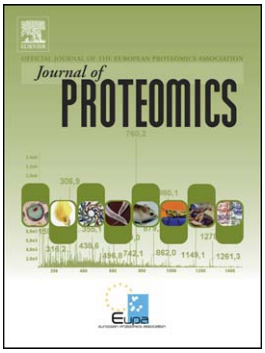


Casein phosphopeptides and $CaCl_2$ increase penicillin production and cause an increment in microbody/peroxisome proteins in *Penicillium chrysogenum*

Rebeca Domínguez-Santos, Katarina Kosalková, Carlos García-Estrada, Carlos Barreiro, Ana Ibáñez, Alejandro Morales, Juan-Francisco Martín

PII: S1874-3919(16)30549-8
DOI: doi:10.1016/j.jprot.2016.12.021
Reference: JPROT 2750

To appear in: *Journal of Proteomics*

Received date: 9 November 2016
Revised date: 15 December 2016
Accepted date: 31 December 2016

Please cite this article as: Domínguez-Santos Rebeca, Kosalková Katarina, García-Estrada Carlos, Barreiro Carlos, Ibáñez Ana, Morales Alejandro, Martín Juan-Francisco, Casein phosphopeptides and $CaCl_2$ increase penicillin production and cause an increment in microbody/peroxisome proteins in *Penicillium chrysogenum*, *Journal of Proteomics* (2017), doi:10.1016/j.jprot.2016.12.021

Casein phosphopeptides and $CaCl_2$ increase penicillin production and cause an increment in microbody/peroxisome proteins in *Penicillium chrysogenum*

Rebeca Domínguez-Santos[a,b], Katarina Kosalková[b], Carlos García-Estrada[b*], Carlos Barreiro[b], Ana Ibáñez[b] , Alejandro Morales[b], Juan-Francisco Martín[a*]

[a] Área de Microbiología, Departamento de Biología Molecular, Universidad de León, Campus de Vegazana s/n, 24071, León, Spain

[b] INBIOTEC, Instituto de Biotecnología de León, Avda. Real nº. 1, Parque Científico de León, 24006, León, Spain

*Corresponding author: Juan Francisco Martín. E-mail: jf.martin@unileon.es

*Co-corresponding author: Carlos García-Estrada. E-mail: carlos.garcia@inbiotec.es / c.gestrada@unileon.es. Phone: +34 987210308. Fax: +34 987210388

Keywords: penicillin; *Penicillium chrysogenum*; *Penicillium rubens*; casein phosphopeptides; calcium; microbodies.

Abstract

Transport of penicillin intermediates and penicillin secretion are still poorly characterized in *Penicillium chrysogenum* (re-identified as *Penicillium rubens*). Calcium (Ca^{2+}) plays an important role in the metabolism of filamentous fungi, and casein phosphopeptides (CPP) are involved in Ca^{2+} internalization. In this study we observe that the effect of $CaCl_2$ and CPP is additive and promotes an increase in penicillin production of up to 10-12 fold. Combination of $CaCl_2$ and CPP greatly promotes expression of the three penicillin biosynthetic genes. Comparative proteomic analysis by 2D-DIGE, identified 39 proteins differentially represented in *P. chrysogenum* Wisconsin 54-1255 after CPP/$CaCl_2$ addition. The most interesting group of overrepresented proteins were a peroxisomal catalase, three proteins of the methylcitrate cycle, two aminotransferases and cystationine β-synthase, which are directly or indirectly related to the formation of penicillin amino acid precursors. Importantly, two of the enzymes of the penicillin pathway (isopenicillin N synthase and isopenicillin N acyltransferase) are clearly induced after CPP/$CaCl_2$ addition. Most of these overrepresented proteins are either authentic peroxisomal proteins or microbody-associated proteins. This evidence suggests that addition of CPP/$CaCl_2$ promotes the formation of penicillin precursors and the penicillin biosynthetic enzymes in peroxisomes and vesicles, which may be involved in transport and secretion of penicillin.

Significance

Penicillin biosynthesis in *Penicillium chrysogenum* is one of the best characterized secondary metabolism processes. However, the mechanism by which penicillin is secreted still remains to be elucidated. Taking into account the role played by Ca^{2+} and CPP in the secretory pathway and considering the positive effect that Ca^{2+} exerts on penicillin production, the analysis of global protein changes produced after CPP/$CaCl_2$ addition is very helpful to decipher the processes related to the biosynthesis and secretion of penicillin.

Introduction

The filamentous fungus *Penicillium chrysogenum*, recently re-identified as *Penicillium rubens* [1], is used in the industrial production of the beta-lactam antibiotic penicillin, which remains as one of the most commonly prescribed drugs. The capability of this microorganism to produce penicillin is evidenced by the fact that antibiotic titers have raised by more than three orders of magnitude during industrial strain improvement programs [2]. One of the best characterized processes of secondary metabolism is the biosynthesis of benzylpenicillin (for a review see [3]) (Figure 1). In short, three amino acids (L-α-aminoadipic acid, L-cysteine and L-valine) are condensed in a non-ribosomal manner by a trimodular and multidomain δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase (ACVS encoded by the *pcbAB* gene), thus forming the tripeptide ACV. Subsequent cyclization of ACV by a reaction catalyzed by the isopenicillin N (IPN) synthase (IPNS encoded by the *pcbC* gene) gives rise to IPN, which is the first compound in the pathway with antimicrobial properties. Finally, the L-α-aminoadipyl side chain of IPN is replaced with aryl side chains (previously activated by specific aryl-CoA ligases) in a reaction catalyzed by the IPN acyltransferase (IAT, encoded by the *penDE* gene), which synthesizes hydrophobic bioactive penicillins (e.g. benzylpenicillin if the side chain is phenylacetyl CoA). The molecular basis underlying the reaction mechanisms of the three enzymes have been analyzed recently [4]. This biosynthetic process is controlled by a complex regulatory network of transcription factors in response to environmental and nutritional conditions [5-17].

Penicillin biosynthesis is compartmentalized between the cytosol and peroxisomes [18,19] (Figure 2). Electron microscopy experiments have confirmed that the enzymes catalyzing the first two steps in the pathway (i.e. ACV and IPN formation) co-localize in cytosol [20], although initial experiments found ACVS attached to or inside vacuoles [21,22]. In fact, precursor amino acids are stored in vacuoles once they are synthesized [22] and therefore, association of ACVS with those organelles is likely. The last step in the pathway (i.e. substitution of the L-α-aminoadipyl side chain of IPN with CoA-activated aryl side chains), which is catalyzed by the IAT, together with the activation of those precursor side chains by Aryl-CoA ligases, take place in peroxisomes [23-25]. This implies that different transport systems must be present to assure the correct flux of precursors, intermediates, enzymes and penicillin through different subcellular compartments. PenV, a vacuolar membrane protein of the MFS (Major Facilitator

Superfamily) class, has been reported to play an important role in the formation of the ACV tripeptide, likely supplying amino acids from the vacuolar lumen to the vacuole-anchored ACVS [26]. PenM, another transporter from the MFS class, is involved in the transport of cytosolic IPN inside the peroxisomal matrix [27], where penicillin is biosynthesized. Incorporation of those side chain precursors (e.g. phenylacetic acid in the case of benzylpenicillin) to the peroxisomal matrix is achieved by means of PaaT, a MFS drug/H+ antiporter with 12 transmembrane spanning domains [28]. This transporter, also known as PenT, was reported to stimulate penicillin production probably through enhancing the translocation of penicillin precursors across fungal cellular membrane [29]. However, no authentic benzylpenicillin transporters that may be located on either the peroxisomal or the fungal membrane have been characterized in *P. chrysogenum* so far, whereas an ABC transporter (encoded by *atrD*) has been found to be related in some way with the secretion of penicillin in *Aspergillus nidulans* [30].

In the absence of scientific evidence for an active transport process for penicillin secretion in *P. chrysogenum*, it has been hypothesized that secretion may occur by an exocytosis mechanism after the fusion of peroxisomes with secretory vesicles [19]. In a recent work, we observed that addition of either 1,3-diaminopropane or spermidine induced penicillin overproduction and led to an increase in the intracellular content of vesicles that derived to vacuoles [31]. Vacuoles and vesicles are known to sequester secondary metabolites to protect host cells from self-toxicity [32]. Many fungal secondary metabolism enzymes are often found in vesicles and vacuoles, including those for the biosynthesis of cyclosporine [33] and aflatoxin [34-36], and also in peroxisomes [37]**.** In a detailed study of the proteins located in peroxisomes, Kiel and coworkers [38] identified by MS/MS a large number of protein that include: 1) authentic peroxisomal matrix proteins, confirmed by the presence of peroxisomal targeting sequences PTS1 or PTS2, and 2) "microbody-associated" proteins, which lack the canonical peroxisome targeting sequences. Both types of proteins co-purified after peroxisome enrichment by discontinuous sucrose gradient ultracentrifugation.

Calcium (Ca^{2+}) is not only an important component of cellular material, but is also a universal signaling molecule, particularly in eukaryotes, including filamentous fungi and plants [39,40]. The cytosolic content of Ca^{2+} is critically important for the function of biosynthetic mechanisms. Ca^{2+} is stored in some organelles, particularly in the endoplasmic reticulum (ER) and in vacuoles [41]. While in the cytosolic Ca^{2+} is in the

micromolar concentration, the level in these storage organelles is in the range of millimolar. Therefore, Ca^{2+} uptake and transport inside the cells is very important. Ca^{2+} is usually complexed with phosphate, with oxalate or other organic acid and in this context, the role of casein phosphopeptides (CPP) is important. Intracellular Ca^{2+} has a particularly important role in the secretory pathway by which many extracellular enzymes and membrane proteins are secreted. In the secretory pathway extracellular or membrane targeted protein are going through the ER and the Golgi system and the intracellular Ca^{2+} level both in the ER or the cytosol are critically important for the targeting of these proteins through the secretory pathway [42].

CPP released by digestion of alpha− and beta-casein are rich in phosphoserine residues (SerP). They stimulate enzyme secretion in the gastrointestinal tract and enhance the immune response in mammals, and are used as food supplements. It is well known that CPP transport Ca^{2+} across the membranes [43]. CPP containing chelated Ca^{2+} drastically increase the secretion of extracellular homologous and heterologous proteins in *Aspergillus* [42,44]. Recent proteomics studies showed that CPP alter drastically the vesicle-mediated secretory pathway in filamentous fungi, apparently because they change the Ca^{2+} concentration in organelles that act as Ca^{2+} reservoirs [42]. It is known that CPP bind to Ca^{2+} and phospholipids in the membrane and allow internalization of calcium into the cell. These results suggest that CPP play an important role in maintaining Ca^{2+} homeostasis and therefore, in the secretion of proteins through vesicles. This same system might affect the secretion of penicillin from peroxisomes, since Ca^{2+} is well known since the mid-fifties to play an important role on the growth of *P. chrysogenum* and in penicillin production, although the basis of the Ca^{2+} role on penicillin production remains obscure.

Therefore, it was important to study the role of Ca^{2+} and CPP in the biosynthesis and secretion of penicillin. In this article we report the synergistic effect of Ca^{2+} and CPP on penicillin production, shedding light on the mechanisms leading to increased penicillin titers by 2D protein analysis. Interestingly we found that most of the proteins that are overrepresented in response to CPP/Ca^{2+} addition correspond to peroxisomal or "microbody-associated" proteins.

Materials and Methods

CE90GMM (CPP)

Commercial CE90GMM obtained from DMV International is a predigested casein (27% degree of hydrolysis) with 15% free amino acids and 19% CPP, the latter consisting of a mixture of short chain peptides (±80% < 1 kDa with an average molecular weight of 0.64 kDa). This product also contains calcium (0.24%), magnesium (0.015%), potassium (1.1%), sodium (1.2%), phosphorus (0.89%) and chloride (0.27%).

Strains and culture conditions.

The filamentous fungus *P. chrysogenum* was used in this work. This ascomycete has been recently re-identified as *P. rubens* [1], but we will keep *P. chrysogenum* along the manuscript for consistency with our previous works. Experiments were carried out with three strains of *P. chrysogenum*; the wild-type NRRL 1951 (very low penicillin producer), the Wisconsin 54-1255 (reference strain for the genome sequencing project and low penicillin producer) and the penicillin high-producer AS-P-78 (kindly provided to us by Antibioticos S.A., León, Spain), which represent different stages of the industrial strain improvement program.

Strains were grown on solid Power sporulation medium [45] for seven days at 28ºC. Conidia from one Petri dish were collected and inoculated into flasks containing 100 ml of defined inoculation (DI) medium with 40g/L glucose [46]. After 24 h of incubation at 25°C and at 250 rpm, 10 ml of the culture in DI medium was added to a 500-ml flask containing 100 ml of defined production medium (MDFP), which was prepared by adding 1 g/L potassium phenylacetate, 30 g/L lactose and 10 g/L sucrose to the DI medium without glucose. MDFP was supplemented with 10 g/L CPP, 2,5 g/L $CaCl_2$ or a mixture of both compounds in order to test their effect on penicillin production. Cultures in MDFP (with or without supplementation) were incubated at 25°C and at 250 rpm for different time points according to each experiment (see below). Three independent experiments were carried out in duplicate.

Quantitation of total penicillins production by bioassay

Total penicillins (including benzylpenicillin and IPN) production by *P. chrysogenum* was assessed by bioassay. Samples were taken at different time-points from cultures of *P. chrysogenum* grown in MDFP with or without CPP and/or $CaCl_2$ supplementation.

Samples were centrifuged at 13,000 x g for 10 min. The mycelium was washed twice with ddH_2O and dry weighted, whereas supernatants were bioassayed by placing 50 µl into holes performed in TSA (Difco) plates previously inoculated with *Micrococcus luteus* ATCC 9341 (test microorganism) at a final OD600 = 0.01. The bioassay was incubated for 2 h at 4ºC and subsequently for 18 h at 30ºC. Every sample was bioassayed in triplicate. Total penicillins production was determined according to the inhibitory halo provided by each sample compared to those of different concentrations of potassium benzylpenicillin.

RNA extraction and semiquantitative RT-PCR assays

RNeasy Mini Kit columns (Qiagen, Hilden, Germany) were used following the manufacturer's instructions to extract RNA from cultures of *P. chrysogenum* Wisconsin 54-1255 grown for 60 h in MDFP with or without CPP/$CaCl_2$ supplementation. Total RNA was treated with ''RQ1 RNase-Free DNase'' (Promega Corporation, Madison, WI, USA), following the manufacturer's instructions and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The absence of contaminant DNA in the samples was tested by PCR.

RT-PCR experiments were carried out with 200 ng of total RNA using the ''SuperScript One-Step RT-PCR with Platinum Taq'' system (Invitrogen Corporation, Carlsbad, CA, USA) under manufacturer's instructions, and several primers (see Supplementary Table S1). RT-PCR parameters were optimized so as to avoid saturation of the final product and were set as follows: 55ºC for 30 min (retrotranscription), 94ºC for 2 min (pre-denaturalization) and 29 cycles comprising three steps: i) 94ºC for 15 s, ii) 55ºC (*penDE* gene) or 60ºC (*pcbAB* and *pcbC* genes) for 30 s and iii) 72ºC for 45 s.

Extraction of proteins from mycelia

Mycelia of *P. chrysogenum* Wisconsin 54-1255 grown for 60 h in MDFP with or without CPP/$CaCl_2$ supplementation were collected by filtration through a nylon cloth (Nytal membrane), washed once with 0.9% NaCl and twice with ddH_2O and stored at -80ºC. Frozen mycelia were ground using liquid nitrogen in a precooled mortar until a fine powder was formed. The powder was resuspended in the appropriate solution according to each experiment (see below).

Western blot

Powdered mycelia was resuspended in TD buffer (50 mM Tris–HCl pH 8.0, 5 mM DTT) and processed as indicated before [13]. Immunological detection of IAT was performed as reported elsewhere [24] using polyclonal antibodies raised against the IAT β-subunit [47]. Immunological detection of α-tubulin was used as control of the amount of loaded protein [13].

Quantitation of RT-PCR and western blot bands

The signals provided by the RT-PCR and western blot were quantified by densitometry using the ‘‘Gel- Pro Analizer’’ software (Media Cybernetics). Expression levels were normalized comparing the intensity of each band (IOD) to that provided by the γ-actin band (in the case of RT-PCR experiments) or to the α-tubulin (for western blot).

DIGE and Protein Identification by MALDI-TOF MS and MS/MS

Sample preparation was based on the protocol described by [2] for *P. chrysogenum*. Briefly, the fine powder generated after grinding in liquid nitrogen two grams of frozen mycelia, which was obtained from 60-h cultures of *P. chrysogenum* Wisconsin 54-1255 grown in MDFP with or without CPP/$CaCl_2$ supplementation as indicated above, was resuspended (ratio 1:5 w/v) in 10 mM potassium phosphate buffer (K_2HPO_4:KH_2PO_4) pH 7.4 supplemented with 0.07% DTT (w/v) and a protease inhibitor mixture (CompleteTM; Roche Applied Science). Subsequently in was precipitated with 20% TCA in acetone containing 0.14% (w/v) DTT. The final protein pellet was solubilized in 500 µl of sample buffer [8 M urea, 2% (w/v) CHAPS, 1 % (v/v) IPG Buffer pH 3–10 non-linear (NL) (GE Healthcare), 60 mM DTT, 0.002% bromphenol blue].

DIGE experiments were performed as previously described [48,49]. In brief, sample impurities were removed by means of 2D-Clean KitTM (GE Healthcare). The labelling ratio used during 30 minutes was 400 pmol/µL of Cy dye per 50 µg and the reaction was stopped by the addition of 10 mM lysine for 10 minutes. Four biological replicates per each condition were used. IEF was performed on 18-cm 3-10NL pH range IPG strips and second dimension was carried out in a 12.5% polyacrylamide SDS-PAGE [Ettan Dalt Six apparatus (GE Healthcare)]. Ettan™ DIGE Imager (GE Healthcare) was used for protein visualization and the image analysis was carried out with DeCyder™ differential analysis software v7.0 (GE Healthcare) to obtain the average ratio, unpaired

Student's t test and ANNOVA values between groups. Protein spots differentially expressed with statistical significance were considered when: i) at least appeared in 70% of gel images, ii) threshold average ratio was 2-fold and iii) p-values were lower than 0.05. False Discovery Rate (FDR) was applied [50].

DIGE gels were stained with Colloidal Coomassie [51], thus allowing spot manual excision. The proteins were processed and digested following previous studies [52]. The samples were analysed with a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (AB Sciex) and the 10 most intense precursor ions from each MS spectra were MS/MS analysed with CID (atmospheric gas was used). Protein candidates combining peptide mass fingerprinting/tandem MS search were considered as identified when the global Mascot score was greater than 82% with a significance level of $p < 0.05$ against the NCBInr database (taxonomy: fungi). Positively identified proteins were uploaded on the BioCyc web server [53] to assign them to specific metabolic pathways.

Results

1. CPP and $CaCl_2$ exert a synergistic effect over the production of penicillin in *P. chrysogenum*

Due to the positive effect previously observed in the metabolism of *A. awamori* after the addition of CPP [44], we decided to test whether a beneficial effect could also be exerted on penicillin production. With this purpose, three strains of *P. chrysogenum*, each producing different amounts of penicillin, were cultured in MDFP in the presence and absence of 1% (w/v) CPP for up to 72 h. In the very low producer wild-type strain NRRL 1951, the presence of CPP gave rise to 12.5- and 10.6-fold increases in volumetric and specific productions, respectively, whereas in the low penicillin producer Wisconsin 54-1255 strain, a significant ~3-fold increase was observed in volumetric and specific penicillin productions, respectively (data not shown). Conversely, only significant 1.48- and 1.30-fold increases in volumetric and specific penicillin productions, respectively, were obtained in the high penicillin producer AS-P-78 (data not shown). The fact that these *P. chrysogenum* strains show different positive effects in response to the addition of CPP may be a consequence of the degree of manipulation undergone by these strains, which represent different stages of the industrial strain improvement program (see Discussion).

In early studies it was reported that the presence of different calcium salts in the culture medium has an effect in penicillin production [54]. Since CPP are able to form complexes with Ca^{2+} and increase calcium bioavailability [43], the effect of the addition of both compounds was assessed on penicillin production.

With this aim, the laboratory reference strain *P. chrysogenum* Wisconsin 54-1255 was cultured in MDFP with or without CPP and/or $CaCl_2$ supplementation for up to 96 h. Samples were taken at different time points along the culture time and dry weight and volumetric and specific productions of total penicillins were assessed (Figure 3).

When $CaCl_2$ was added to the culture medium, no differences in dry weight were observed regarding control values (MDFP without supplementation). Conversely, the addition of CPP to the culture medium gave rise to a significant 1.31-1.45-fold increase in dry weight at 84 h and 96 h, respectively. Interestingly, supplementation of both CPP and $CaCl_2$ together produced a significant ~1.70-fold increase in biomass at early time-points. This effect of increased biomass remained until the end of the culture time and reached the maximum difference at 72 h (1.88-fold increase). After this time point, the increase in biomass was similar to that observed with the addition of CPP alone (1.31 fold at 84 h and 1.45 fold at 96 h) (Figure 3A).

The volumetric production of total penicillins was also assessed (Figure 3B). The addition of $CaCl_2$ to the culture medium provided significant modifications in penicillin production at 48 h (1.90-fold increase) and 72 h (1.70-fold increase). When CPP were added to the culture medium, significant increases in penicillin production were observed after 60 h till the end of the culture time. These values ranged from 1.60-fold increase at 72 h to 2.3 fold increase at 96 h. Strikingly, the supplementation of both CPP and $CaCl_2$ together led to significant increases in total penicillins production since early time-points, thus suggesting that addition of both CPP and $CaCl_2$ facilitate the internalization of the calcium ions since the very beginning of the culture. Values ranged from 1.9-fold increase at 48 h to 5.1-fold increase at 96 h.

Total penicillins specific production was estimated according to volumetric production and dry weight data (Figure 3C). After the addition of $CaCl_2$ to the culture medium, significant increases in penicillin production were observed at 48 h (1.90-fold increase) and 72 h (1.60-fold increase). Supplementation with CPP led to a significant improvement in penicillin titers after 72 h. The increased production ranged from 1.30

fold at 72 h to 1.40 fold at 84 h and 96 h. In concordance with volumetric production data, penicillin specific production values laid bare the synergistic effect of CPP and $CaCl_2$ supplementation, mainly after 60 h in culture, with increases in penicillin production ranging from 2 fold at 60 h to 3.2 fold at 96 h.

2. Proteomics wide analysis of the synergistic effect exerted by CPP and $CaCl_2$.

In order to carry out a deep analysis about the mechanisms triggered by CPP/$CaCl_2$ in *P. chrysogenum* Wisconsin 54-1255, a wide proteome comparative analysis was conducted. With this purpose, protein samples were obtained from 60-h cultures grown in MDFP with or without CPP/$CaCl_2$ supplementation, and analyzed by 2D-DIGE (Figure 4). Fifty four spots were found differentially represented (at least two-fold) between the two conditions. These spots included 39 different proteins, which were identified by MALDI-TOF MS and MS/MS analysis (Table 1 and Supplementary Table S2) and functionally classified according to their metabolic roles. The main findings are summarized below.

Penicillin biosynthetic enzymes: One of the most striking effects of CPP/$CaCl_2$ is the induction of two penicillin biosynthetic enzymes. Spot 39P contains the enzyme involved in the biosynthesis of IPN (IPN synthase), which is 2.18 times overrepresented under these conditions. In addition, spot 54P, which includes the IAT in charge of catalyzing the last step of penicillin biosynthesis, is also overrepresented (2.07 times). The first enzyme of the pathway, ACVS, is not detected in the DIGE gels due to its big molecular mass (425 kDa) and therefore, the positive effect of CPP/$CaCl_2$ on the expression of the gene encoding ACVS cannot be ruled out (see next section).

Carbohydrate metabolism and energy: Some enzymes from glycolysis were differentially expressed after the addition of CPP/$CaCl_2$. Spot 16P contains a probable phosphoglycerate mutase, which is 2.54 times overrepresented under these conditions. Additionally, spot 42C, which contains a probable glyceraldehyde-3-phosphate dehydrogenase, is 2.03 times overrepresented under control conditions. These two enzymes are involved in the metabolism of three carbon intermediates in the late steps of the glycolysis and serve to synthesize acetyl-CoA and propionyl-CoA, which are important precursors for the homocitrate synthase and the methylcitrate synthase, respectively. In addition, the glyceraldehyde-3-phosphate dehydrogenase has been described to be involved in fusion of vesicles to the plasma membrane [42,55,56].

Some of the proteins induced after the addition of CPP/$CaCl_2$ are involved in the tricarboxylic acid cycle. Spots 31P and 35P, which in comparison with control conditions are overrepresented 2.18 and 2.31 times, respectively, include a probable citrate synthase, the latter showing 55% identity with the *P. chrysogenum* methylcitrate synthase encoded by the *mcsA* gene and 19% identity with the *P. chrysogenum* homocitrate synthase encoded by the *lys1* gene. Spot 41P include a probable malate dehydrogenase, which is 3.49 times overrepresented due to CPP/$CaCl_2$ supplementation.

Spot 37P is 2.23-fold overrepresented after CPP/$CaCl_2$ supplementation. This spot 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% similarity, 84% identity), the latter catalyzing the oxidation of formate to bicarbonate originating NADH (required for penicillin biosynthesis). Formate serves 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 [57]. Interestingly, this protein is overrepresented in penicillin high-producing strains of *P. chrysogenum* [2].

It is worth mentioning that several enzymes of the methylcitrate cycle (conversion of propionate into pyruvate and succinate) are induced by the presence of CPP/$CaCl_2$. Spot 15P, which contains a probable 2-methylisocitrate lyase Icl2, an enzyme converting (2S,3R)-2-methylisocitrate into pyruvate and (or plus) succinate, is 5.23 times overrepresented under these conditions. Spot 28P is 3.18 times overrepresented, and includes a probable 2-methylcitrate dehydratase PrpD, an enzyme catalyzing the conversion of (2S,3R)-2-methylcitrate into (2S,3R)-2-methylisocitrate. Finally, spots 32P, 33P and 34P (3.12, 3.72 and 2.84 times overrepresented) include three protein species of a probable methylcitrate synthase *mcsA* (the first enzyme of the methylcitrate cycle, see Discussion). This enzyme converts propanoyl-CoA into (2S,3R)-2-methylcitrate and its overexpression has been reported to relieve the inhibition of sterigmatocystin production by propionate [58].

Amino acids metabolism and penicillin biosynthetic precursors: Supplementation of media with CPP/$CaCl_2$ induced the expression of some enzymes involved in different amino acids metabolic pathways leading to the synthesis of glutamate and/or α-

aminoadipate. Spot 22P (3.3 times overrepresented) contains a probable delta-1-pyrroline-5carboxylate dehydrogenase (P5Cdh), which participates in ornitine and proline catabolism to glutamate, and shows 47% similarity (27% identity) to the piperideine-6-carboxylate dehydrogenase (P6Cdh) involved in cephamycin C biosynthesis in *Streptomyces clavuligerus*. The gene encoding this enzyme (*pcd*) is located in the cephamycin C gene cluster of *S. clavuligerus*. Indeed, a P6C dehydrogenase was previously proposed to be involved in an alternative pathway for α-aminoadipic acid biosynthesis in *P. chrysogenum* [59]. A probable alanine transaminase ALA2 is included in spot 25P (2.06 times overrepresented). This protein catalyzes in a reversible manner the transfer of an amino group from L-alanine to α-ketoglutarate giving rise to pyruvate and L-glutamate. This enzyme is likely to transfer the amino group from alanine to α-ketoadipate to form α-aminoadipate, a transamination step of the lysine pathway that has not been characterized before. Spot 38P contains a probable cytosolic and/or peroxisomal aspartate transaminase Aat2, which is 2.32 times overrepresented. This enzyme catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate. This enzyme has been shown to be a peroxisomal enzyme in *Saccharomyces cerevisiae* and in plants under some nutritional conditions [60,61]. Although glutamate is not a direct penicillin precursor amino acid, it has been reported to induce penicillin biosynthesis [62] (see Discussion).

Some other enzymes involved in the metabolism of penicillin precursor amino acids are also induced by CPP/$CaCl_2$. Spot 21P includes a probable cystathione beta-synthase CysB and is 2.08 times overrepresented. This enzyme participates in the biosynthesis of cystathionine, a precursor of the cysteine in the ACV tripeptide. On the other hand, spot 19P (2.03 times overrepresented) contains a probable methylmalonate semialdehyde dehydrogenase (MMSDH), which is involved in the last steps of valine catabolism (namely the conversion of α-methylmalonyl semialdehyde into propionyl-CoA) and probably, also in a parallel reaction oxidizing methyl-α-aminoadipate semialdehyde (see Discussion).

Oxidative stress response: Spots 17P and 18P include protein species of a probable catalase Cta1P, which is 2.84- and 2.69-fold induced, respectively, after the addition of CPP/$CaCl_2$. It has been reported that this type of peroxisomal catalase mediates high resistance to fungal oxidative stress (see Discussion).

Peroxisomal or "microbody-associated" proteins: A very interesting observation is the finding that most of the proteins differentially represented in response to CPP/$CaCl_2$ are authentic peroxisomal matrix or microbody-associated proteins. Kiel and coworkers [38] identified in *P.chrysogenum* a large number of proteins that were purified by sucrose gradient centrifugation following peroxisome enrichment. Some of these proteins were identified biochemically and genetically because they contain the peroxisomal targeting sequences PTS1 or PTS2, whereas several other proteins lacked these sequences and are designated here as "microbody-associated". In our study, 7 spots overrepresented in the presence of CPP/$CaCl_2$ contain 4 different proteins (Table 1) that coincide with those known as real peroxisomal matrix proteins [38]. These proteins include the IAT, which is the last enzyme of the penicillin biosynthetic pathway. On the other hand, 23 differentially represented spots contain 16 different proteins identified by Kiel and coworkers [38] as "microbody-associated" proteins (Table 1). In this second group of proteins we found the IPN synthase, the second enzyme of the penicillin biosynthetic pathway, fact that provides support for the possible attachment of this enzyme to peroxisomes or secretory vesicles. This has been proposed recently in a penicillin high-producer strain of *P. chrysogenum*, where electron microscopy studies have revealed that IPN synthase is concentrated at the polyribosomes arranged in the peripheral cytoplasm and along the tonoplast as well as around the peroxisomes [63]. Other proteins of this second group include the protein encoded by an acetate-inducible gene (aciA), 2-methylisocitrate lyase Icl2, methylcitrate synthase McsA, alanine transaminase or methylmalonate semialdehyde dehydrogenase among others. The relevance of this finding in relation to penicillin biosynthesis is analyzed in detail in the Discussion section.

3. CPP and $CaCl_2$ increases the expression of the penicillin biosynthetic genes.

In order to confirm whether CPP/$CaCl_2$ plays a positive role in the expression of the three penicillin biosynthetic genes, the transcription pattern of *pcbAB, pcbC* and *penDE* was analyzed. Total RNA was extracted from samples taken at 60 h from *P. chrysogenum* Wisconsin 54-1255 cultured in MDFP with or without CPP/$CaCl_2$ supplementation and expression was analyzed by semiquantitative RT-PCR. Expression of the gene encoding γ-actin was used as internal control of the total RNA amount loaded in the gel. Results (Figure 5A) indicated that the addition of CPP/$CaCl_2$ produced

a significant increase in the expression of the three biosynthetic genes; 40% for *pcbAB*, 35% for *pcbC* and 48% for *penDE*.

The levels of the last enzyme involved in penicillin biosynthesis (IAT encoded by *penDE*) were quantified by inmunodetection. Proteins were extracted from samples taken at 60 h from *P. chrysogenum* Wisconsin 54-1255 cultured in MDFP with or without the addition of CPP/$CaCl_2$ and IAT levels were analyzed by western blot. The levels of α-tubulin were used as internal control of the total protein amount loaded in the gel. As seen in Figure 5B, IAT levels underwent a 2.5-fold increase due to CPP/$CaCl_2$ supplementation.

These results confirm that one of the main mechanisms triggered by CPP/$CaCl_2$ is related to the enhanced expression of the penicillin biosynthetic gene cluster, which is finally responsible for increased penicillin titers.

Discussion

The present work shows that Ca^{2+}, in addition to CPP, stimulates drastically (up to 5.1 fold) penicillin biosynthesis in *P. chrysogenum*. Proteomics studies were carried out in order to characterize the mechanism of action of Ca^{2+} (in the form of $CaCl_2$) and CPP. Essentially, we observed that there are at least seven different effects of the CPP/$CaCl_2$ on the proteome: 1) the first one is related to the formation of energy and NADH, required in large amounts for penicillin biosynthesis. This is the case of the formate dehydrogenase. Formate is known to serve as additional carbon source for penicillin biosynthesis [57] and therefore, this enzyme catabolizing the formation of CO_2 and NADH is critically important for optimizing penicillin biosynthesis; 2) a second group of proteins that are clearly overrepresented in response to the CPP/ $CaCl_2$ are enzymes related to glycolysis and methylcitrate cycle. The two enzymes of the glycolysis might serve to stimulate the synthesis of propionyl-CoA, which is required for the methylcitrate cycle; 3) the formation of α-ketoadipate and α-aminoadipate in relation to penicillin biosynthesis has remained obscure for many years. α-aminoadipate has been shown to be limiting for penicillin biosynthesis [64]. These two intermediates of penicillin biosynthesis are formed from the lysine pathway by condensation of the five carbon α-ketoglutarate with acetyl-CoA by the enzyme homocitrate synthase. Homocitrate is then converted to homoisocitrate, α-ketoadipate, and finally α-aminoadipate. However, the results of this work point out that the seven carbon

compounds, which are the direct precursors of the α-ketoadipate and α-aminoadipate, might also be formed by the methylcitrate pathway. The oxidative decarboxylation of α-methyl-dicarboxylic acid release CO_2 and forms α-ketoacids with one carbon less than the substrate. The seven carbon compound methylcitrate is formed by condensation of propionyl-CoA with oxalacetate in a cycle which is parallel to the citric acid cycle and both may share several enzymes, since some enzymes of the citric acid cycle and the methylcitrate cycle show broad substrate specificity and are active in both cycles [65]. Several of the enzymes overrepresented in the CPP/ $CaCl_2$-supplemented cultures correspond to methylcitrate synthases and other enzymes (e.g. a methylcitrate dehydratase) of the methylcitrate cycle. The role of the methylcitrate cycle in relation to secondary metabolism is still obscure. It has been reported that blockage of methylcitrate cycle inhibits polyketide production in *Aspergillus nidulans* [58], thus suggesting that it provides short chain fatty acids for polyketide biosynthesis. These authors also hypothesized that excess buildup of propionyl-Co in *mcsA* (encoding methylcitrate synthase) mutants also interferes with polyketide synthase activity. Earlier, it had been reported that propionate (100 mM) not only impaired hyphal growth of *A. nidulans*, but also synthesis of the green polyketide-derived pigment of the conidia [66]; 4) some aminases and aminotransferases (e.g. Pc12g09430 or Pc22g19440) that are overrepresented in the CPP/ $CaCl_2$ supplemented cultures are likely to be involved in the transfer of the amino group from alanine or aspartate to form glutamate. Although glutamate is not a penicillin precursor and does not modify the intracellular pools of α-aminoadipate and valine, a regulatory role has been proposed for L-glutamate, since this amino acid is able to induce the intracellular concentration of ACVS, thus contributing to increase penicillin titers [62]. Moreover, glutamic acid may serve as effector molecule in the regulation of the transamination reactions that convert α-ketoadipate to α-aminoadipate, the latter being one of the precursors in the formation of ACV. In this article we have also observed that transcription of the *pcbAB* gene (encoding ACVS) increases after CPP/$CaCl_2$ supplementation; 5) cystathione may represent an important intermediate metabolite that helps to maintain appropriate levels of cysteine pools, the latter amino acid being one of the penicillin building blocks for the penicillin intermediate tripeptide ACV. In fact, it has been observed that moderate expression of cystathionine-gamma-lyase, an enzyme that synthesizes cysteine from cystathionine, stimulates the biosynthesis of cephalosporin (another ACV-derived beta-lactam

antibiotic) in the filamentous fungus *Acremonium chrysogenum* [67]. In addition, the penicillin high-producing *P. chrysogenum* AS-P-78 strain showed increased levels of the cystathionine beta synthase [2]. It is well-known that one of the factors that greatly influences penicillin production is cysteine concentration [68] and therefore, the positive role exerted by CPP/$CaCl_2$ in cystathione biosynthesis may be related to the beneficial effect observed on penicillin production; 6) we have also observed that CPP/$CaCl_2$ stimulates the expression of MMSDH. Overrepresentation of this protein may increase the release of semialdehyde of α-methyladipate. Similar reactions are likely to be involved in the conversion of 2-methyl-isocitrate semialdehyde to α-ketoadipate. This enzyme removes a carboxylic COOH group adjacent to the 2-methyl residue and this reaction takes place also in the conversion of α-methylisocitrate to α-ketoadipate, which may be released as its CoA derivative by incorporation of CoA-SH during the decarboxylation reaction, as it also occurs with the MMSDH. α-ketoadipyl-CoA may be the authentic precursor of the side chain of IPN; 7) another overrepresented protein observed after CPP/$CaCl_2$ addition is a peroxisomal catalase, Cta1P. This enzyme is specifically induced under conditions of high peroxisomal activity [69] and mediates high resistance to oxidative stress in the fungal pathogen *Candida glabrata* [70]. Since the last step of penicillin biosynthesis takes place inside peroxisomes [19,71] and oxidative stress response has been one of the main adaptive modifications affecting high-producing strains [2], induction of Cta1P by CPP/$CaCl_2$ may be a mechanism positively contributing to boost penicillin titers.

It is known that secretion of penicillin in hyphae takes place largely in the subapical region, not far from the hyphal tip, but it does not occur in a significant extent in the old hyphae [72]. Proliferation of peroxisomes, which are required for efficient penicillin production, occurs at the tip of the growing hyphae [73]. This suggests that, as it occurs with the secretion of the extracellular enzymes, the subapical region, which is very rich in vesicles, plays an important role in the secretion of penicillin, and indicates that penicillin might be transported by specialized vesicles from peroxisomes to the membranes to be finally exported by an exocytosis process [19,74].

The finding that most of the proteins overrepresented in cultures supplemented with CPP/$CaCl_2$ correspond to authentic peroxisome matrix or to microbody-associated proteins is really interesting. It seems likely that the increase in Ca^{2+} concentration is involved in the formation of peroxisomal and vesicle proteins; therefore, this explains

the observed increase in peroxisomal proteins (Table 1). As shown in this table, most of the overrepresented proteins in the CPP/$CaCl_2$ cultures are microbody-associated proteins, which are likely to be located in vesicle systems that co-purify with authentic peroxisomal proteins.

Conclusions

In this study we shed light on the effect of Ca^{2+} and CPP on the production of penicillin. There is a strong stimulation of expression of the penicillin biosynthetic genes and of the formation of peroxisomal and microbody-associated proteins, including two enzymes for penicillin biosynthesis. Out of the 39 differentially represented proteins in cells supplemented with Ca^{2+} and CPP there are several enzymes related to the formation of the penicillin amino acids precursors and three enzymes of the methylcitrate cycle. Most of the overrepresented proteins belong either to the peroxisomal matrix, such as the catalase Cta1P, or are proteins associated to microbodies, which are known to co-purify with peroxisomes. Two of these proteins, the last enzymes of the penicillin pathway, IPNS and IAT, are located in the cytosol, associated with polyribosomes, and in the peroxisomes, respectively. The proteomics data are supported by transcriptional studies of the three genes involved in penicillin biosynthesis and by immunoblotting quantification of the IAT in gels. In summary, it seems that the primary effect of Ca^{2+} together with CPP (known to be involved in Ca^{2+} internalization), stimulates the formation of peroxisomes and microbodies, which are involved in transport of penicillin biosynthetic intermediates and finally in penicillin secretion.

Acknowledgements

We especially thank P. Liras (University of León, Spain) for valuable scientific discussions. We acknowledge the excellent technical assistance of B. Martín and J. Merino (INBIOTEC, Spain). R. Domínguez-Santos was granted a fellowship from Junta de Castilla y León (ORDEN EDU/1204/2010) cofinanced by the Fondo Social Europeo. This research did not receive any other specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] Houbraken J, Frisvad JC, Samson RA. Fleming's penicillin producing strain is not *Penicillium chrysogenum* but *P. rubens*. IMA Fungus 2011;2:87-95.

[2] Jami MS, Barreiro C, García-Estrada C, Martín JF. Proteome analysis of the penicillin producer *Penicillium chrysogenum*: characterization of protein changes during the industrial strain improvement. Mol Cell Proteomics 2010;9:1182–98.

[3] García-Estrada C, Martín JF. Penicillins. In: Martin JF, García-Estrada C, Zeilinger S, editors. Biosynthesis and Molecular Genetics of Fungal Secondary Metabolites, vol I. New York: Springer US; 2014, p.17-42.

[4] Martín JF, Liras P. Insights into the structure and Molecular Mechanisms of β-lactam synthesizing enzymes in fungi. In: Brahmachati G, Demain AL, Adrio JL, editors. Biotechnology of microbial enzymes. NewYork: Elsevier; 2016, p.215-41.

[5] Feng B, Friedlin E, Marzluf GA. A reporter gene analysis of penicillin biosynthesis gene expression in *Penicillium chrysogenum* and its regulation by nitrogen and glucose catabolite repression. Appl Environ Microbiol 1994;60:4432-9.

[6] Haas H, Marzluf GA. NRE, the major nitrogen regulatory protein of *Penicillium chrysogenum*, binds specifically to elements in the intergenic promoter regions of nitrate assimilation and penicillin biosynthetic gene clusters. Curr Genet 1995;28:177-83.

[7] Suárez T, Peñalva MA. Characterization of a *Penicillium chrysogenum* gene encoding a PacC transcription factor and its binding sites in the divergent *pcbAB-pcbC* promoter of the penicillin biosynthetic cluster. Mol Microbiol 1996;20:529-40.

[8] Kosalková K, Marcos AT, Fierro F, Hernando-Rico V, Gutiérrez S, Martín JF. A novel heptameric sequence (TTAGTAA) is the binding site for a protein required for high level expression of *pcbAB*, the first gene of the penicillin biosynthesis in *Penicillium chrysogenum*. J Biol Chem 2000;275:2423-30.

[9] Kosalková K, García-Estrada C, Ullán RV, Godio RP, Feltrer R, Teijeira F, Mauriz E, Martín JF. The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. Biochimie 2009;91:214-25.

[10] Martín JF. Molecular control of expression of penicillin biosynthesis genes in fungi: Regulatory proteins interact with a bi-directional promoter region. J Bacteriol 2000;182:2355-62.

[11] Brakhage AA, Spröte P, Al-Abdallah Q, Gehrke A, Plattner H, Tüncher A. Regulation of penicillin biosynthesis in filamentous fungi. Adv Biochem Eng Biotechnol 2004;88:45-90.

[12] Hoff B, Kamerewerd J, Sigl C, Mitterbauer R, Zadra I, Kürnsteiner H, Kück U. Two components of a velvet-like complex control hyphal morphogenesis, conidiophore development and penicillin biosynthesis in *Penicillium chrysogenum*. Eukaryot Cell 2010;9:1236-50.

[13] Domínguez-Santos R, Martín JF, Kosalková K, Prieto C, Ullán RV, García-Estrada C. The regulatory factor PcRFX1 controls the expression of the three genes of β-lactam biosynthesis in *Penicillium chrysogenum*. Fungal Genet Biol 2012;49:866-81.

[14] Domínguez-Santos R, García-Estrada C, Kosalková K, Prieto C, Santamarta I, Martín JF. PcFKH1, a novel regulatory factor from the forkhead family, controls the biosynthesis of penicillin in *Penicillium chrysogenum*. Biochimie 2015;115:162-76.

[15] Kopke K., Hoff B., Bloemendal S., Katschorowski A., Kamerewerd J., Kück U. Members of the *Penicillium chrysogenum* velvet complex play functionally opposing roles in the regulation of penicillin biosynthesis and conidiation. Eukaryot Cell 2013;12:299-310.

[16] Cepeda-García C, Domínguez-Santos R, García-Rico RO, García-Estrada C, Cajiao A, Fierro F, Martín JF. Direct involvement of the CreA transcription factor in penicillin biosynthesis and expression of the *pcbAB* gene in *Penicillium chrysogenum*. Appl Microbiol Biotechnol 2014;98:7113-24.

[17] Martín JF. Key role of LaeA and velvet complex proteins on expression of β-lactam and PR-toxin genes in *Penicillium chrysogenum*: cross-talk regulation of secondary metabolite pathways. J Ind Microbiol Biotechnol 2016.

[18] Evers ME, Trip H, van den Berg MA, Bovenberg RA, Driessen AJ. Compartmentalization and transport in beta-lactam antibiotics biosynthesis. Adv Biochem Eng Biotechnol 2004;88:111-35.

[19] Martín JF, Ullán RV, García-Estrada C. Regulation and compartmentalization of β-lactam biosynthesis. Microb Biotechnol 2010;3:285-99.

[20] van der Lende TR, van de Kamp M, Berg M, Sjollema K, Bovenberg RA, Veenhuis M, Konings WN, Driessen AJ. delta-(L-alpha-Aminoadipyl)-L-cysteinyl-D-valine synthetase, that mediates the first committed step in penicillin biosynthesis, is a cytosolic enzyme. Fungal Genet Biol 2002;37:49-55.

[21] Müller WH, van der Krift TP, Krouwer AJ, Wösten HA, van der Voort LH, Smaal EB, Verkleij AJ. Localization of the pathway of the penicillin biosynthesis in *Penicillium chrysogenum*. EMBO J 1991;10:489-95.

[22] Lendenfeld T, Ghali D, Wolschek M, Kubicek-Pranz EM, Kubicek CP. Subcellular compartmentation of penicillin biosynthesis in *Penicillium chrysogenum*. The amino acid precursors are derived from the vacuole. J Biol Chem 1993;268:665-71.

[23] Lamas-Maceiras M, Vaca I, Rodríguez E, Casqueiro J, Martín JF. Amplification and disruption of the phenylacetyl-CoA ligase gene of *Penicillium chrysogenum* encoding an aryl-capping enzyme that supplies phenylacetic acid to the isopenicillin N acyltransferase. Biochem J 2006;395:147-55.

[24] García-Estrada C, Vaca I, Fierro F, Sjollema K, Veenhuis M, Martín JF. The unprocessed preprotein form IAT^{C103S} of the isopenicillin N acyltransferase is transported inside peroxisomes and regulates its self-processing. Fungal Genet Biol 2008;45:1043-52.

[25] Yu ZL, Liu J, Wang FQ, Dai M, Zhao BH, He JG, Zhang H. Cloning and characterization of a novel CoA-ligase gene from *Penicillium chrysogenum*. Folia Microbiol (Praha) 2011;56:246-52.

[26] Fernández-Aguado M, Teijeira F, Martín JF, Ullán RV. A vacuolar membrane protein affects drastically the biosynthesis of the ACV tripeptide and the beta-lactam pathway of *Penicillium chrysogenum*. Appl Microbiol Biotechnol 2013;97:795-808.

[27] Fernández-Aguado M, Martín JF, Rodríguez-Castro R, García-Estrada C, Albillos SM, Teijeira F, Ullán RV. New insights into the isopenicillin N transport in *Penicillium chrysogenum* Metabolic Engineering 2014;22:89-103.

[28] Fernández-Aguado M, Ullán RV, Teijeira F, Rodríguez-Castro R, Martín JF. The transport of phenylacetic acid across the peroxisomal membrane is mediated by the PaaT protein in *Penicillium chrysogenum*. Appl Microbiol Biotechnol 2013;97:3073-84.

[29] Yang J, Xu X, Liu G. Amplification of an MFS transporter encoding gene *penT* significantly stimulates penicillin production and enhances the sensitivity of *Penicillium chrysogenum* to phenylacetic acid. J Genet Genomics 2012;39:593-602.

[30] Andrade AC, Van Nistelrooy JG, Peery RB, Skatrud PL, De Waard MA. The role of ABC transporters from *Aspergillus nidulans* in protection against cytotoxic agents and in antibiotic production. Mol Gen Genet 2000;263:966-77.

[31] García-Estrada C, Barreiro C, Jami MS, Martín-González J, Martín JF. The inducers 1,3-diaminopropane and spermidine cause the reprogramming of metabolism in *Penicillium chrysogenum*, leading to multiple vesicles and penicillin overproduction. J Proteomics 2013;85:129-59.

[32] Sirikantaramas S, Yamazaki M, Saito K. Mechanisms of resistance to self-produced toxic secondary metabolites in plants. Phytochem Rev 2008;7:467–477.

[33] Hoppert M, Gentzsch C, Schorgendorfer K. Structure and localization of cyclosporin synthetase, the key enzyme of cyclosporin biosynthesis in *Tolypocladium inflatum*. Arch Microbiol 2001;176:285–93.

[34] Hong SY, Linz JE. Functional expression and subcellular localization of the aflatoxin pathway enzyme Ver-1 fused to enhanced green fluorescent protein. Appl Environ Microbiol 2008;74:6385–96.

[35] Hong SY, Linz JE. Functional expression and sub-cellular localization of the early aflatoxin pathway enzyme Nor-1 in *Aspergillus parasiticus*. Mycol Res 2009;113:591–601.

[36] Lee LW, Chiou CH, Klomparens KL, Cary JW, Linz JE. Subcellular localization of aflatoxin biosynthetic enzymes Nor-1, Ver-1, and OmtA in time-dependent fractionated colonies of *Aspergillus parasiticus*. Arch Microbiol 2004;181:204–14.

[37] Martín JF, Ullán RV, García-Estrada C. Role of peroxisomes in the biosynthesis and secretion of β-lactams and other secondary metabolites. J Ind Microbiol Biotechnol 2012;39:367-82.

[38] Kiel JA, van den Berg MA, Fusetti F, Poolman B, Bovenberg RA, Veenhuis M, van der Klei IJ. Matching the proteome to the genome: the microbody of penicillin-producing *Penicillium chrysogenum* cells. Funct Integr Genomics 2009;9:167-84.

[39] Case RM, Eisner D, Gurney A, Jones O, Muallem S, Verkhratsky A. Evolution of calcium homeostasis: from birth of the first cell to an omnipresent signaling system. Cell Calcium 2007;42:345–50.

[40] Clapham DE. Calcium signaling. Cell 2007;131:1047–58.

[41] Pittman JK. Vacuolar Ca(2+) uptake. Cell Calcium 2011;50:139-46.

[42] Martín JF. Calcium-containing phosphopeptides pave the secretory pathway for efficient protein traffic and secretion in fungi. Microb Cell Fact 2014;13:117.

[43] Kitts DD, Yuan YV. Caseinophosphopeptides and calcium bioavailability. Trends Food Sc Technol 1992;3:31–35.

[44] Kosalková K, García-Estrada C, Barreiro C, Flórez MG, Jami MS, Paniagua MA, Martín JF. Casein phosphopeptides drastically increase the secretion of extracellular proteins in *Aspergillus awamori*. Proteomics studies reveal changes in the secretory pathway. Microb Cell Fact 2012;11:5.

[45] Fierro F, Montenegro E, Gutiérrez S, Martín JF. Mutants blocked in penicillin biosynthesis show a deletion of the entire penicillin gene cluster at a specific site within a conserved hexanucleotide sequence. Appl Microbiol Biotechnol 1996;44:597–604.

[46] Casqueiro J, Gutiérrez S, Bañuelos O, Hijarrubia MJ, Martín JF. Gene targeting in *Penicillium chrysogenum*: disruption of the *lys2* gene leads to penicillin overproduction. J Bacteriol 1999;181:1181-8.

[47] Fernández FJ, Cardoza RE, Montenegro E, Velasco J, Gutiérrez S, Martín JF. The isopenicillin N acyltransferases of *Aspergillus nidulans* and *Penicillium chrysogenum* differ in their ability to maintain the 40-kDa alphabeta heterodimer in an undissociated form. Eur J Biochem 2003;270:1958-68.

[48] Martínez-Gomariz M, Perumal P, Mekala S, Nombela C, Chaffin WL, Gil C. Proteomic analysis of cytoplasmic and surface proteins from yeast cells, hyphae, and biofilms of *Candida albicans*. Proteomics 2009;9:2230–52.

[49] Vasco-Cárdenas MF, Baños S, Ramos A, Martín JF, Barreiro C. Proteome response of Corynebacterium glutamicum to high concentration of industrially relevant C4 and C5 dicarboxylic acids. J Proteomics 2013;85:65–88.

[50] Bland JM, Altman DG. Multiple significance tests: the Bonferroni method. BMJ 1995;310:170.

[51] Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 2004;25:1327-33.

[52] Havlis J, Thomas H, Sebela M, Shevchenko A. Fast-response proteomics by accelerated in-gel digestion of proteins. Anal Chem 2003;75:1300–6.

[53] Caspi R, Altman T, Billington R, Dreher K, Foerster H, Fulcher CA, Holland TA, Keseler IM, Kothari A, Kubo A, Krummenacker M, Latendresse M, Mueller LA, Ong Q, Paley S, Subhraveti P, Weaver DS, Weerasinghe D, Zhang P, Karp PD. The MetaCycdatabase of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. Nucleic Acids Res 2014;42:D459-71.

[54] El-Marsafy M, Abdel-Akher M, El-Saied H. Effect of media composition on the penicillin production. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg 1977;132:117-22.

[55] Hessler RJ, Blackwood RA, Brock TG, Francis JW, Harsh DM, Smolen JE. Identification of glyceraldehyde-3-phosphate dehydrogenase as a Ca2+-dependent fusogen in human neutrophil cytosol. J Leukoc Biol 1998;63:331-6.

[56] Tisdale EJ. Glyceraldehyde-3-phosphate dehydrogenase is required for vesicular transport in the early secretory pathway. J Biol Chem 2001;276:2480-6.

[57] Harris DM, van der Krogt ZA, van Gulik WM, van Dijken JP, Pronk JT. Formate as an auxiliary substrate for glucose-limited cultivation of *Penicillium chrysogenum*: impact on penicillin G production and biomass yield. Appl Environ Microbiol 2007;73:5020-5.

[58] Zhang YQ, Keller NP. Blockage of methylcitrate cycle inhibits polyketide production in *Aspergillus nidulans*. Mol Microbiol 2004;52:541-50.

[59] Esmahan C, Alvarez E, Montenegro E, Martin JF. Catabolism of lysine in *Penicillium chrysogenum* leads to formation of 2-aminoadipic acid, a precursor of penicillin biosynthesis. Appl Environ Microbiol 1994;60:1705-10.

[60] Verleur N, Elgersma Y, Van Roermund CW, Tabak HF, Wanders RJ. Cytosolic aspartate aminotransferase encoded by the AAT2 gene is targeted to the peroxisomes in oleate-grown *Saccharomyces cerevisiae*. Eur J Biochem 1997;247:972-80.

[61] Gebhardt JS, Wadsworth GJ, Matthews BF. Characterization of a single soybean cDNA encoding cytosolic and glyoxysomal isoenzymes of aspartate aminotransferase. Plant Mol Biol 1998;37:99-108.

[62] Lara F, del Carmen Mateos R, Vázquez G, Sánchez S. Induction of penicillin biosynthesis by L-glutamate in *Penicillium chrysogenum*. Biochem Biophys Res Commun 1982;105:172-8.

[63] Kurzątkowski W, Staniszewska M, Bondaryk M, Gębska-Kuczerowska A. Compartmentalization in penicillin G biosynthesis by *Penicillium chrysogenum* PQ-96. Pol J Microbiol 2014;63:399-408.

[64] Hönlinger C, Kubicek CP. Regulation of delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine and isopenicillin N biosynthesis in *Penicillium chrysogenum* by the alpha-aminoadipate pool size. FEMS Microbiol Lett 1989;53:71-5.

[65] Kobayashi K, Hattori T, Honda Y, Kirimura K. Gene identification and functional analysis of methylcitrate synthase in citric acid-producing *Aspergillus niger* WU-2223L. Biosci Biotechnol Biochem 2013;77:1492-8.

[66] Brock M, Fischer R, Linder D, Buckel W. Methylcitrate synthase from *Aspergillus nidulans*: implications for propionate as an antifungal agent. Mol Microbiol 2000;35:961-73.

[67] Kosalková K, Marcos AT, Martín JF. A moderate amplification of the *mecB* gene encoding cystathionine-gamma-lyase stimulates cephalosporin biosynthesis in *Acremonium chrysogenum*. J Ind Microbiol Biotechnol 2001;27:252-8.

[68] Nasution U, van Gulik WM, Ras C, Proell A, Heijnen JJ. A metabolome study of the steady-state relation between central metabolism, amino acid biosynthesis and penicillin production in *Penicillium chrysogenum*. Metab Eng 2008;10:10-23.

[69] Filipits M, Simon MM, Rapatz W, Hamilton B, Ruis H. A *Saccharomyces cerevisiae* upstream activating sequence mediates induction of peroxisome proliferation by fatty acids. Gene 1993;132:49-55.

[70] Cuéllar-Cruz M, Briones-Martin-del-Campo M, Cañas-Villamar I, Montalvo-Arredondo J, Riego-Ruiz L, Castaño I, De Las Peñas A. High resistance to oxidative stress in the fungal pathogen *Candida glabrata* is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. Eukaryot Cell 2008;7:814-25.

[71] Müller WH, Bovenberg RA, Groothuis MH, Kattevilder F, Smaal EB, Van der Voort LH, Verkleij AJ. Involvement of microbodies in penicillin biosynthesis. Biochim Biophys Acta 1992;1116:210-3.

[72] Paul GC, Thomas CR. A structured model for hyphal differentiation and penicillin production using *Penicillium chrysogenum*. Biotechnol Bioeng 1996;51:558-72.

[73] Meijer WH, Gidijala L, Fekken S, Kiel JA, van den Berg MA, Lascaris R, Bovenberg RA, van der Klei IJ. Peroxisomes are required for efficient penicillin biosynthesis in *Penicillium chrysogenum*. Appl Environ Microbiol 2010;76:5702-9.

[74] Roze LV, Chanda A, Linz JE. Compartmentalization and molecular traffic in secondary metabolism: a new understanding of established cellular processes. Fungal Genet Biol 2011;48:35-48.

Figure captions

Figure 1. Benzylpenicillin biosynthetic pathway.

Figure 2. Schematic representation of the compartmentalization of the benzylpenicillin biosynthetic pathway in *P. chrysogenum*. α-AA: α-aminoadipic acid; ACV: δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine; ACVS: ACV synthetase; CoA: Coenzyme A; Cys: Cysteine; IAT: isopenicillin N acyltransferase; IPN: isopenicillin N; IPNS: IPN synthase; M: mitochondrion; N: nucleus; P: peroxisome; PAA: phenylacetic acid; PA-CoA: phenylacetyl CoA; PCL: phenylaectyl CoA ligase; PenG: benzylpenicillin; Val: Valine.

Figure 3. Comparative analysis of A) growth (represented as dry weight), B) volumetric production of total penicillins, and C) specific production of total penicillins, after the addition at inoculation time of 1% CPP, 0.25% $CaCl_2$, or a mixture of both (CPP + $CaCl_2$) to control cultures of *P. chrysogenum* Wisconsin 54-1255 (MDFP). Results are represented as the mean ± standard deviation from three independent experiments carried out in duplicate. Statistical significance by ANOVA test is represented above error bars as follows: *** ($0.001 \geq P$); ** ($0.01 \geq P > 0.001$); * ($0.05 \geq P > 0.01$).

Figure 4: A) PCA (principal component analysis) showing the clustering of the 8 individual Cy3- and Cy5-labeled DIGE spot maps in two groups differentiated by two-principal component. Each datum pointed in the PCA plots describes the global expression values for the subset of proteins whose ratios varied 2.0-fold or more (ANOVA $p > 0.05$) in one biological replicate. Description of symbols is shown in the top-right side for every condition [red: control; blue: CPP/$CaCl_2$ supplementation]. Shadowed regions clearly group the plot in the different analyzed conditions. B) Example of a 2D-DIGE gel. Some proteins related to increased penicillin titers after CPP/$CaCl_2$ spplementation are highlighted (see text). Cy3 (red spots) labels proteins under control condition, whereas Cy5 (green spots) stains those proteins representing the CPP/$CaCl_2$ supplementation condition. pI range (3-10 NL) and molecular weight (kDa) are shown. Spot names are in concordance with Table 1 and Supplementary Table S2.

Figure 5. Analysis of the expression of the penicillin biosynthetic genes after the addition of a mixture of 1% CPP and 0.25% $CaCl_2$ (CPP + $CaCl_2$) to cultures of *P.*

chrysogenum Wisconsin 54-1255 grown in MDFP medium. A) Relative IOD provided in the RT-PCR experiments by the penicillin biosynthetic genes. B) Relative IOD provided in the western blot experiments by the β-subunit of IAT. Results are represented as the mean ± standard deviation from three independent measurements and the statistical significance by ANOVA test was at least ($0.05 \geq P$). Control values were set to 100%.

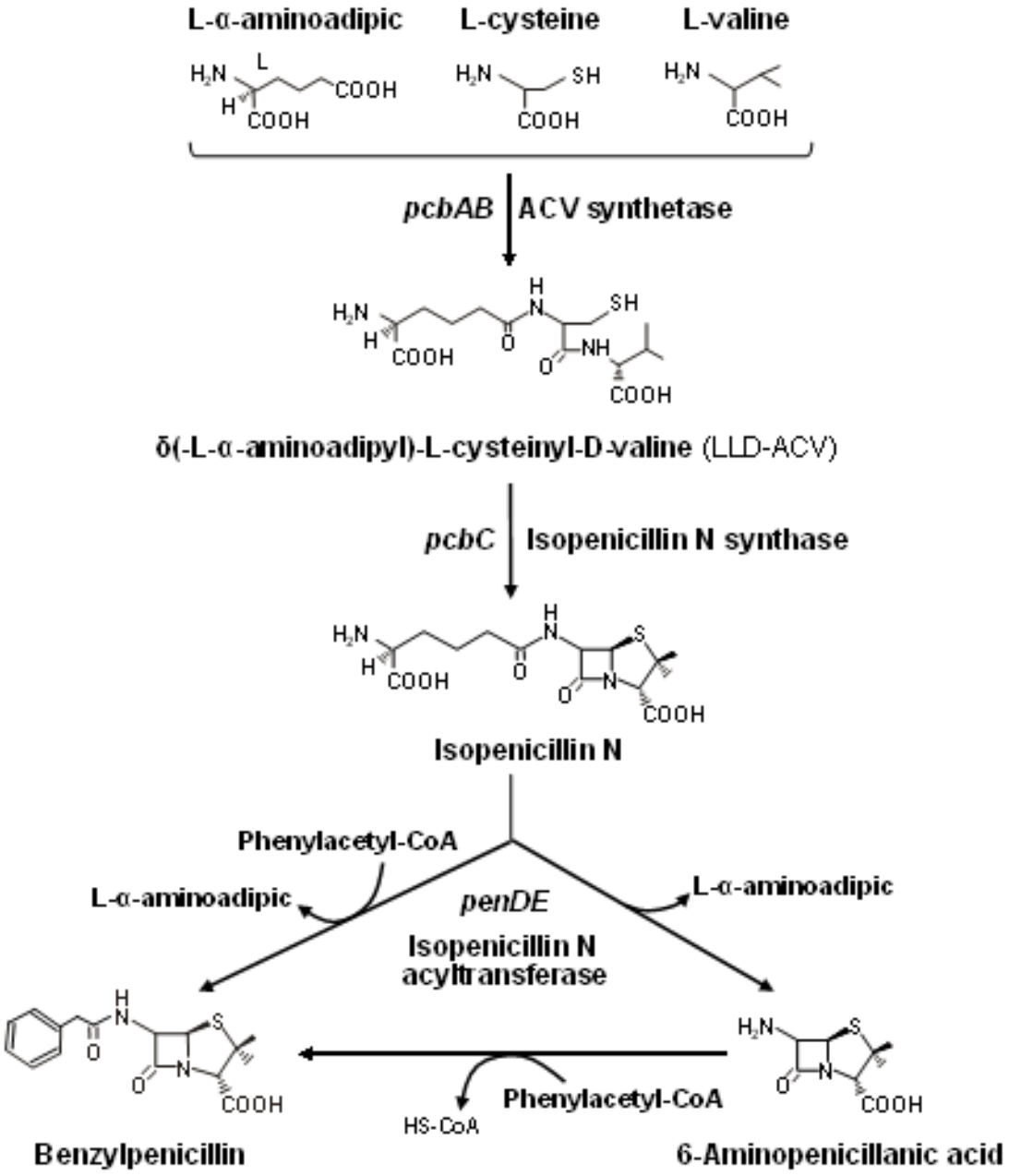


Figure 1. Domínguez-Santos et al.

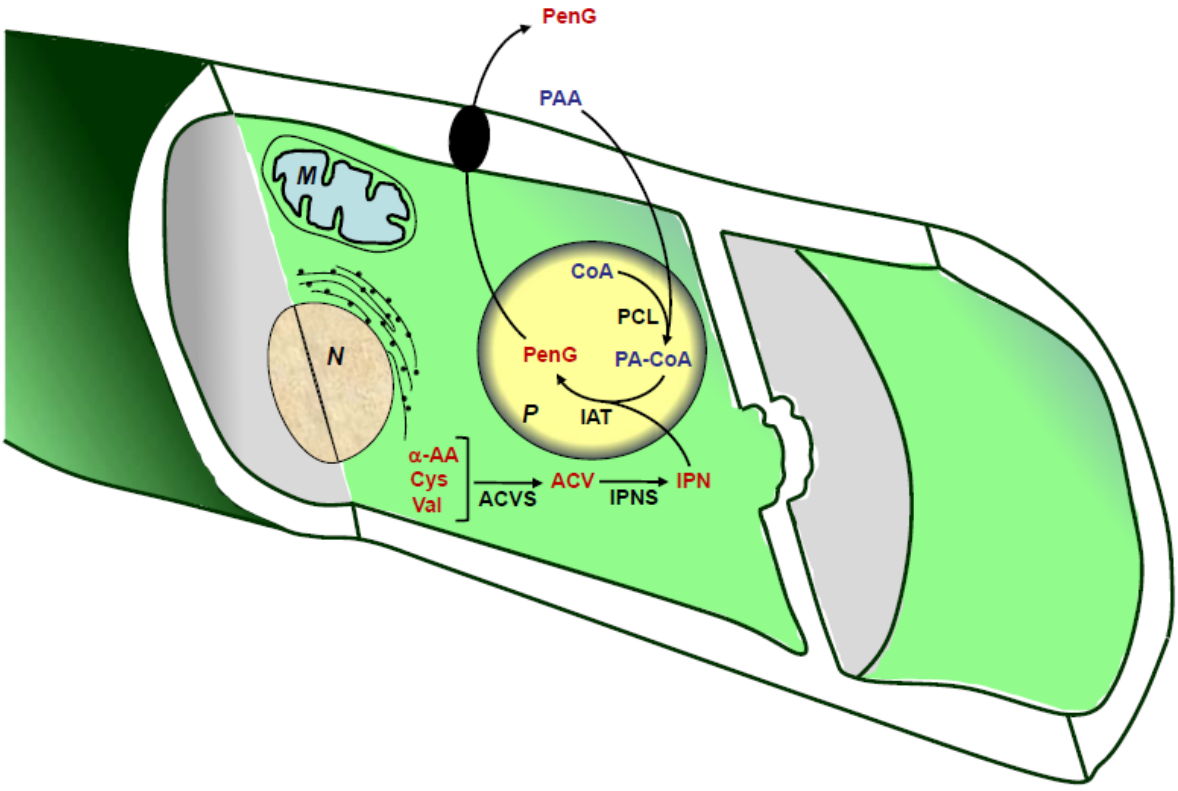


Figure 2. Domínguez-Santos et al.

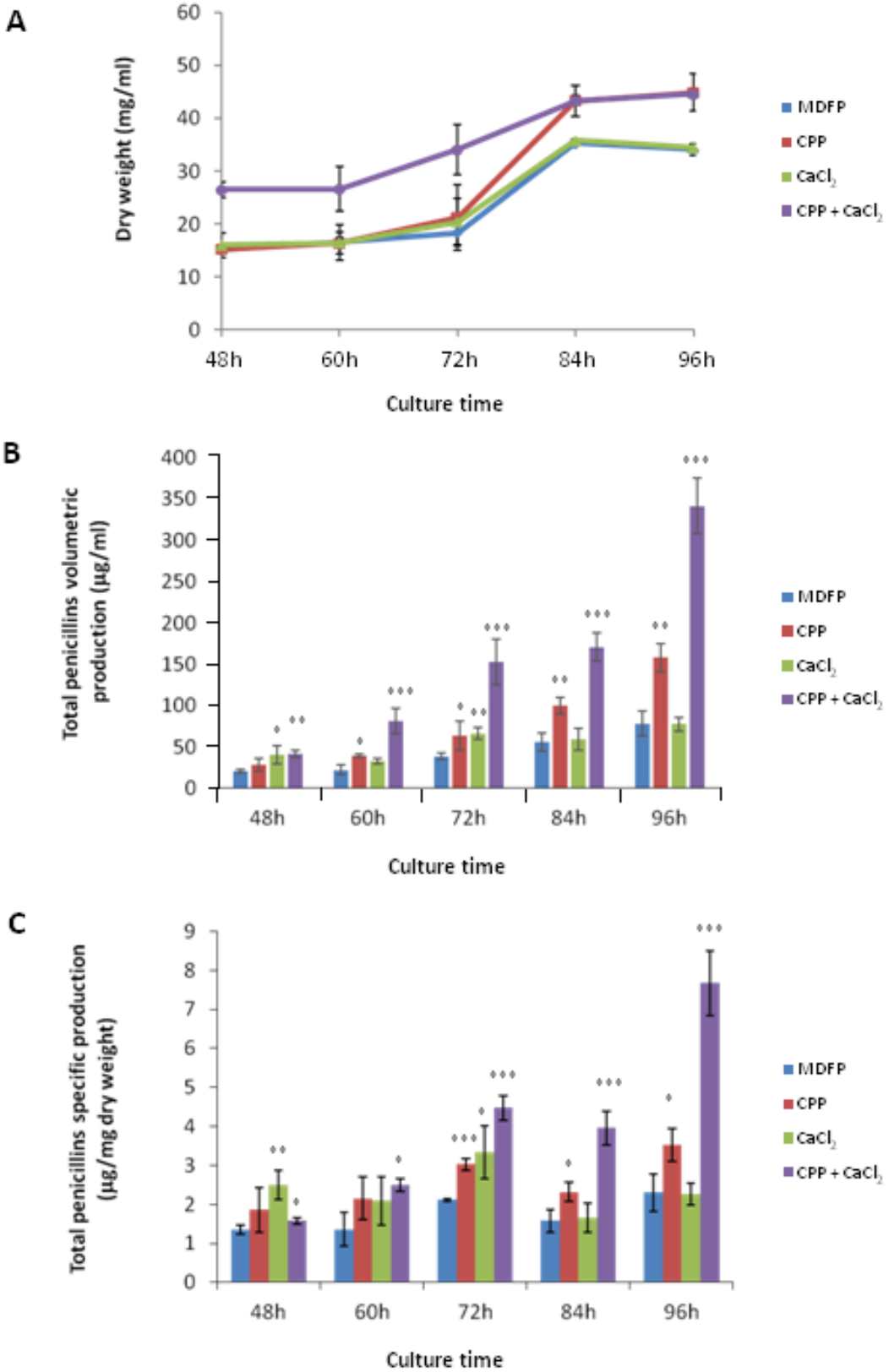


Figure 3. Domínguez-Santos et al.

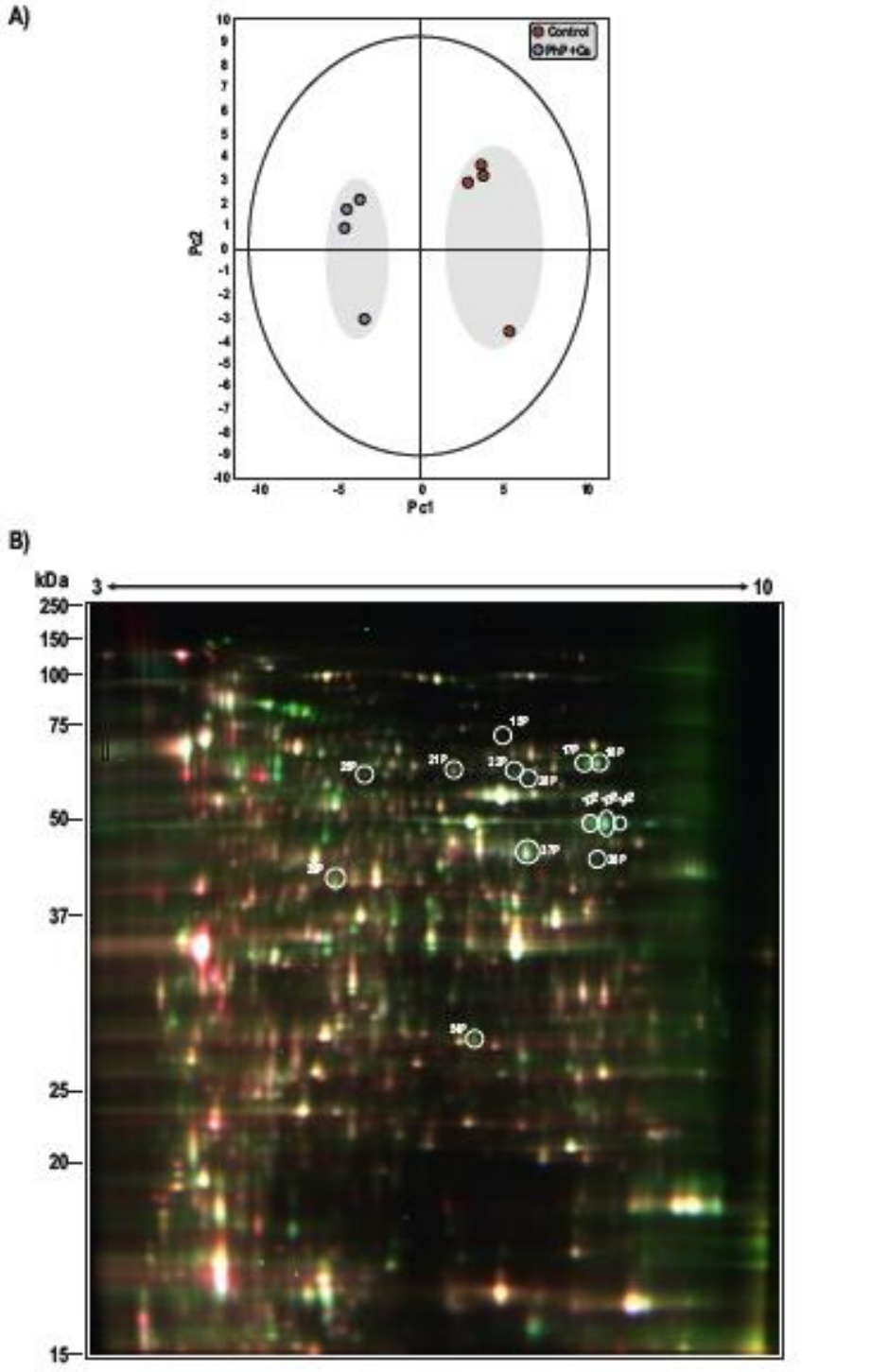


Figure 4. Domínguez-Santos *et al.*

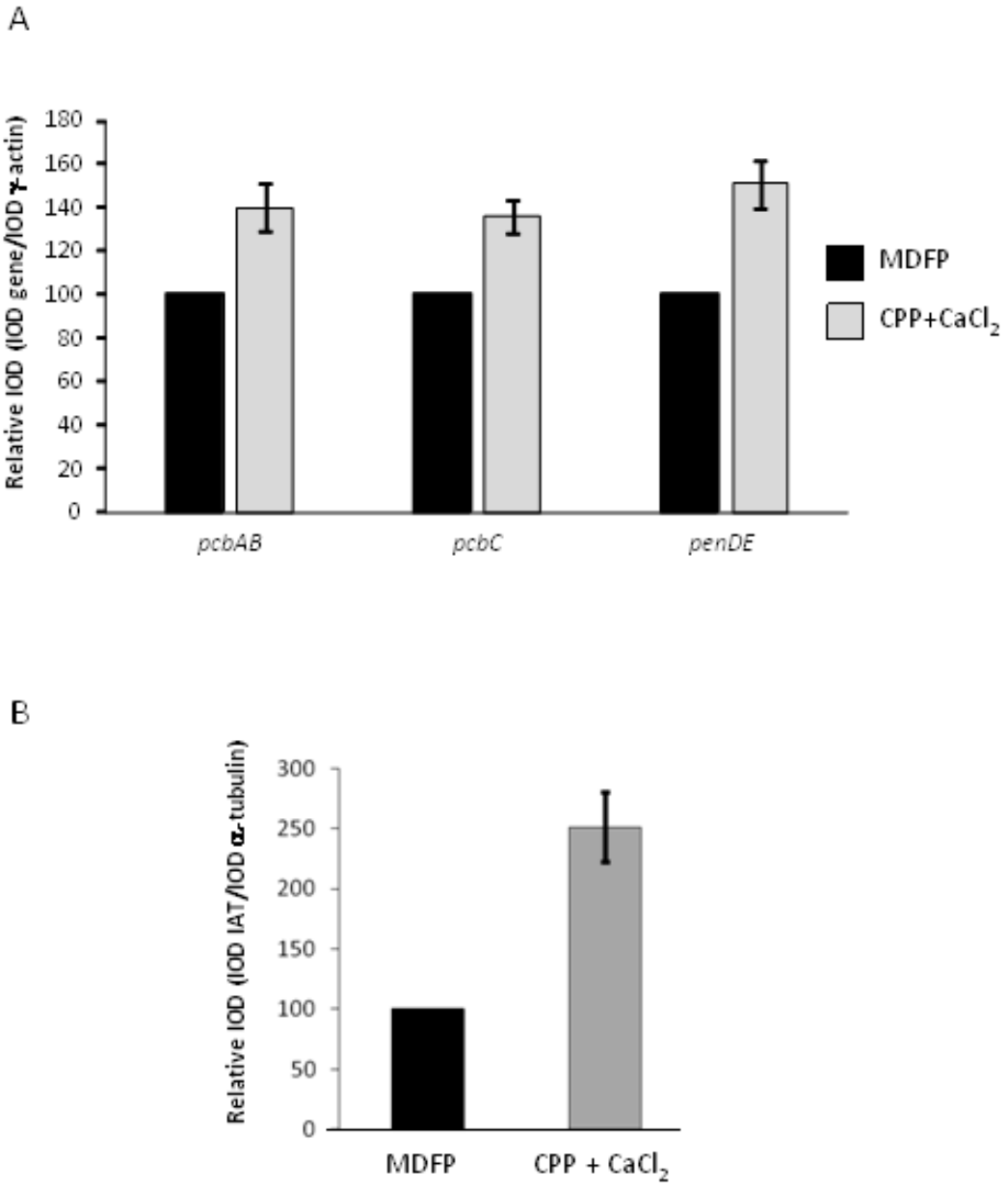


Figure 5. Domínguez-Santos et al.

Table 1. Spots identified by mass spectrometry due to their differential expression observed by 2D-DIGE with or without CPP/$CaCl_2$ supplementation. Spot name labels P or C mean up-regulated after CPP/$CaCl_2$ addition (P, green letters), or up-regulated under contol conditions (C, red letters), respectively. Those proteins located within peroxisomes or associated with microbodies are indicated in the table.

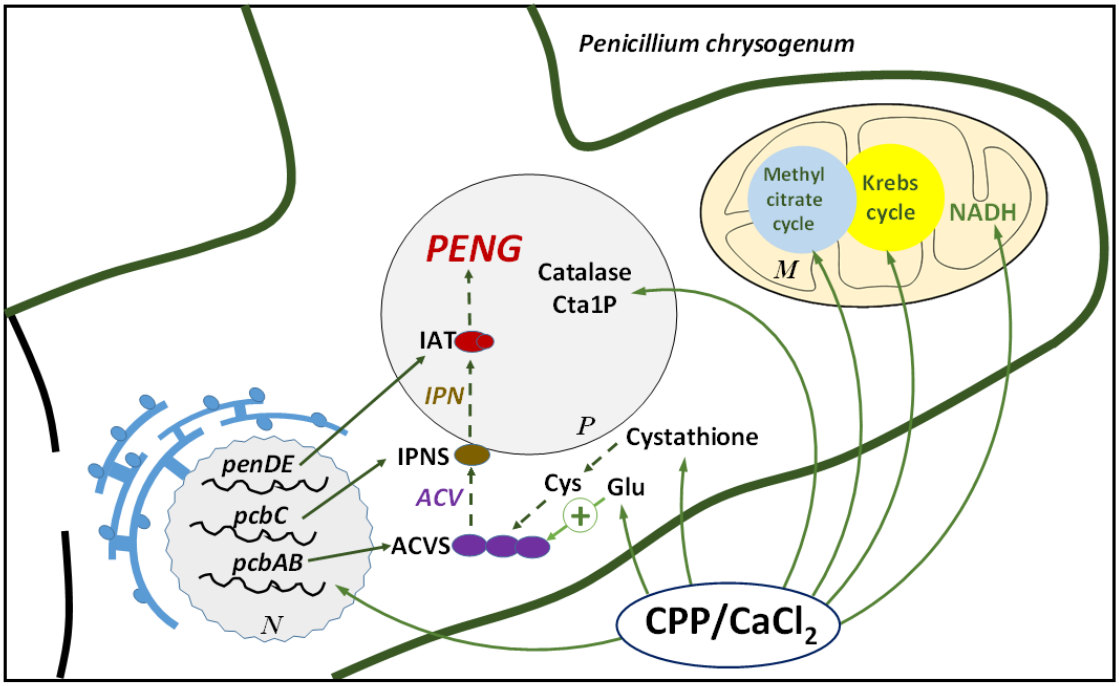
Spot	ORF	Similarity	Fold change	P-value	Peroxisomal (P) or microbody-associated (MA)[a]
1P	Pc12g11270	strong similarity to hypothetical protein contig1492_0.tfa_1860cg - Aspergillus fumigatus	**2.32**	0.012	-
2C	Pc18g02900	lysophospholipase phospholipase B plb1-Penicillium chrysogenum	**-2.21**	0.0095	-
3C	Pc16g11860	strong similarity to catalase R catR -Aspergillus niger	**-2.22**	0.009	-
4P	Pc22g19990	strong similarity to endonuclease SceI 75 kDa subunit Ens1 - Saccharomyces cerevisiae	**3.64**	0.024	MA
5P	Pc22g19990	strong similarity to endonuclease SceI 75 kDa subunit Ens1 - Saccharomyces cerevisiae	**3.51**	0.0092	MA
6P	Pc22g19990	strong similarity to endonuclease SceI 75 kDa subunit Ens1 - Saccharomyces cerevisiae	**3.73**	0.0063	MA
7C	Pc06g00710	strong similarity to 150 kDa oxygen regulated protein ORP150 - Rattus norvegicus	**-2.42**	0.011	MA
8P	Pc22g10220	strong similarity to dnaK-type molecular chaperone Ssb2 - Saccharomyces cerevisiae	**2.51**	0.024	MA
9P	Pc16g09030	strong similarity to polyadenylate-binding protein Pabp - Saccharomyces cerevisiae	**2.33**	0.017	MA
10P	Pc20g10240	strong similarity to serine-type carboxypeptidase precursor cpdS - Aspergillus phoenicis	**3.55**	0.009	-
11C	Pc22g09380	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - Aspergillus fumigatus	**-2.12**	0.0062	-

12P	Pc22g10020	strong similarity to alcohol dehydrogenase alkJ - Pseudomonas oleovorans	**3.07**	0.0098	-
13C	Pc22g09380	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - Aspergillus fumigatus	**-2.13**	0.0092	-
14P	Pc22g10020	strong similarity to alcohol dehydrogenase alkJ - Pseudomonas oleovorans	**2.40**	0.011	-
15P	Pc22g23150	strong similarity to 2-methylisocitrate lyase Icl2 - Saccharomyces cerevisiae	**5.23**	0.0091	MA
16P	Pc12g16040	strong similarity to phosphoglycerate mutase pgm - Bacillus subtilis	**2.54**	0.012	-
17P	Pc22g21240	strong similarity to catalase cta1p- Schizosaccharomyces pombe	**2.84**	0.01	P
18P	Pc22g21240	strong similarity to catalase cta1p- Schizosaccharomyces pombe	**2.69**	0.0095	P
19P	Pc22g02810	strong similarity to methylmalonate-semialdehyde dehydrogenase MMSDH - Rattus norvegicus	**2.03**	0.0092	MA
20C	Pc22g17420	strong similarity to hypothetical protein contig_1_153_scaffold_12.tfa_500cg - Aspergillus nidulans	**-3.13**	0.011	-
21P	Pc13g05320	strong similarity to hypothetical cystathione beta-synthase cysB - Dictyostelium discoideum	**2.08**	0.031	-
22P	Pc12g08900	strong similarity to delta-1-pyrroline-5-carboxylate dehydrogenase p5cdh - Homo sapiens	**3.30**	0.0062	-
23C	Pc22g17870	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - Schizosaccharomyces pombe	**-2.91**	0.0092	-
24C	Pc22g17420	strong similarity to hypothetical protein contig_1_153_scaffold_12.tfa_500cg - Aspergillus nidulans	**-2.92**	0.04	-
25P	Pc12g09430	strong similarity to alanine transaminase ALA2 - Hordeum vulgare	**2.06**	0.013	MA
26C	Pc22g17420	strong similarity to hypothetical protein contig_1_153_scaffold_12.tfa_500cg - Aspergillus nidulans	**-3.16**	0.012	-
27P	Pc20g08320	strong similarity to hypothetical protein FLJ10830 - Homo sapiens	**4.27**	0.0098	MA
28P	Pc20g13350	strong similarity to 2-methylcitrate dehydratase PrpD - Salmonella typhimurium	**3.18**	0.0092	-
29C	Pc22g17870	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - Schizosaccharomyces pombe	**-2.92**	0.0063	-
30P	Pc22g09630	strong similarity to hypothetical protein gi_19111905 - Schizosaccharomyces pombe	**2.94**	0.009	-

31P	Pc12g05400	strong similarity to citrate synthase citA -Aspergillus niger	**2.18**	0.018	P
32P	Pc20g13510	strong similarity to methylcitrate synthase mcsA - Aspergillus nidulans	**3.12**	0.012	MA
33P	Pc20g13510	strong similarity to methylcitrate synthase mcsA - Aspergillus nidulans	**3.73**	0.012	MA
34P	Pc20g13510	strong similarity to methylcitrate synthase mcsA - Aspergillus nidulans	**2.84**	0.012	MA
35P	Pc12g05400	strong similarity to citrate synthase citA -Aspergillus niger	**2.31**	0.011	P
36P	Pc21g01100	strong similarity to isovaleryl-coenzyme A dehydrogenase like protein An07g04280 - Aspergillus niger	**2.33**	0.012	-
37P	Pc12g04310	strong similarity to acetate-inducible gene aciA - Aspergillus nidulans	**2.23**	0.012	MA
38P	Pc22g19440	strong similarity to cytosolic aspartate transaminase Aat2 - Saccharomyces cerevisiae	**2.32**	0.03	P
39P	Pc21g21380	isopenicillin N synthase ips/PcbC-Penicillium chrysogenum	**2.18**	0.0092	MA
40P	Pc12g11200	strong similarity to Arp2/3 complex 34 kD subunit BCKDH - Gallus gallus	**2.36**	0.017	-
41P	Pc12g04750	strong similarity to malate dehydrogenase precursor MDH - Mus musculus	**3.49**	0.0062	P
42C	Pc21g14560	strong similarity to glyceraldehyde-3-phosphate dehydrogenase gpdA - Aspergillus niger	**-2.03**	0.019	MA
43C	Pc22g11240	strong similarity to heat shock protein 70 hsp70 - Ajellomyces capsulatus	**-2.18**	0.017	MA
44P	Pc16g09250	strong similarity to cytochrome-b5 reductase Mcr1 - Saccharomyces cerevisiae	**2.75**	0.022	-
45P	Pc16g09250	strong similarity to cytochrome-b5 reductase Mcr1 - Saccharomyces cerevisiae	**3.13**	0.018	-
46C	Pc22g11240	strong similarity to heat shock protein 70 hsp70 - Ajellomyces capsulatus	**-2.32**	0.0092	MA
47C	Pc22g19990	strong similarity to endonuclease SceI 75 kDa subunit Ens1 - Saccharomyces cerevisiae	**-3.63**	0.0062	MA
48C	Pc20g11630	gamma-actin act-Penicillium chrysogenum	**-2.00**	0.016	MA
49P	Pc21g02210	strong similarity to hypothetical protein contig31_part_ii.tfa_2190wg - Aspergillus fumigatus	**2.27**	0.013	-
50C	Pc13g03800	strong similarity to precursor of mitochondrial nuclease Nuc1 - Saccharomyces cerevisiae	**-2.06**	0.01	-
51C	Pc20g11630	gamma-actin act-Penicillium chrysogenum	**-2.44**	0.0063	MA
52C	Pc12g05480	similarity to core protein II of ubiquinol--cytochrome c reductase CAA42214.1 - Bos primigenius taurus	**-2.32**	0.013	MA

53C	Pc12g05480	similarity to core protein II of ubiquinol--cytochrome c reductase CAA42214.1 - Bos primigenius taurus	**-2.23**	0.03	MA
54P	Pc21g21370	acyl-coenzyme A:isopenicillin N acyltransferase (acyltransferase) AAT/PenDE- Penicillium chrysogenum	**2.07**	0.037	P

[a] **According to data reported by Kiel et al., 2009**



Graphical abstract

Highlights

- Casein phosphopeptides (CPP) and calcium ($CaCl_2$) increase penicillin production.
- These compounds exert a synergistic effect at different subcellular levels.
- The expression of penicillin biosynthetic genes is strongly stimulated by CPP/$CaCl_2$.
- Most of the proteins induced by CPP/$CaCl_2$ are peroxisomal or microbody-associated.