenase YEL071w - <i>Saccharomyces cerevisiae</i>	59.7	5.8	55.5	5.5	22	38	227	62	General metabolism
94	Pc12g14680	gij211582891	strong similarity to hypothetical lysosomal peptstatin insensitive protease CLN2 - <i>Canis lupus</i>	65.7	4.8	52.8	4.7	14	51	456	38	Proteases
	Pc22g17870	gij211592713	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>	41.2	4.6	52.8	4.7	15	50	275	38	Cell wall morphogenesis
	Pc21g12590	gij211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	54.7	5.2	53.4	5.1	19	46	541	46	Utilization of nutrients
95	Pc15g01030	gij211584950	strong similarity to 1,3-beta-glucanoyltransferase gel1 - <i>A. fumigatus</i>	49.5	5.2	53.4	5.1	12	53	159	40	Cell wall morphogenesis
	Pc15g01030	gij211584950	strong similarity to 1,3-beta-glucanoyltransferase gel1 - <i>A. fumigatus</i>	49.5	5.2	53.6	5.0	13	52	338	44	Cell wall morphogenesis
96	Pc21g12590	gij211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	54.7	5.2	53.6	5.0	15	50	263	40	Utilization of nutrients
97	Pc22g22810	gij211593192	strong similarity to sulphhydryl oxidase Sox from patent EP565172 A1 - <i>A. niger</i>	42.3	4.9	53.4	4.9	15	50	252	41	General metabolism
98	Pc12g09430	gij211582389	strong similarity to alanine transaminase ALA2 - <i>Hordeum vulgare</i>	60.3	6.4	54.2	5.7	8	57	140	22	General metabolism
99	Pc22g17870	gij211592713	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>	41.2	4.6	52.4	4.6	13	52	341	35	Cell wall morphogenesis
	Pc21g23210	gij211590999	strong similarity to cell wall protein Cht1 - <i>Saccharomyces cerevisiae</i>	36.1	4.7	52.4	4.6	13	52	146	43	Cell wall morphogenesis
100	Pc15g01880	gij211585025	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	50.7	5.1	53.8	5.2	18	47	424	45	Cell rescue, defense and virulence
	Pc21g12590	gij211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	54.7	5.2	53.8	5.2	11	54	116	22	Utilization of nutrients
101	Pc21g23210	gij211590999	strong similarity to cell wall protein Cht1 - <i>Saccharomyces cerevisiae</i>	36.1	4.7	51.5	4.4	16	47	432	62	Cell wall morphogenesis
102	Pc21g09780	gij211589725	strong similarity to fructosyl amine oxygen oxidoreductase - <i>A. fumigatus</i>	50.2	5.8	53.1	6.0	15	46	192	39	Utilization of nutrients
103	Pc21g23210	gij211590999	strong similarity to cell wall protein Cht1 - <i>Saccharomyces cerevisiae</i>	36.1	4.7	51.0	4.4	14	51	269	53	Cell wall morphogenesis
104	Pc22g17870	gij211592713	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>	41.2	4.6	52.1	4.7	15	46	591	37	Cell wall morphogenesis
105	Pc16g13280	gij211586250	strong similarity to glutathione reductase Gr1 - <i>Saccharomyces cerevisiae</i>	51.5	6.2	51.9	6.8	22	40	857	59	Cell rescue, defense and virulence

106	Pc20g02250	gj211587518	similarity to chitinase - <i>Chelonius</i> sp.	49.0	4.9	51.8	4.8	12	53	126	31	Cell wall morphogenesis
107	Pc18g05530	gj211587113	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	55.6	4.8	51.5	4.8	13	52	547	27	Utilization of nutrients
108	Pc22g17560	gj211592684	glutamate dehydrogenase <i>gdhA</i> - <i>Penicillium chrysogenum</i>	50.0	6.0	51.3	6.2	31	30	791	73	General metabolism
109	Pc20g04720	gj211587749	strong similarity to precursor of dihydroloipoamide dehydrogenase Lpd1 - <i>Saccharomyces cerevisiae</i>	54.6	7.7	51.3	6.5	25	39	757	52	General metabolism
110	Pc22g17560	gj211592684	glutamate dehydrogenase <i>gdhA</i> - <i>Penicillium chrysogenum</i>	50.0	6.0	50.7	6.4	32	28	872	76	General metabolism
111	Pc21g12590	gj211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	54.7	5.2	49.7	5.1	17	46	621	39	Utilization of nutrients
112	Pc20g04720	gj211587749	strong similarity to precursor of dihydroloipoamide dehydrogenase Lpd1 - <i>Saccharomyces cerevisiae</i>	54.6	7.7	50.7	4.3	15	50	202	35	General metabolism
	Pc21g23210	gj211590999	strong similarity to cell wall protein Ctn1 - <i>Saccharomyces cerevisiae</i>	36.1	4.7	50.7	4.3	13	52	154	43	Cell wall morphogenesis
113	Pc21g12590	gj211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	54.7	5.2	49.5	5.0	18	45	705	43	Utilization of nutrients
114	Pc20g04720	gj211587749	strong similarity to precursor of dihydroloipoamide dehydrogenase Lpd1 - <i>Saccharomyces cerevisiae</i>	54.6	7.7	50.0	6.6	33	28	992	68	General metabolism
115	Pc21g23210	gj211590999	strong similarity to cell wall protein Ctn1 - <i>Saccharomyces cerevisiae</i>	36.1	4.7	50.6	4.3	15	49	301	60	Cell wall morphogenesis
	Pc20g04720	gj211587749	strong similarity to precursor of dihydroloipoamide dehydrogenase Lpd1 - <i>Saccharomyces cerevisiae</i>	54.6	7.7	50.6	4.3	13	52	270	29	General metabolism
116	Pc21g23210	gj211590999	strong similarity to cell wall protein Ctn1 - <i>Saccharomyces cerevisiae</i>	36.1	4.7	50.7	4.3	14	50	297	53	Cell wall morphogenesis
117	Pc12g04370	gj211581925	strong similarity to phosphoserine transaminase Ser1 - <i>Saccharomyces cerevisiae</i>	48.4	5.5	50.4	5.8	11	47	160	30	General metabolism
118	Pc21g12590	gj211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	54.7	5.2	48.2	5.1	20	43	666	46	Utilization of nutrients
119	Pc22g01850	gj211591246	glucan 1,4-alpha-glucosidase BAC8255- <i>Penicillium chrysogenum</i>	52.4	5.0	49.6	5.2	18	47	565	58	Utilization of nutrients
	Pc22g09680	gj211591995	similarity to hypothetical protein PA4204 - <i>Pseudomonas aeruginosa</i>	43.6	5.2	49.6	5.2	9	50	131	47	General metabolism
120	Pc22g222810	gj211593192	strong similarity to sulphhydryl oxidase Sox from patent EP565172. A1 - <i>A. niger</i>	42.3	4.9	48.3	4.9	18	43	575	53	General metabolism
121	Pc22g222810	gj211593192	strong similarity to sulphhydryl oxidase Sox from patent EP565172. A1 - <i>A. niger</i>	42.3	4.9	47.4	4.9	18	43	570	47	General metabolism
122	Pc22g09680	gj211591995	similarity to hypothetical protein PA4204 - <i>Pseudomonas aeruginosa</i>	43.6	5.2	48.9	5.3	19	44	612	62	General metabolism
123	Pc22g222810	gj211593192	strong similarity to sulphhydryl oxidase Sox from patent EP565172. A1 - <i>A. niger</i>	42.3	4.9	47.1	5.0	18	42	647	49	General metabolism
124	Pc22g23810	gj211593289	strong similarity to fumarate hydratase <i>fumR</i> - <i>Rhizopus oryzae</i>	58.1	7.7	48.8	6.0	11	54	312	37	General metabolism
125	Pc22g09680	gj211591995	similarity to hypothetical protein PA4204 - <i>Pseudomonas aeruginosa</i>	43.6	5.2	47.6	5.2	17	44	873	66	General metabolism
126	Pc22g23810	gj211593289	strong similarity to fumarate hydratase <i>fumR</i> - <i>Rhizopus oryzae</i>	58.1	7.7	48.7	6.1	21	41	617	55	General metabolism
	Pc22g09580	gj211591986	strong similarity to acid phosphatase <i>aphA</i> - <i>A. ficuum</i>	55.1	4.8	48.8	6.1	10	52	374	39	Utilization of nutrients
127	Pc18g05530	gj211587113	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	55.6	4.8	48.8	6.1	10	55	200	22	Utilization of nutrients

128	Pc22g09680	gij211591995	similarity to hypothetical protein PA4204 - <i>Pseudomonas aeruginosa</i>	43.6	5.2	48.1	5.1	20	42	819	74	General metabolism
129	Pc22g09580	gij211591986	strong similarity to acid phosphatase aphA - <i>A. ficum</i>	55.1	4.8	46.4	4.7	15	50	615	53	Utilization of nutrients
	Pc18g05530	gij211587113	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobaacter oxidans</i>	55.6	4.8	46.4	4.7	12	53	330	25	Utilization of nutrients
130	Pc22g09680	gij211591995	similarity to hypothetical protein PA4204 - <i>Pseudomonas aeruginosa</i>	43.6	5.2	48.3	5.2	16	48	534	58	General metabolism
131	Pc20g03610	gij211587647	strong similarity to precursor of mitochondrial isocitrate dehydrogenase icdA - <i>A. niger</i>	54.4	8.4	48.3	5.9	24	40	694	52	General metabolism
132	Pc20g03610	gij211587647	strong similarity to precursor of mitochondrial isocitrate dehydrogenase icdA - <i>A. niger</i>	54.4	8.4	48.3	5.8	19	46	496	35	General metabolism
133	Pc12g14860	gij211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	45.9	5.2	48.2	5.3	13	52	280	42	Utilization of nutrients
	Pc14g01740	gij211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47.3	5.3	48.2	5.3	13	52	104	44	General metabolism
134	Pc14g01740	gij211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47.3	5.3	48.3	5.4	20	42	809	57	General metabolism
135	Pc14g01740	gij211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47.3	5.3	47.0	5.6	23	39	871	65	General metabolism
136	Pc22g01850	gij211591246	glucan 1,4-alpha-glucosidase BAC8255- <i>Penicillium chrysogenum</i>	52.4	5.0	47.6	5.1	27	38	811	69	Utilization of nutrients
137	Pc14g01740	gij211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47.3	5.3	48.0	5.7	25	35	897	63	General metabolism
	Pc18g05530	gij211587113	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobaacter oxidans</i>	55.6	4.8	47.8	4.6	11	54	371	25	Utilization of nutrients
138	Pc22g09580	gij211591986	strong similarity to acid phosphatase aphA - <i>A. ficum</i>	55.1	4.8	47.8	4.6	9	56	173	35	Utilization of nutrients
	Pc13g09520	gij211583995	strong similarity to chitinase 1 precursor cts1 - <i>Coccidioides immitis</i>	44.1	4.5	47.8	4.6	11	54	163	40	Cell wall morphogenesis
139	Pc14g01740	gij211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47.3	5.3	47.9	5.5	24	37	881	63	General metabolism
140	Pc14g01740	gij211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47.3	5.3	47.8	5.5	25	37	940	65	General metabolism
141	Pc18g05530	gij211587113	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobaacter oxidans</i>	55.6	4.8	47.4	4.7	13	52	427	27	Utilization of nutrients
	Pc22g09580	gij211591986	strong similarity to acid phosphatase aphA - <i>A. ficum</i>	55.1	4.8	47.4	4.7	10	55	246	37	Utilization of nutrients
142	Pc18g05530	gij211587113	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobaacter oxidans</i>	55.6	4.8	47.0	4.8	16	49	631	36	Utilization of nutrients
	Pc22g09580	gij211591986	strong similarity to acid phosphatase aphA - <i>A. ficum</i>	55.1	4.8	47.0	4.8	10	55	178	37	Utilization of nutrients
143	Pc16g05080	gij211585478	strong similarity to adenosylhomocysteinase - <i>Homo sapiens</i>	49.5	5.6	47.6	5.9	24	39	614	42	General metabolism
144	Pc12g10940	gij211582533	strong similarity to phosphogluconate dehydrogenase Gnd1 - <i>Saccharomyces cerevisiae</i>	56.1	5.9	47.3	6.0	27	37	616	63	General metabolism
145	Pc22g09580	gij211591986	strong similarity to acid phosphatase aphA - <i>A. ficum</i>	55.1	4.8	46.7	6.1	8	57	231	30	Utilization of nutrients
	Pc18g05530	gij211587113	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobaacter oxidans</i>	55.6	4.8	46.7	6.1	10	55	114	22	Utilization of nutrients
	Pc13g04930	gij211583548	strong similarity to hypothetical protein contig_1_128_scaffold_9.tfa_680cg - <i>A. nidulans</i>	40.4	5.9	46.7	6.1	5	60	112	32	Unknown

146	Pc21g23240	gj211591002	strong similarity to alpha-isopropylmalate synthase Leu4 - <i>Saccharomyces cerevisiae</i>	70.7	5.4	47.1	5.3	20	41	495	29	General metabolism
147	Pc12g09030	gj211582350	strong similarity to fumarylacetate - <i>Homo sapiens</i>	46.6	6.0	47.0	6.3	16	47	386	53	General metabolism
148	Pc21g12590	gj211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	54.7	5.2	46.8	5.1	18	47	467	43	Utilization of nutrients
	Pc15g01030	gj211584950	strong similarity to 1,3-beta-glucanoyltransferase gel1 - <i>A. fumigatus</i>	49.5	5.2	46.8	5.1	11	54	315	36	Cell wall morphogenesis
149	Pc20g13360	gj211588551	strong similarity to exo-alpha 1,4-polygalacturonase PGX1 - <i>Cochliobolus carbonum</i>	42.3	5.1	46.6	4.9	18	47	327	61	Plant cell wall degradation
150	Pc15g01030	gj211584950	strong similarity to 1,3-beta-glucanoyltransferase gel1 - <i>A. fumigatus</i>	49.54	5.16	45.88	5.12	19	42	703	53	Cell wall morphogenesis
151	Pc15g01030	gj211584950	strong similarity to 1,3-beta-glucanoyltransferase gel1 - <i>A. fumigatus</i>	49.54	5.16	45.63	5.05	13	51	490	46	Cell wall morphogenesis
152	Pc22g09680	gj211591995	similarity to hypothetical protein PM4204 - <i>Pseudomonas aeruginosa</i>	43.61	5.19	45.06	5.22	13	52	319	58	General metabolism
153	Pc12g02970	gj211581802	strong similarity to hypothetical protein contig1492_0.tfa_4570cg - <i>A. fumigatus</i>	45.76	4.96	43.87	5.02	13	52	325	52	Cell wall morphogenesis
	Pc15g01030	gj211584950	strong similarity to 1,3-beta-glucanoyltransferase gel1 - <i>A. fumigatus</i>	49.54	5.16	43.87	5.02	10	55	118	30	Cell wall morphogenesis
154	Pc22g20290	gj211592946	strong similarity to polygalacturonase pgal - <i>A. niger</i>	39.01	5.47	43.56	4.87	16	49	913	67	Plant cell wall degradation
155	Pc22g20290	gj211592946	strong similarity to polygalacturonase pgal - <i>A. niger</i>	39.01	5.47	41.57	5.53	17	45	856	65	Plant cell wall degradation
156	Pc22g20290	gj211592946	strong similarity to polygalacturonase pgal - <i>A. niger</i>	39.01	5.47	42.32	5.38	19	46	966	67	Plant cell wall degradation
157	Pc22g20290	gj211592946	strong similarity to polygalacturonase pgal - <i>A. niger</i>	39.01	5.47	42.40	5.72	15	50	837	58	Plant cell wall degradation
	Pc18g05720	gj211587132	strong similarity to hypothetical protein contig_1_89_scaffold_6.tfa_270cg - <i>A. nidulans</i>	47.66	7.60	42.40	5.72	9	56	141	31	General metabolism
158	Pc12g02970	gj211581802	strong similarity to hypothetical protein contig1492_0.tfa_4570cg - <i>A. fumigatus</i>	45.76	4.96	42.63	4.93	15	47	587	52	Cell wall morphogenesis
159	Pc12g02970	gj211581802	strong similarity to hypothetical protein contig1492_0.tfa_4570cg - <i>A. fumigatus</i>	45.76	4.96	42.17	5.10	18	42	739	60	Cell wall morphogenesis
160	Pc12g02970	gj211581802	strong similarity to hypothetical protein contig1492_0.tfa_4570cg - <i>A. fumigatus</i>	45.76	4.96	42.55	5.15	13	48	502	52	Cell wall morphogenesis
161	Pc12g02970	gj211581802	strong similarity to hypothetical protein contig1492_0.tfa_4570cg - <i>A. fumigatus</i>	45.76	4.96	41.80	5.04	17	45	542	50	Cell wall morphogenesis
162	Pc12g02970	gj211581802	strong similarity to hypothetical protein contig1492_0.tfa_4570cg - <i>A. fumigatus</i>	45.76	4.96	42.63	4.84	14	49	320	48	Cell wall morphogenesis
	Pc16g14030	gj211586324	similarity to G1/S transition control protein-binding protein RbAp48 - <i>Mus musculus</i>	39.84	5.05	42.63	4.84	8	57	151	35	Control of cellular functions
163	Pc12g02970	gj211581802	strong similarity to hypothetical protein contig1492_0.tfa_4570cg - <i>A. fumigatus</i>	45.76	4.96	41.95	4.98	21	38	813	61	Cell wall morphogenesis
164	Pc12g04310	gj211581920	strong similarity to acetate-inducible gene aciA - <i>A. nidulans</i>	50.35	6.25	42.25	6.42	23	42	368	53	Utilization of nutrients
165	Pc12g04310	gj211581920	strong similarity to acetate-inducible gene aciA - <i>A. nidulans</i>	50.35	6.25	42.02	6.30	26	37	591	59	Utilization of nutrients

166	Pc12g04310	gj211581920	strong similarity to acetate-inducible gene <i>aciA</i> - <i>A. nidulans</i>	50.35	6.25	42.02	6.55	26	37	570	60	Utilization of nutrients
167	Pc16g02790	gj211585272	strong similarity to aspartate transaminase like protein An08g01000 - <i>A. niger</i>	41.33	6.13	41.87	6.48	16	49	365	49	General metabolism
168	Pc12g05380	gj211582002	strong similarity to coproporphyrinogen oxidase III Hem13 - <i>Saccharomyces cerevisiae</i>	50.01	7.16	40.83	6.11	17	47	400	31	General metabolism
169	Pc22g13130	gj211592257	strong similarity to mitochondrial aspartate aminotransferase mAspAT - <i>Mus musculus</i>	46.88	9.07	40.25	5.46	19	46	470	54	General metabolism
170	Pc20g08410	gj211588099	strong similarity to UDP-glucose 4-epimerase Gal10 - <i>Saccharomyces cerevisiae</i>	36.25	5.67	40.32	6.03	10	31	237	48	General metabolism
171	Pc22g13130	gj211592257	strong similarity to mitochondrial aspartate aminotransferase mAspAT - <i>Mus musculus</i>	46.88	9.07	39.75	4.20	20	44	567	59	General metabolism
172	Pc22g13130	gj211592257	strong similarity to mitochondrial aspartate aminotransferase mAspAT - <i>Mus musculus</i>	46.88	9.07	39.89	4.27	21	41	664	66	General metabolism
173	Pc16g08460	gj211585789	strong similarity to sorbitol dehydrogenase <i>gutB</i> - <i>Bacillus subtilis</i>	37.49	5.88	39.47	4.92	16	49	385	65	Utilization of nutrients
			strong similarity to ketol-acid reductoisomerase <i>liv-2</i> - <i>Neurospora crassa</i>	44.15	8.79	39.47	4.92	17	48	239	51	General metabolism
174	Pc13g15810	gj211584596	strong similarity to ketol-acid reductoisomerase <i>liv-2</i> - <i>Neurospora crassa</i>	44.15	8.79	39.54	6.32	13	52	167	35	General metabolism
175	Pc16g08460	gj211585789	strong similarity to sorbitol dehydrogenase <i>gutB</i> - <i>Bacillus subtilis</i>	37.49	5.88	39.61	6.47	22	41	753	84	Utilization of nutrients
176	Pc22g16780	gj211592608	strong similarity to branched-chain-amino-acid aminotransferase Bat2 - <i>Saccharomyces cerevisiae</i>	46.00	8.18	38.97	5.33	19	46	417	54	General metabolism
177	Pc12g09820	gj211582426	strong similarity to glyoxalase I Glc1 - <i>Saccharomyces cerevisiae</i>	35.17	5.45	38.63	5.21	20	45	463	58	Cell rescue, defense and virulence
			strong similarity to D-arabinose dehydrogenase Ara1 - <i>Saccharomyces cerevisiae</i>	36.63	5.54	38.63	5.21	10	55	234	38	General metabolism
178	Pc22g16780	gj211592608	strong similarity to branched-chain-amino-acid aminotransferase Bat2 - <i>Saccharomyces cerevisiae</i>	46.00	8.18	38.49	6.10	8	21	295	22	General metabolism
179	Pc12g09820	gj211582426	strong similarity to glyoxalase I Glc1 - <i>Saccharomyces cerevisiae</i>	35.17	5.45	37.94	5.67	18	35	477	64	Cell rescue, defense and virulence
180	Pc20g15580	gj211588765	strong similarity to NADPH-dependent aldehyde reductase - <i>Sporobolomyces salmonicolor</i>	37.33	6.14	38.01	6.50	9	56	261	38	General metabolism
			strong similarity to hypothetical membrane protein YDL237w - <i>Saccharomyces cerevisiae</i>	35.04	4.54	37.60	4.71	11	54	332	42	Unknown
181	Pc13g09680	gj211584011	strong similarity to aspartyl proteinase candidapepsin - <i>Candida albicans</i>	43.59	5.08	37.60	4.71	12	53	247	50	Proteases
			strong similarity to hypothetical protein config_1_7_scaffold_1.tfa_1160wg - <i>A. nidulans</i>	26.00	4.69	37.60	4.71	3	62	150	21	Cell rescue, defense and virulence
182	Pc20g05320	gj211587803	strong similarity to phosphomutase homolog Pmu1 - <i>Saccharomyces cerevisiae</i>	33.70	5.18	37.94	5.34	12	34	173	59	Utilization of nutrients
183	Pc22g09580	gj211591986	strong similarity to acid phosphatase <i>aphA</i> - <i>A. ficuum</i>	55.07	4.84	37.00	5.37	7	58	146	23	Utilization of nutrients
			strong similarity to pectate lyase <i>plyA</i> - <i>A. niger</i>	34.41	5.28	37.00	5.07	7	43	238	31	Plant cell wall degradation
184	Pc13g10260	gj211584068	strong similarity to hypothetical protein config_1_84_scaffold_5.tfa_850cg - <i>A. nidulans</i>	37.98	5.00	37.00	5.07	12	39	196	51	Unknown
185		gj119482686	ATP phosphoribosyltransferase - <i>Neosartorya fischeri</i> NRRL 181	32.56	5.70	36.84	5.78	4	37	127	29	General metabolism

186	Pc12g10800	gj211582519	strong similarity to endo 1,5-alpha-arabinanase abnA - <i>A. niger</i>	36.06	6.19	36.63	6.71	17	42	550	78	Plant cell wall degradation
187	Pc22g24890	gj211593394	strong similarity to pectate lyase plyA - <i>A. niger</i>	34.41	5.28	36.47	5.29	16	49	589	60	Plant cell wall degradation
188	Pc22g24890	gj211593394	strong similarity to pectate lyase plyA - <i>A. niger</i>	34.41	5.28	36.26	5.33	13	33	487	54	Plant cell wall degradation
189	Pc22g24890	gj211593394	strong similarity to pectate lyase plyA - <i>A. niger</i>	34.41	5.28	35.34	5.19	16	47	707	63	Plant cell wall degradation
190	Pc22g24890	gj211593394	strong similarity to pectate lyase plyA - <i>A. niger</i>	34.41	5.28	36.06	5.53	14	50	611	64	Plant cell wall degradation
191	Pc22g24890	gj211593394	strong similarity to pectate lyase plyA - <i>A. niger</i>	34.41	5.28	35.95	4.99	13	52	395	65	Plant cell wall degradation
192	Pc22g24890	gj211593394	strong similarity to pectate lyase plyA - <i>A. niger</i>	34.41	5.28	35.90	5.07	18	45	723	71	Plant cell wall degradation
	Pc22g24890	gj211593394	strong similarity to pectate lyase plyA - <i>A. niger</i>	34.41	5.28	36.06	4.92	9	56	283	35	Plant cell wall degradation
193	Pc12g07820	gj211582235	similarity to chitosanase csnA - <i>A. oryzae</i>	32.60	4.94	36.06	4.92	8	57	196	39	Cell wall morphogenesis
	Pc20g07020	gj211587962	endo-1,4-beta-xylanase A precursor XylP- <i>Penicillium chrysogenum</i>	35.58	4.90	36.06	4.92	12	53	160	44	Plant cell wall degradation
194	Pc22g24890	gj211593394	strong similarity to pectate lyase plyA - <i>A. niger</i>	34.41	5.28	35.59	5.54	13	38	505	48	Plant cell wall degradation
195	Pc22g04850	gj211591539	strong similarity to D-arabinose dehydrogenase Ara1 - <i>Saccharomyces cerevisiae</i>	36.63	5.54	35.39	5.72	20	43	690	61	General metabolism
196	Pc22g03140	gj211591374	strong similarity to Gbeta like protein cpcB - <i>A. nidulans</i>	35.35	6.31	35.54	6.66	13	31	237	48	Control of cellular functions
197	Pc18g05530	gj211587113	similarity to 6-hydroxy-D-nicotine oxidase 6-HDO - <i>Arthrobacter oxidans</i>	55.61	4.82	34.99	4.73	12	53	469	26	Utilization of nutrients
198	Pc20g02780	gj211587567	strong similarity to hypothetical protein PA3829 - <i>Pseudomonas aeruginosa</i>	33.82	5.39	35.04	5.47	15	47	454	58	Plant cell wall degradation
199	Pc21g16950	gj211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	35.59	5.45	34.64	5.34	15	31	272	54	General metabolism
200	Pc14g00690	gj211584684	strong similarity to pyruvate dehydrogenase beta chain precursor Pdb1 - <i>Saccharomyces cerevisiae</i>	39.29	8.22	34.39	5.44	17	48	419	60	General metabolism
201	Pc21g16950	gj211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	35.59	5.45	34.29	5.49	17	30	539	57	General metabolism
202	Pc21g14560	gj211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase gpdA - <i>A. niger</i>	36.15	6.23	34.44	6.73	22	42	639	84	General metabolism
203	Pc13g08730	gj211583918	strong similarity to 1,3-beta-glucanoyltransferase bg11 - <i>A. fumigatus</i>	34.67	5.00	34.19	5.09	11	21	316	50	Cell wall morphogenesis
204	Pc21g16950	gj211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	35.59	5.45	34.14	5.37	13	52	398	48	General metabolism
205	Pc12g08930	gj211582340	strong similarity to hypothetical protein contig1492_0.1fa_1890cg - <i>A. fumigatus</i>	34.14	6.11	33.85	6.40	15	27	406	74	Utilization of nutrients
206	Pc22g24890	gj211593394	strong similarity to pectate lyase plyA - <i>A. niger</i>	34.41	5.28	33.80	4.73	12	53	351	56	Plant cell wall degradation
	Pc20g07010	gj211587961	strong similarity to ferulic acid esterase A faeA - <i>A. niger</i>	30.55	4.86	33.80	4.73	9	56	306	30	Plant cell wall degradation

207	Pc16g09710	gij211585905	strong similarity to proliferating cell nuclear antigen pcn1p - <i>Schizosaccharomyces pombe</i>	33.50	4.93	33.27	4.48	13	52	254	44	Control of cellular functions
208	Pc20g07010	gij211587961	strong similarity to ferulic acid esterase A faeA - <i>A. niger</i>	30.55	4.86	33.17	4.70	9	54	468	30	Plant cell wall degradation
209	Pc16g09710	gij211585905	strong similarity to proliferating cell nuclear antigen pcn1p - <i>Schizosaccharomyces pombe</i>	33.50	4.93	32.94	4.73	11	54	358	53	Control of cellular functions
210	Pc20g07230	gij211587983	strong similarity to inorganic pyrophosphatase <i>lpp1</i> - <i>Saccharomyces cerevisiae</i>	32.85	5.14	32.75	5.22	18	44	469	72	General metabolism
211	Pc06g00430	gij211581271	strong similarity to ribonuclease T2 precursor mIB - <i>A. onyzae</i>	28.75	4.28	32.33	4.37	8	39	381	36	Cell rescue, defense and virulence
	Pc12g05820	gij211582045	strong similarity to esterase D ESD - <i>Homo sapiens</i>	31.36	5.89	32.33	4.37	11	23	314	60	General metabolism
212	Pc13g08730	gij211583918	strong similarity to 1,3-beta-glucanoyltransferase bg11 - <i>A. fumigatus</i>	34.67	5.00	32.65	5.10	14	49	479	66	Cell wall morphogenesis
213	Pc12g05820	gij211582045	strong similarity to esterase D ESD - <i>Homo sapiens</i>	31.36	5.89	32.47	6.20	19	43	720	87	General metabolism
214	Pc14g01140	gij211584729	strong similarity to proteasome 20S subunit Pre10 - <i>Saccharomyces cerevisiae</i>	28.88	5.13	32.37	5.29	9	37	188	40	General metabolism
215	Pc18g02740	gij211586848	strong similarity to regucalcin also known as senescence marker protein-30 like protein An04g03420 - <i>A. niger</i>	34.52	5.01	32.05	5.07	23	41	704	73	Control of cellular functions
	Pc13g08730	gij211583918	strong similarity to 1,3-beta-glucanoyltransferase bg11 - <i>A. fumigatus</i>	34.67	5.00	32.05	5.07	10	55	205	45	Cell wall morphogenesis
216	Pc13g08730	gij211583918	strong similarity to 1,3-beta-glucanoyltransferase bg11 - <i>A. fumigatus</i>	34.67	5.00	32.10	5.19	12	32	331	53	Cell wall morphogenesis
217	Pc20g15730	gij211588780	strong similarity to endo 1,5-alpha-arabinanase abnA - <i>A. niger</i>	34.78	5.35	32.01	5.39	3	62	194	20	Plant cell wall degradation
218	Pc13g08730	gij211583918	strong similarity to 1,3-beta-glucanoyltransferase bg11 - <i>A. fumigatus</i>	34.67	5.00	31.87	5.02	18	45	541	73	Cell wall morphogenesis
	Pc18g02740	gij211586848	strong similarity to regucalcin also known as senescence marker protein-30 like protein An04g03420 - <i>A. niger</i>	34.52	5.01	31.87	5.02	12	53	95	38	Control of cellular functions
219	Pc12g01330	gij211581651	strong similarity to hypothetical protein contig31_part_ii.ifa.1360wg - <i>A. fumigatus</i>	36.19	5.30	31.82	5.30	6	35	157	25	Plant cell wall degradation
220	Pc13g08730	gij211583918	strong similarity to 1,3-beta-glucanoyltransferase bg11 - <i>A. fumigatus</i>	34.67	5.00	31.23	5.10	22	38	685	82	Cell wall morphogenesis
221	Pc13g15920	gij211584606	strong similarity to endo 1,5-alpha-arabinanase abnA - <i>A. niger</i>	34.33	5.53	31.55	5.38	5	49	239	23	Plant cell wall degradation
222	Pc22g05880	gij211591637	strong similarity to hypothetical protein 1117_scaffold_1.ifa_300cg - <i>Fusarium graminearum</i>	25.56	4.47	30.79	4.48	8	57	543	49	Plant cell wall degradation
223	Pc20g07030	gij211587963	strong similarity to 1,4-beta-D-arabinoxylan arabinofuranohydrolase axhA - <i>A. niger</i>	35.60	5.04	31.05	4.54	11	54	306	55	Plant cell wall degradation
224	Pc13g15920	gij211584606	strong similarity to endo 1,5-alpha-arabinanase abnA - <i>A. niger</i>	34.33	5.53	30.97	5.38	4	45	113	24	Plant cell wall degradation
225	Pc20g07030	gij211587963	strong similarity to 1,4-beta-D-arabinoxylan arabinofuranohydrolase axhA - <i>A. niger</i>	35.60	5.04	30.74	4.86	11	50	562	55	Plant cell wall degradation
226	Pc21g20440	gij211590742	strong similarity to ribose 5-phosphate isomerase RPI - <i>Mus musculus</i>	29.00	5.76	30.66	5.92	8	37	206	26	General metabolism
227	Pc13g04420	gij211583497	orotidine 5-phosphate decarboxylase pyrG - <i>Penicillium chrysogenum</i>	29.94	5.59	30.35	5.68	18	33	645	71	General metabolism
228	Pc06g00430	gij211581271	strong similarity to ribonuclease T2 precursor mIB - <i>A. onyzae</i>	28.75	4.28	29.32	4.38	10	39	668	39	Cell rescue, defense and virulence

229	Pc22g25470	gi211593449	strong similarity to So1 - <i>Saccharomyces cerevisiae</i>	29.06	5.51	28.74	5.79	14	51	285	70	General metabolism
230	Pc12g00830	gi211581603	strong similarity to sorbitol utilization protein sou2 - <i>Candida albicans</i>	28.71	5.33	28.21	5.52	17	39	393	74	Utilization of nutrients
231	Pc13g09540	gi211583997	strong similarity to proteasome 20S subunit Sc1 - <i>Saccharomyces cerevisiae</i>	27.77	5.78	28.00	6.04	16	43	603	66	General metabolism
232	Pc21g23210	gi211590999	strong similarity to cell wall protein Cht1 - <i>Saccharomyces cerevisiae</i>	36.15	4.72	27.88	4.71	7	56	155	20	Cell wall morphogenesis
233	Pc12g00830	gi211581603	strong similarity to sorbitol utilization protein sou2 - <i>Candida albicans</i>	28.71	5.33	27.56	5.38	14	28	237	72	Utilization of nutrients
234	Pc12g00830	gi211581603	strong similarity to sorbitol utilization protein sou2 - <i>Candida albicans</i>	28.71	5.33	27.29	5.52	18	41	606	80	Utilization of nutrients
235	Pc21g23210	gi211590999	strong similarity to cell wall protein Cht1 - <i>Saccharomyces cerevisiae</i>	36.15	4.72	27.64	4.81	17	48	359	65	Cell wall morphogenesis
236	Pc22g22290	gi211593141	strong similarity to IgE-binding protein - <i>A. fumigatus</i>	20.16	4.79	25.66	4.76	2	51	200	16	Unknown
237	Pc14g01230	gi211584738	strong similarity to proteasome 20S subunit Pre7 - <i>Saccharomyces cerevisiae</i>	28.06	5.53	25.29	5.51	10	29	218	52	General metabolism
238	Pc12g00830	gi211581603	strong similarity to sorbitol utilization protein sou2 - <i>Candida albicans</i>	28.71	5.33	25.00	5.96	12	52	312	49	Utilization of nutrients
239	Pc20g04810	gi211587757	strong similarity to estrogen receptor-binding cyclophilin cypD - <i>Bos primigenius taurus</i>	41.31	5.91	24.41	5.50	12	53	360	38	Control of cellular functions
240	Pc20g04810	gi211587757	strong similarity to estrogen receptor-binding cyclophilin cypD - <i>Bos primigenius taurus</i>	41.31	5.91	24.28	5.22	7	15	138	28	Control of cellular functions
241	Pc20g04810	gi211587757	strong similarity to estrogen receptor-binding cyclophilin cypD - <i>Bos primigenius taurus</i>	41.31	5.91	23.87	5.22	11	38	403	32	Control of cellular functions
242	Pc12g13600	gi211582786	strong similarity to hypothetical necrosis and ethylene inducing protein BH0395 - <i>Bacillus halodurans</i>	26.26	5.22	23.51	4.76	7	58	315	46	Cell rescue, defense and virulence
243	Pc22g22290	gi211593141	strong similarity to IgE-binding protein - <i>A. fumigatus</i>	20.16	4.79	23.51	4.76	2	63	154	16	Unknown
244	Pc21g14220	gi211590148	strong similarity to estrogen receptor-binding cyclophilin cypD - <i>Bos primigenius taurus</i>	41.31	5.91	23.39	5.23	12	33	391	40	Control of cellular functions
245	Pc12g14640	gi211582887	strong similarity to hypothetical protein contig1495_2.fga_630cg - <i>A. fumigatus</i>	22.21	4.51	21.00	4.52	8	57	289	40	Unknown
246	Pc12g14640	gi211582887	strong similarity to hypothetical protein An06g00160 - <i>A. niger</i>	17.18	5.48	19.84	5.48	5	40	224	18	Unknown
247	Pc12g10010	gi211582445	strong similarity to hypothetical protein An06g00160 - <i>A. niger</i>	17.18	5.48	19.31	5.48	5	60	220	18	Unknown
248	Pc12g10010	gi211582445	strong similarity to protein copper regulated - <i>A. nidulans</i>	16.75	4.59	19.22	4.52	5	60	173	44	Unknown
249	Pc22g00190	gi211591082	strong similarity to protein copper regulated - <i>A. nidulans</i>	16.75	4.59	18.68	4.67	5	19	171	60	Unknown
250	Pc22g23760	gi211593284	strong similarity to hypothetical cell wall protein binB - <i>A. nidulans</i>	19.11	5.61	17.85	5.42	2	43	277	18	Unknown
251	Pc22g09580	gi211591986	strong similarity to type 2 peroxiredoxin like protein An12g08570 - <i>A. niger</i>	18.70	4.90	18.61	4.84	7	44	117	44	Cell rescue, defense and virulence
252	Pc12g10010	gi211582445	strong similarity to acid phosphatase aphA - <i>A. ficuum</i>	55.07	4.84	18.47	5.74	5	60	296	12	Utilization of nutrients
253	Pc22g09580	gi211591986	strong similarity to protein copper regulated - <i>A. nidulans</i>	16.75	4.59	18.45	4.59	5	25	139	60	Unknown
254	Pc22g09580	gi211591986	strong similarity to acid phosphatase aphA - <i>A. ficuum</i>	55.07	4.84	18.39	5.89	5	45	154	11	Utilization of nutrients
255	Pc13g08810	gi211583926	strong similarity to acid phosphatase aphA - <i>A. ficuum</i>	55.07	4.84	18.30	5.74	5	42	136	12	Utilization of nutrients
256	Pc13g08810	gi211583926	strong similarity to elongation factor 1 beta EF-1 - <i>Oryctolagus cuniculus</i>	25.05	4.44	18.24	5.14	3	62	131	23	General metabolism

256	Pc12g10010	gij211582445	strong similarity to protein copper regulated - <i>A. nidulans</i>	16.75	4.59	18.24	5.34	7	58	310	67	Unknown
257		gij144952798	16 kDa allergen - <i>Penicillium chrysogenum</i>	16.45	5.20	18.21	4.67	4	61	278	21	Unknown
258	Pc12g02950	gij211581800	hypothetical protein - <i>Penicillium chrysogenum</i>	20.33	5.08	18.01	5.11	4	61	101	32	Unknown
259	Pc16g00900	gij211585131	strong similarity to hypothetical protein contig10.tfa_380cg - <i>A. nidulans</i>	16.12	4.58	18.00	5.35	9	56	485	81	Unknown
260	Pc13g06930	gij211583746	strong similarity to hypothetical protein contig_1_7_scaffold_1.tfa_1010wg - <i>A. nidulans</i>	15.47	4.03	17.58	4.63	3	62	171	31	Unknown
261	Pc16g00900	gij211585131	strong similarity to hypothetical protein contig10.tfa_380cg - <i>A. nidulans</i>	16.12	4.58	17.30	4.53	8	44	461	81	Unknown
262	Pc13g06930	gij211583746	strong similarity to hypothetical protein contig_1_7_scaffold_1.tfa_1010wg - <i>A. nidulans</i>	15.47	4.03	17.00	4.20	4	59	121	31	Unknown
263	Pc16g00900	gij211585131	strong similarity to hypothetical protein contig10.tfa_380cg - <i>A. nidulans</i>	16.12	4.58	17.08	4.46	5	30	165	35	Unknown
264	Pc20g15140	gij211588723	strong similarity to secreted serine protease 19 kDa CS antigen CS-Ag - <i>Coccidioides immitis</i>	15.68	4.70	17.08	4.53	4	52	117	30	Proteases
265	Pc22g24890	gij211593394	strong similarity to pectate lyase plyA - <i>A. niger</i>	34.41	5.28	31.73	6.11	7	39	160	33	Plant cell wall degradation
266	Pc21g12590	gij211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobaacter oxidans</i>	54.72	5.18	18.15	5.44	13	52	302	27	Utilization of nutrients
267	Pc21g02110	gij211588987	strong similarity to hypothetical protein mg01387.1 - <i>Magnaporthe grisea</i>	48.82	4.89	18.15	5.44	5	60	148	29	Control of cellular functions
267	Pc21g12590	gij211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobaacter oxidans</i>	54.72	5.18	53.60	4.93	19	44	640	45	Utilization of nutrients
268	Pc21g12590	gij211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobaacter oxidans</i>	54.72	5.18	52.07	5.04	16	49	492	31	Utilization of nutrients
269	Pc21g12590	gij211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobaacter oxidans</i>	54.72	5.18	55.66	5.04	20	45	523	47	Utilization of nutrients
270	Pc15g00210	gij211584871	similarity to glucan 1,3-beta-glucosidase Bgl2 - <i>Saccharomyces cerevisiae</i>	48.50	4.47	50.00	5.08	9	56	353	27	Plant cell wall degradation
270	Pc16g06700	gij211585628	strong similarity to hypothetical protein contig_1_7_scaffold_1.tfa_1160wg - <i>A. nidulans</i>	26.00	4.69	50.00	5.08	5	60	138	22	Cell rescue, defense and virulence
271	Pc21g02370	gij211589012	strong similarity to aspergillopepsin aprS - <i>A. phoenicis</i>	45.99	4.82	43.40	4.46	8	52	322	27	Proteases
271	Pc20g07340	gij211587992	weak similarity to endoglucanase A eglA - <i>A. niger</i>	36.30	4.37	43.40	4.46	5	57	127	28	Plant cell wall degradation
272	Pc15g00210	gij211584871	similarity to glucan 1,3-beta-glucosidase Bgl2 - <i>Saccharomyces cerevisiae</i>	48.50	4.47	37.67	4.36	6	35	246	19	Plant cell wall degradation
272	Pc16g06700	gij211585628	strong similarity to hypothetical protein contig_1_7_scaffold_1.tfa_1160wg - <i>A. nidulans</i>	26.00	4.69	37.67	4.36	3	40	145	21	Cell rescue, defense and virulence
273	Pc13g13110	gij211584345	strong similarity to hypothetical endoglucanase IV - <i>Trichoderma reesei</i>	40.35	5.50	39.54	4.73	6	50	168	30	Plant cell wall degradation
274	Pc16g04220	gij211585400	strong similarity to trehalose metabolism factor Pnu1 - <i>Saccharomyces cerevisiae</i>	39.75	6.05	52.07	6.36	11	37	314	27	Utilization of nutrients
275	Pc16g11860	gij211586109	strong similarity to catalase R catR - <i>A. niger</i>	79.9	5.4	85.70	4.80	20	45	446	35	Cell rescue, defense and virulence
276	Pc18g01940	gij211586771	strong similarity to beta-glucosidase 1 bglI - <i>A. aculeatus</i>	94.66	4.83	109.55	4.85	11	54	176	18	Plant cell wall degradation
277	Pc18g02580	gij211586833	strong similarity to acid phosphatase AFPhoA - <i>A. ficium</i>	50.21	4.98	87.81	5.18	5	32	231	13	Utilization of nutrients
278	Pc14g01740	gij211584789	enolase BAC82549 - <i>Penicillium chrysogenum</i>	47.3	5.3	18.43	4.86	23	39	871	65	General metabolism
279	Pc22g00190	gij211591082	strong similarity to hypothetical cell wall protein binB - <i>A. nidulans</i>	19.11	5.61	18.56	5.44	2	22	172	18	Unknown

Supplementary Table 4. Different proteins identified in the extracellular proteome of *P. chrysogenum* Wisconsin 54-1255. The number of isoforms is indicated for each one of these proteins, which have been ordered according to their ORF name. Prediction of signal peptides and the possibility of secretion through a non-classical secretory mechanism are also indicated.

Protein (ORF name)	Accession Number	Description	Spot	Isoforms	Function	Signal peptide probability	Signal peptide	Non-classically secreted protein
Pc06g00430	gil211581271	strong similarity to ribonuclease T2 precursor <i>rntB</i> - <i>Aspergillus oryzae</i>	211 228 230	2	Cell rescue, defense and	0.9950	YES	
Pc12g00830	gil211581603	strong similarity to sorbitol utilization protein <i>sou2</i> - <i>Candida albicans</i>	233 234 238	4	Utilization of nutrients	0.0210	NO	NO
Pc12g01330	gil211581651	strong similarity to hypothetical protein contig31_part_ii.tfa_1360wg - <i>Aspergillus fumigatus</i>	219	1	Plant cell wall degradation	1.0000	YES	
Pc12g02950	gil211581800	hypothetical protein [<i>Penicillium chrysogenum</i>]	258 153 158	1	Unknown	0.9980	YES	
Pc12g02970	gil211581802	strong similarity to hypothetical protein contig1492_0.tfa_4570cg - <i>Aspergillus fumigatus</i>	159 160 161 162 163 164	7	Cell wall morphogenesis	1.0000	YES	
Pc12g04310	gil211581920	strong similarity to acetate-inducible gene <i>aciA</i> - <i>Aspergillus nidulans</i>	165 166	3	Utilization of nutrients	0.0020	NO	YES
Pc12g04370	gil211581925	strong similarity to phosphoserine transaminase Ser1 - <i>Saccharomyces cerevisiae</i>	117	1	General metabolism	0.0140	NO	NO
Pc12g05380	gil211582002	strong similarity to coproporphyrinogen oxidase III Hem13 - <i>Saccharomyces cerevisiae</i>	168	1	General metabolism	0.0000	NO	YES
Pc12g05820	gil211582045	strong similarity to esterase D ESD - <i>Homo sapiens</i>	211 213	2	General metabolism	0.1650	NO	YES
Pc12g07820	gil211582235	similarity to chitosanase <i>csnA</i> - <i>Aspergillus oryzae</i>	193	1	Cell wall morphogenesis	0.9980	YES	
Pc12g08930	gil211582340	strong similarity to hypothetical protein contig1492_0.tfa_1890cg - <i>Aspergillus fumigatus</i>	205	1	Utilization of nutrients	0.0020	NO	YES
Pc12g09030	gil211582350	strong similarity to fumarylacetoacetase - <i>Homo sapiens</i>	147	1	General metabolism	0.1500	NO	YES
Pc12g09430	gil211582389	strong similarity to alanine transaminase ALA2 - <i>Hordeum</i>	98	1	General metabolism	0.1210	NO	NO
Pc12g09820	gil211582426	strong similarity to glyoxalase I Glo1 - <i>Saccharomyces cerevisiae</i>	177 179 247	2	Cell rescue, defense and	0.0010	NO	NO
Pc12g10010	gil211582445	strong similarity to protein copper regulated - <i>Aspergillus nidulans</i>	248 252 256	4	Unknown	0.9990	YES	
Pc12g10800	gil211582519	strong similarity to endo 1,5-alpha-arabinanase <i>abnA</i> - <i>Aspergillus niger</i>	186	1	Plant cell wall degradation	0.9940	YES	
Pc12g10940	gil211582533	strong similarity to phosphogluconate dehydrogenase Gnd1 - <i>Saccharomyces cerevisiae</i>	144	1	General metabolism	0.0570	NO	NO
Pc12g13400	gil211582767	strong similarity to cephalosporin esterase - <i>Rhodospiridium toruloides</i>	53	1	Cell rescue, defense and	0.9810	YES	
Pc12g13600	gil211582786	strong similarity to hypothetical necrosis and ethylene inducing protein BH0395 - <i>Bacillus halodurans</i>	242	1	Cell rescue, defense and virulence	0.9970	YES	
Pc12g14640	gil211582887	strong similarity to hypothetical protein An06g00160 - <i>Aspergillus niger</i>	245 246	2	Unknown	0.9940	YES	

Pc12g14680	gi 211582891	strong similarity to hypothetical lysosomal pepstatin insensitive protease CLN2 - <i>Canis lupus</i>	94	1	Proteases	1.0000	YES	
			75					
Pc12g14860	gi 211582909	extracellular acid phosphatase PhoA- <i>Penicillium chrysogenum</i>	76 79 86 133	5	Utilization of nutrients	0.9990	YES	
Pc12g15470	gi 211582966	strong similarity to hypothetical protein contig1492_0.tfa_1880cg - <i>Aspergillus fumigatus</i>	2 3 4	3	Utilization of nutrients	0.9980	YES	
Pc13g04420	gi 211583497	orotidine 5-phosphate decarboxylase pyrG- <i>Penicillium chrysogenum</i>	227	1	General metabolism	0.0010	NO	YES
Pc13g04930	gi 211583548	strong similarity to hypothetical protein contig_1_128_scaffold_9.tfa_680cg - <i>Aspergillus nidulans</i>	145	1	Unknown	1.0000	YES	
Pc13g06930	gi 211583746	strong similarity to hypothetical protein contig_1_7_scaffold_1.tfa_1010wg -	260 262 203 212 215 216 218 220	2	Unknown	1.0000	YES	
Pc13g08730	gi 211583918	strong similarity to 1,3-beta-glucanosyltransferase bgt1 - <i>Aspergillus fumigatus</i>	215 216 218 220	6	Cell wall morphogenesis	1.0000	YES	
Pc13g08810	gi 211583926	strong similarity to elongation factor 1 beta EF-1 - <i>Oryctolagus cuniculus</i>	255	1	General metabolism	0.0150	NO	NO
Pc13g09520	gi 211583995	strong similarity to chitinase 1 precursor cts1 - <i>Coccidioides immitis</i>	138	1	Cell wall morphogenesis	0.0030	NO	YES
Pc13g09540	gi 211583997	strong similarity to proteasome 20S subunit Scl1 - <i>Saccharomyces cerevisiae</i>	231	1	General metabolism	0.0000	NO	NO
Pc13g09680	gi 211584011	strong similarity to aspartyl proteinase candidapepsin - <i>Candida albicans</i>	181	1	Proteases	1.0000	YES	
Pc13g10260	gi 211584068	strong similarity to hypothetical protein contig_1_84_scaffold_5.tfa_850cg - <i>Aspergillus nidulans</i>	184	1	Unknown	0.0000	NO	NO
Pc13g13110	gi 211584345	strong similarity to hypothetical endoglucanase IV - <i>Trichoderma</i>	273	1	Plant cell wall degradation	0.9910	YES	
Pc13g15810	gi 211584596	strong similarity to ketol-acid reductoisomerase ilv-2 - <i>Neurospora</i>	173 174	2	General metabolism	0.9990	YES	
Pc13g15920	gi 211584606	strong similarity to endo 1,5-alpha arabinanase abnA - <i>Aspergillus niger</i>	221 224 69 77 78 80	2	Plant cell wall degradation	0.8760	YES	
Pc14g00170	gi 211584633	strong similarity to hypothetical protein contig1490_3.tfa_630cg - <i>Aspergillus fumigatus</i>	77 78 80	4	Cell rescue, defense and virulence	1.0000	YES	
Pc14g00370	gi 211584653	strong similarity to rhamnogalacturonase B precursor rhgB - <i>Aspergillus aculeatus</i>	88	1	Plant cell wall degradation	0.9990	YES	
Pc14g00690	gi 211584684	strong similarity to pyruvate dehydrogenase beta chain precursor Pdb1 - <i>Saccharomyces cerevisiae</i>	200	1	General metabolism	0.7480	NO	YES
Pc14g01140	gi 211584729	strong similarity to proteasome 20S subunit Pre10 - <i>Saccharomyces cerevisiae</i>	214	1	General metabolism	0.0000	NO	NO
Pc14g01230	gi 211584738	strong similarity to proteasome 20S subunit Pre7 - <i>Saccharomyces cerevisiae</i>	237 133 134 135 137 139 140 278	1	General metabolism	0.0050	NO	YES
Pc14g01740	gi 211584789	enolase BAC82549- <i>Penicillium chrysogenum</i>	135 137 139 140 278	7	General metabolism	0.0000	NO	NO

Pc15g00210	gil211584871	similarity to glucan 1,3-beta-glucosidase Bgl2 - <i>Saccharomyces</i>	270 272 95 96	2	Plant cell wall degradation	0.9990	YES	
Pc15g01030	gil211584950	strong similarity to 1,3-beta-glucanotransferase <i>gel1</i> - <i>Aspergillus fumigatus</i>	148 150 151 153 81	6	Cell wall morphogenesis	0.9710	YES	
Pc15g01880	gil211585025	similarity to non-hemolytic phospholipase C - <i>Burkholderia pseudomallei</i>	82 83 100 259	4	Cell rescue, defense and virulence	0.9990	YES	
Pc16g00900	gil211585131	strong similarity to hypothetical protein contig10.tfa_380cg - <i>Aspergillus fumigatus</i>	261 263	3	Unknown	1.0000	YES	
Pc16g02790	gil211585272	strong similarity to aspartate transaminase like protein An08g01000 - <i>Aspergillus niger</i>	167	1	General metabolism	0.0020	NO	YES
Pc16g04220	gil211585400	strong similarity to trehalose metabolism factor Pmu1 -	84 274	2	Utilization of nutrients	0.9990	YES	
Pc16g04640	gil211585438	similarity to L-galactonolactone oxidase Alo - <i>Saccharomyces</i>	47	1	Cell rescue, defense and	0.9980	YES	
Pc16g05080	gil211585478	strong similarity to adenosylhomocysteinase - <i>Homo sapiens</i>	143	1	General metabolism	0.0040	NO	YES
Pc16g06700	gil211585628	strong similarity to hypothetical protein contig_1_7_scaffold_1.tfa_1160wg - <i>Aspergillus nidulans</i>	181 270 272 173	3	Cell rescue, defense and virulence	0.9910	YES	
Pc16g08460	gil211585789	strong similarity to sorbitol dehydrogenase <i>gutB</i> - <i>Bacillus</i>	175	2	Utilization of nutrients	0.0000	NO	NO
Pc16g09710	gil211585905	strong similarity to proliferating cell nuclear antigen <i>pcn1p</i> -	207 209 12 13 14 15 16 17 18 19 20 21 22 23 24 26 27 29 31 275	2	Control of cellular functions	0.0000	NO	YES
Pc16g11860	gil211586109	strong similarity to catalase R <i>catR</i> - <i>Aspergillus niger</i>	20 21 22 23 24 26 27 29 31 275	18	Cell rescue, defense and virulence	0.0000	NO	YES
Pc16g13280	gil211586250	strong similarity to glutathione reductase Glr1 - <i>Saccharomyces cerevisiae</i>	105	1	Cell rescue, defense and virulence	0.0010	NO	NO
Pc16g14030	gil211586324	similarity to G1/S transition control protein-binding protein RbAp48 - <i>Mus musculus</i>	162	1	Control of cellular functions	0.0810	NO	YES
Pc18g00980	gil211586677	strong similarity to hypothetical trunk lateral cell specific gene HrTLC1 - <i>Halocynthia roretzi</i>	85 87 90 66	3	Cell rescue, defense and virulence	0.0460	NO	YES
Pc18g01390	gil211586717	strong similarity to phosphoglucomutase <i>pgmB</i> - <i>Aspergillus nidulans</i>	67 7 71	4	General metabolism	0.0000	NO	YES

Pc18g01940	gi 211586771	strong similarity to beta-glucosidase 1 <i>bgl1</i> - <i>Aspergillus aculeatus</i>	276 6 10 11 34 38 43 277	1	Plant cell wall degradation	0.9700	YES	
Pc18g02580	gi 211586833	strong similarity to acid phosphatase AFPhoA - <i>Aspergillus ficuum</i>	215 218	7	Utilization of nutrients	1.0000	YES	
Pc18g02740	gi 211586848	strong similarity to regucalcin also known as senescence marker protein-	1	2	Control of cellular functions	0.0000	NO	NO
Pc18g02900	gi 211586864	lysophospholipase phospholipase B <i>plb1</i> - <i>Penicillium chrysogenum</i>	107 127 129 138 141 142 145 197	1	Cell rescue, defense and	0.9990	YES	
Pc18g05530	gi 211587113	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter</i> <i>oxidans</i>	157	8	Utilization of nutrients	0.9390	YES	
Pc18g05720	gi 211587132	strong similarity to hypothetical protein contig_1_89_scaffold_6.tfa_270cg - <i>Aspergillus nidulans</i>	106	1	General metabolism	0.0000	NO	YES
Pc20g02250	gi 211587518	similarity to chitinase - <i>Chelonius</i> sp.	198	1	Cell wall	0.9980	YES	
Pc20g02780	gi 211587567	strong similarity to hypothetical protein PA3829 - <i>Pseudomonas aeruginosa</i>	92	1	Plant cell wall degradation	0.0000	NO	YES
Pc20g03330	gi 211587620	strong similarity to glucose-6- phosphate 1-dehydrogenase <i>gsdA</i> - <i>Aspergillus niger</i>	131 132 109	1	General metabolism	0.0010	NO	NO
Pc20g03610	gi 211587647	strong similarity to precursor of mitochondrial isocitrate	112 114 115 239	2	General metabolism	0.2800	NO	YES
Pc20g04720	gi 211587749	strong similarity to precursor of dihydrolipoamide dehydrogenase <i>Lpd1</i> - <i>Saccharomyces cerevisiae</i>	240 241 243	4	General metabolism	0.0690	NO	YES
Pc20g04810	gi 211587757	strong similarity to estrogen receptor- binding cyclophilin <i>cypD</i> - <i>Bos</i> <i>primigenius taurus</i>	182	4	Control of cellular functions	0.0000	NO	NO
Pc20g05320	gi 211587803	strong similarity to phosphomutase homolog Pmu1 - <i>Saccharomyces</i> <i>cerevisiae</i>	206 208	1	Utilization of nutrients	0.0160	NO	NO
Pc20g07010	gi 211587961	strong similarity to ferulic acid esterase A <i>faeA</i> - <i>Aspergillus niger</i>	193	2	Plant cell wall degradation	0.9980	YES	
Pc20g07020	gi 211587962	endo-1,4-beta-xylanase A precursor <i>XylP</i> - <i>Penicillium chrysogenum</i>	223 225	1	Plant cell wall degradation	0.9890	YES	
Pc20g07030	gi 211587963	strong similarity to 1,4-beta-D- arabinoxylan arabinofuranohydrolase	210	2	Plant cell wall degradation	0.9980	YES	
Pc20g07230	gi 211587983	strong similarity to inorganic pyrophosphatase <i>lpp1</i> - <i>Saccharomyces cerevisiae</i>	271	1	General metabolism	0.0000	NO	NO
Pc20g07340	gi 211587992	weak similarity to endoglucanase A <i>eglA</i> - <i>Aspergillus niger</i>	170	1	Plant cell wall degradation	0.9780	YES	
Pc20g08410	gi 211588099	strong similarity to UDP-glucose 4- epimerase Gal10 - <i>Saccharomyces</i> <i>cerevisiae</i>	41	1	General metabolism	0.1540	NO	NO
Pc20g09560	gi 211588186	strong similarity to glucose oxidase GOX - <i>Penicillium amagasakiense</i>	56	1	Cell rescue, defense and	0.9930	YES	
Pc20g10240	gi 211588252	strong similarity to serine-type carboxypeptidase precursor <i>cpdS</i> - <i>Aspergillus phoenicis</i>	51 52 55 56	1	Proteases	1.0000	YES	
Pc20g10360	gi 211588263	beta-N-acetylhexosaminidase <i>hex</i> - <i>Penicillium chrysogenum</i> (68 kDa allergen)		4	Cell wall morphogenesis	0.9970	YES	

Pc20g11630	gil211588384	gamma-actin <i>act-Penicillium chrysogenum</i>	36	1	Cytoskeleton	0.0000	NO	YES
Pc20g13360	gil211588551	strong similarity to exo-alpha 1,4-polygalacturonase PGX1 - <i>Cochliobolus carbonum</i>	149	1	Plant cell wall degradation	1.0000	YES	
Pc20g15140	gil211588723	strong similarity to secreted serine protease 19 kDa CS antigen CS-Ag - <i>Coccidioides immitis</i>	264	1	Proteases	1.0000	YES	
Pc20g15580	gil211588765	strong similarity to NADPH-dependent aldehyde reductase - <i>Sporobolomyces salmonicolor</i>	180	1	General metabolism	0.0030	NO	NO
Pc20g15730	gil211588780	strong similarity to endo 1,5-alpha-arabinanase <i>abnA</i> - <i>Aspergillus niger</i>	217	1	Plant cell wall degradation	0.9950	YES	
Pc21g02110	gil211588987	strong similarity to hypothetical protein mg01387.1 - <i>Magnaporthe grisea</i>	266	1	Control of cellular functions	1.0000	YES	
Pc21g02370	gil211589012	strong similarity to aspergillopepsin <i>apnS</i> - <i>Aspergillus phoenicis</i>	271	1	Proteases	1.0000	YES	
Pc21g06670	gil211589422	strong similarity to carboxypeptidase S1 - <i>Penicillium janthinellum</i>	22	1	Proteases	0.9770	YES	
Pc21g09780	gil211589725	strong similarity to fructosyl amine oxygen oxidoreductase - <i>Aspergillus fumigatus</i>	102	1	Utilization of nutrients	0.6370	YES	
Pc21g09860	gil211589733	strong similarity to hypothetical amidase - <i>Schizosaccharomyces pombe</i>	39	1	General metabolism	0.9990	YES	
			89					
			95					
			96					
			100					
			111					
Pc21g12590	gil211589997	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	113	12	Utilization of nutrients	0.9990	YES	
			118					
			148					
			266					
			267					
			268					
			269					
Pc21g14220	gil211590148	strong similarity to hypothetical protein contig1495_2.tfa_630cg - <i>Aspergillus fumigatus</i>	244	1	Unknown	0.9990	YES	
Pc21g14560	gil211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase <i>gpdA</i> - <i>Aspergillus niger</i>	202	1	General metabolism	0.0000	NO	YES
Pc21g14970	gil211590220	strong similarity to protein D-lactate dehydrogenase YEL071w - <i>Saccharomyces cerevisiae</i>	93	1	General metabolism	0.4280	NO	YES
Pc21g16950	gil211590406	strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>	199	3	General metabolism	0.0010	NO	NO
			201					
			204					
Pc21g20440	gil211590742	strong similarity to ribose 5-phosphate isomerase RPI - <i>Mus musculus</i>	226	1	General metabolism	0.0000	NO	NO
Pc21g22000	gil211590882	strong similarity to 1,3-beta-glucanosyltransferase <i>gel1</i> - <i>Aspergillus fumigatus</i>	61	1	Cell wall morphogenesis	0.9970	YES	
			22					
			40					
			74					
			99					
			101					
Pc21g23210	gil211590999	strong similarity to cell wall protein Crh1 - <i>Saccharomyces cerevisiae</i>	103	11	Cell wall morphogenesis	1.0000	YES	
			112					
			115					
			116					
			232					
			235					

Pc21g23240	gil211591002	strong similarity to alpha-isopropylmalate synthase Leu4 - <i>Saccharomyces cerevisiae</i>	146	1	General metabolism	0.0000	NO	NO
Pc22g00190	gil211591082	strong similarity to hypothetical cell wall protein <i>binB</i> - <i>Aspergillus</i>	249 279	2	Unknown	0.9960	YES	
Pc22g01750	gil211591236	strong similarity to hypothetical amidase - <i>Schizosaccharomyces</i>	44 46	2	General metabolism	0.0000	NO	YES
Pc22g01850	gil211591246	glucan 1,4-alpha-glucosidase BAC8255- <i>Penicillium chrysogenum</i>	119 136 13 16 17	2	Utilization of nutrients	0.9970	YES	
Pc22g02000	gil211591261	strong similarity to mitochondrial aconitate hydratase Aco1 - <i>Saccharomyces cerevisiae</i>	25 28 30 62	7	General metabolism	0.2920	NO	NO
Pc22g02800	gil211591341	strong similarity to calcium-binding protein precursor <i>cnx1p</i> -	59 63	2	Control of cellular functions	0.9980	YES	
Pc22g03140	gil211591374	strong similarity to Gbeta like protein <i>cpcB</i> - <i>Aspergillus nidulans</i>	196	1	Control of cellular functions	0.0050	NO	NO
Pc22g04850	gil211591539	strong similarity to D-arabinose dehydrogenase Ara1 -	177 195	2	General metabolism	0.1470	NO	NO
Pc22g05880	gil211591637	strong similarity to hypothetical protein 1117_scaffold_1.tfa_300cg - <i>Fusarium graminearum</i>	222	1	Plant cell wall degradation	0.9990	YES	
Pc22g06490	gil211591694	strong similarity to alkaline phosphatase - <i>Neurospora crassa</i>	5 7 8 9 32 33 35	4	Utilization of nutrients	0.9990	YES	
Pc22g09380	gil211591967	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanotransferase <i>gel3</i> - <i>Aspergillus fumigatus</i>	37 48 50 54 57	8	Cell wall morphogenesis	1.0000	YES	
Pc22g09390	gil211591968	strong similarity to mannitol dehydrogenase <i>mtlD</i> - <i>Pseudomonas</i>	68 70 60 127 129 138 141	2	Utilization of nutrients	0.0000	NO	NO
Pc22g09580	gil211591986	strong similarity to acid phosphatase <i>aphA</i> - <i>Aspergillus ficuum</i>	142 145 183 251 253 254 119 122	11	Utilization of nutrients	0.7250	YES	
Pc22g09680	gil211591995	similarity to hypothetical protein PA4204 - <i>Pseudomonas aeruginosa</i>	125 128 130 152	6	General metabolism	0.9930	YES	
Pc22g12670	gil211592227	strong similarity to hypothetical protein contig_1_67_scaffold_4.tfa_440wg - <i>Aspergillus nidulans</i>	74	1	Utilization of nutrients	0.9980	YES	
Pc22g13130	gil211592257	strong similarity to mitochondrial aspartate aminotransferase <i>mAspAT</i> - <i>Mus musculus</i>	169 171 172	3	General metabolism	0.0190	NO	YES
Pc22g14360	gil211592377	strong similarity to hypothetical membrane protein YDL237w - <i>Saccharomyces cerevisiae</i>	181	1	Unknown	1.0000	YES	
Pc22g15910	gil211592524	strong similarity to cytoplasmic form of aminopeptidase like protein An03g04230 - <i>Aspergillus niger</i>	45	1	Proteases	0.0000	NO	NO
Pc22g16510	gil211592582	strong similarity to isoamyl alcohol oxidase <i>mreA</i> - <i>Aspergillus oryzae</i>	49 58 59	3	General metabolism	1.0000	YES	

Pc22g16780	gil211592608	strong similarity to branched-chain-amino-acid aminotransferase Bat2 -	176	2	General metabolism	0.8010	YES	
Pc22g17560	gil211592684	glutamate dehydrogenase <i>gdhA</i> - <i>Penicillium chrysogenum</i>	178 108 110 72	2	General metabolism	0.0000	NO	NO
Pc22g17870	gil211592713	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>	73 94 99 104 22	5	Cell wall morphogenesis	0.9920	YES	
Pc22g19230	gil211592843	strong similarity to endo-beta-1,4-glucanase A <i>eglA</i> - <i>Aspergillus nidulans</i>	23 40 42	4	Plant cell wall degradation	0.9930	YES	
Pc22g19730	gil211592892	strong similarity to glucose-6-phosphate isomerase Pgi1 - <i>Saccharomyces cerevisiae</i>	91	1	Utilization of nutrients	0.0000	NO	NO
Pc22g20290	gil211592946	strong similarity to polygalacturonase <i>pgal</i> - <i>Aspergillus niger</i>	154 155 156 157	4	Plant cell wall degradation	1.0000	YES	
Pc22g22290	gil211593141	strong similarity to IgE-binding protein - <i>Aspergillus fumigatus</i>	236 242	2	Unknown	0.9990	YES	
Pc22g22710	gil211593182	strong similarity to dihydroxy-acid dehydratase <i>llv3</i> - <i>Saccharomyces</i>	64 65 97	2	General metabolism	0.0440	NO	YES
Pc22g22810	gil211593192	strong similarity to sulphhydryl oxidase Sox from patent EP565172-A1 - <i>Aspergillus niger</i>	120 121 123	4	General metabolism	1.0000	YES	
Pc22g23760	gil211593284	strong similarity to type 2 peroxiredoxin like protein An12g08570 - <i>Aspergillus niger</i>	250	1	Cell rescue, defense and virulence	0.0000	NO	YES
Pc22g23810	gil211593289	strong similarity to fumarate hydratase <i>fumR</i> - <i>Rhizopus oryzae</i>	124 126 184 187 188 189 190	2	General metabolism	0.6190	YES	
Pc22g24890	gil211593394	strong similarity to pectate lyase <i>plyA</i> - <i>Aspergillus niger</i>	191 192 193 194 206 265	11	Plant cell wall degradation	1.0000	YES	
	gil211593449	strong similarity to Sol1 - <i>Saccharomyces cerevisiae</i>	229	1	General metabolism	0.0010	NO	YES
Pc22g25470	gil119482686	ATP phosphoribosyltransferase - <i>Neosartorya fischeri</i> NRRL 181	185	1	General metabolism	0.0000	NO	YES
	gil144952798	16 kDa allergen [<i>Penicillium chrysogenum</i>]	257	1	Unknown (First 149 aa of	0.9990	YES	

Supplementary Table 5. Proteins found in this work in the extracellular proteome of *P. chrysogenum* that have been identified by LC-MS/MS analysis in the microbody matrix fraction of *P. chrysogenum*

Spot	ORF name	Accession Number	Description	Function	Notes	Signal peptide probability	Signal peptide	Non-classically secreted protein
92	Pc20g03330	gil211587620	strong similarity to glucose-6-phosphate 1-dehydrogenase <i>gsdA</i> - <i>Aspergillus niger</i>	General metabolism. Pentose phosphate pathway	PTS1	0.0010	NO	NO
131	Pc20g03610	gil211587647	strong similarity to precursor of mitochondrial isocitrate dehydrogenase <i>icdA</i> - <i>Aspergillus</i>	General metabolism. Citric acid and glyoxylate cycles	PTS1	0.2800	NO	YES
36	Pc20g11630	gil211588384	gamma-actin act- <i>Penicillium chrysogenum</i>	Cytoskeleton		0.0000	NO	YES
239								
240	Pc20g04810	gil211587757	strong similarity to estrogen receptor-binding cyclophilin <i>cypD</i> - <i>Bos primigenius taurus</i>	Control of cellular functions. Chaperones and cell signaling		0.0000	NO	NO
241								
243								
255	Pc13g08810	gil211583926	strong similarity to elongation factor 1 beta EF-1 - <i>Oryctolagus cuniculus</i>	General metabolism. Translation		0.0150	NO	NO
196	Pc22g03140	gil211591374	strong similarity to Gbeta like protein <i>cpcB</i> - <i>Aspergillus nidulans</i>	Control of cellular functions. Response to amino acid		0.0050	NO	NO
169								
171	Pc22g13130	gil211592257	strong similarity to mitochondrial aspartate aminotransferase <i>mAspAT</i> - <i>Mus musculus</i>	General metabolism. Amino acids metabolism	Mitochondrial	0.0190	NO	YES
172								
168	Pc12g05380	gil211582002	strong similarity to coproporphyrinogen oxidase III Hem13 - <i>Saccharomyces cerevisiae</i>	General metabolism. Heme biosynthetic pathway	Mitochondrial	0.0000	NO	YES
109								
112	Pc20g04720	gil211587749	strong similarity to precursor of dihydrolipoamide dehydrogenase <i>Lpd1</i> - <i>Saccharomyces cerevisiae</i>	General metabolism. Energy	Mitochondrial	0.0690	NO	YES
114								
115								
64	Pc22g22710	gil211593182	strong similarity to dihydroxy-acid dehydratase <i>Ilv3</i> - <i>Saccharomyces cerevisiae</i>	General metabolism. Branched-chain amino acids	Mitochondrial	0.0440	NO	YES
65								
124	Pc22g23810	gil211593289	strong similarity to fumarate hydratase <i>fumR</i> - <i>Rhizopus oryzae</i>	General metabolism. TCA cycle	Mitochondrial	0.6190	YES	
126								
173	Pc13g15810	gil211584596	strong similarity to ketol-acid reductoisomerase <i>ilv-2</i> - <i>Neurospora crassa</i>	General metabolism. Branched-chain amino acids	Mitochondrial	0.9990	YES	
174								
13								
16								
17								
25	Pc22g02000	gil211591261	strong similarity to mitochondrial aconitate hydratase <i>Aco1</i> - <i>Saccharomyces cerevisiae</i>	General metabolism. TCA cycle	Mitochondrial	0.2920	NO	NO
28								
30								
62								
181	Pc13g09680	gil211584011	strong similarity to aspartyl proteinase candidapepsin - <i>Candida albicans</i>	Protease. Virulence	Vacuole and secreted	1.0000	YES	
214	Pc14g01140	gil211584729	strong similarity to proteasome 20S subunit Pre10 - <i>Saccharomyces cerevisiae</i>	General metabolism. Protein fate		0.0000	NO	NO
231	Pc13g09540	gil211583997	strong similarity to proteasome 20S subunit Scl1 - <i>Saccharomyces cerevisiae</i>	General metabolism. Protein fate		0.0000	NO	NO
143	Pc16g05080	gil211585478	strong similarity to adenosylhomocysteinase - <i>Homo sapiens</i>	General metabolism.		0.0040	NO	YES
133								
134								
135								
137	Pc14g01740	gil211584789	enolase BAC82549- <i>Penicillium chrysogenum</i>	General metabolism. Glycolysis		0.0000	NO	NO
139								
140								
278								
211	Pc12g05820	gil211582045	strong similarity to esterase D ESD - <i>Homo sapiens</i>	General metabolism		0.1650	NO	YES
213								
105	Pc16g13280	gil211586250	strong similarity to glutathione reductase <i>Glrl</i> - <i>Saccharomyces cerevisiae</i>	Cell rescue, defense and virulence		0.0010	NO	NO
202	Pc21g14560	gil211590181	strong similarity to glyceraldehyde-3-phosphate dehydrogenase <i>gpdA</i> - <i>Aspergillus niger</i>	General metabolism. Glycolysis. Virulence		0.0000	NO	YES
210	Pc20g07230	gil211587983	strong similarity to inorganic pyrophosphatase <i>lpp1</i> - <i>Saccharomyces cerevisiae</i>	General metabolism. Phosphate control		0.0000	NO	NO
164								
165	Pc12g04310	gil211581920	strong similarity to acetate-inducible gene <i>aciA</i> - <i>Aspergillus nidulans</i>	Utilization of nutrients. Formate oxidation		0.0020	NO	YES
166								
150	Pc12g10940	gil211582533	strong similarity to phosphogluconate dehydrogenase <i>Gnd1</i> - <i>Saccharomyces cerevisiae</i>	General metabolism. Pentose phosphate pathway		0.0570	NO	NO
199								
201	Pc21g16950	gil211590406	strong similarity to transaldolase <i>Tal1</i> - <i>Saccharomyces cerevisiae</i>	General metabolism. Pentose phosphate pathway		0.0010	NO	NO
204								
260	Pc13g06930	gil211583746	strong similarity to hypothetical protein contig_1_7_scaffold_1.tfa_1010wg -	Unknown		1.0000	YES	
262								