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TESIS DOCTORAL

Caracterización de nuevos reguladores de los genes de biosíntesis de penicilina en *Penicillium chrysogenum*

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Memoria presentada por Rebeca Domínguez Santos
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A mi familia

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Abreviaturas y acrónimos

1,3-DAP: 1, 3-diaminopropano

6-APA: ácido 6-aminopenicilánico

ACV: δ-(L-α-aminoadipil)-L-cisteinil-D-valina

ACVS: ACV sintasa

ADN: ácido desoxirribonucleico

AMPc: adenosín monofosfato cíclico

ARN: ácido ribonucleico

ARNi: ARN de interferencia

ARNm: ARN mensajero

ARNr: ARN ribosomal

ATP: adenosín trifosfato

bHLH: región básica hélice-vuelta-hélice

Bis-ACV: ACV en su forma oxidada

CoA: coenzima A

CPC: cefalosporina C

CSL: líquido de maceración del maíz

C-terminal: carboxi-terminal

Cys: L-cisteina

DAC-AT: deacetilcefalosporina acetiltransferasa

DAC: desacetilcefalosporina C

DAOC: desacetoxicefalosporina C

EH: deacetoxicefalosporina sintasa (expandasa/hidroxilasa)

EMSA: ensayo de retraso de la movilidad electroforética

FHA: dominio de asociación a *forkhead*

FKH: dominio C-terminal de unión al ADN

GDP: guanosín difosfato

GTP: guanosín trifosfato

His: L-Histidina

i.e.: es decir

IAT: IPN aciltransferasa

IPN: isopenicilina N

IPNS: IPN sintasa

kDa: kilodaltons

kpb: kilopares de bases

MFS: Major Facilitator Superfamily

mM: miliMolar

NADPH: nicotinamida adenina dinucleótido fosfato

nm: nanométros

nt: nucleótido

N-terminal: amino-terminal

ORF: marco de lectura abierto

p. ej.: por ejemplo

PAA: ácido fenilacético

pb: pares de bases

PBPs: proteínas de unión a penicilina

PCL, PhlA: fenilacetil-CoA ligasa

PenG: penicilina G

PenN: penicilina N

PPTasa: 4`-fosfopantenteinil transferasa

proIAT: proaciltransferasa de IPN

TrxAB: tiorredoxina disulfuro reductasa dependiente de NADPH

Val: L-valina

αAA: L-α-aminoadípico

Índice

1. Introducción

1.1. Introducción a los antibióticos β-lactámicos

El descubrimiento de los antibióticos es uno de los hitos más transcendentes no sólo de la historia de la medicina, sino también de la historia de la humanidad, puesto que con ellos se han podido hacer frente a las enfermedades infecciosas reduciendo drásticamente el índice de mortalidad.

Algunos hongos filamentosos y bacterias producen compuestos orgánicos bioactivos de bajo peso molecular que a bajas concentraciones, inhiben selectivamente el crecimiento de microorganismos, pudiendo considerarse a estos compuestos como antibióticos.

Los antibióticos son considerados metabolitos secundarios, ya que no son necesarios para el crecimiento, desarrollo o reproducción de los microorganismos que los producen. La función biológica de estos compuestos, en los microorganismos productores, se atribuye a que confieren una ventaja ecológica frente a otros microorganismos cuando deben competir por los nutrientes del medio ambiente (Martín y Demain, 1980).

1.2. Clasificación y estructura química de los antibióticos β-lactámicos

Los antibióticos β-lactámicos, grupo de antibióticos en los que se engloban las penicilinas y cefalosporinas, son moléculas peptídicas con una estructura química común, consistente en un anillo β-lactámico de 4 átomos (3 átomos de carbono y 1 átomo de nitrógeno) constituido por la condensación no ribosómica de tres aminoácidos: L-valina, L-cisteína y ácido L-α-aminoadípico. A diferencia de las monolactamas, que sólo presentan el anillo β-lactámico, estos compuestos están formados por un sistema bicíclico, siendo la estructura de este segundo anillo la que permite su clasificación en penicilinas, cefalosporinas, clavamas y carbapenemas,

además de las anteriormente mencionadas monolactamas (Aharonowitz y col., 1992) (Tabla 1).

En el caso de las penicilinas, el núcleo central es el ácido 6-aminopenicilánico (6-APA), formado por el anillo β-lactámico unido a un segundo anillo tiazolidínico. En cambio, las cefalosporinas y las cefamicinas contienen como núcleo central el ácido 7-aminocefalosporánico formado por el anillo β-lactámico acoplado a un anillo dihidrotiazínico (Figura 1).

Los antibióticos β-lactámicos poseen cadenas laterales que confieren un carácter hidrofóbico o hidrofílico. Las penicilinas con cadena lateral hidrofóbica (p. ej. la bencilpenicilina o penicilina G y la fenoximetilpenicilina o penicilina V presentan una cadena lateral de ácido fenilacético y ácido fenoxiacético, respectivamente) son producidas exclusivamente por hongos filamentosos del género *Penicillium* (*Penicillium. chrysogenum*, *Penicillium nalgiovense*) y *Aspergillus* (*Aspergillus nidulans*). Por el contrario, las penicilinas hidrofílicas (p. ej. la isopenicilina N, presenta cadena lateral de ácido L-α-aminoadípico) son sintetizadas por hongos filamentosos (*Acremonium chrysogenum*), actinobacterias (*Streptomyces* sp.) y algunas bacterias Gram negativas. Las cefalosporinas son producidas por hongos (*A. chrysogenum*, *Paecilomyces persicinus*, *Kallichroma tethys* y algunos otros deuteromicetos), actinomicetos Gram positivos (*Streptomyces clavuligerus* o *Nocardia lactamdurans*) y bacterias Gram negativas (*Lysobacter lactamgenus*) (Liras, 1999; Brakhage y col., 2009; Martín y col., 2010).

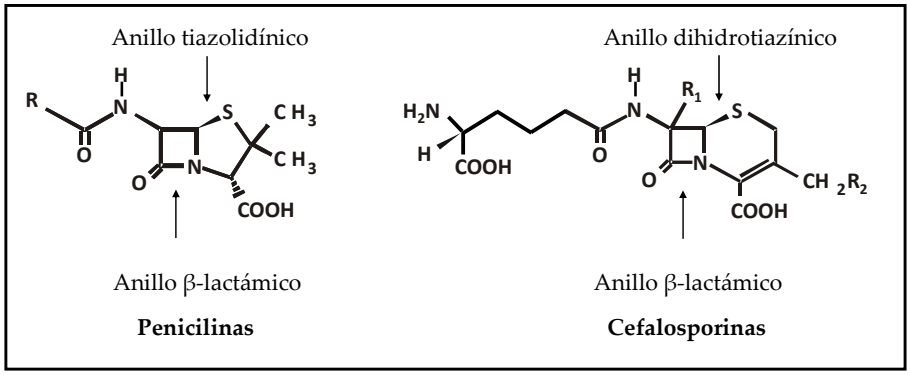
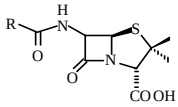
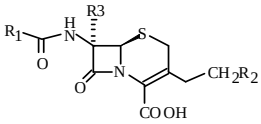
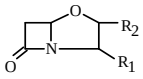
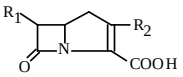
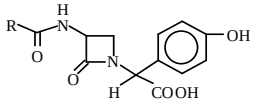
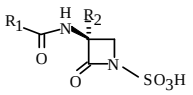


Figura 1. Estructura química general de penicilinas y cefalosporinas.

Tabla 1. Clasificación de los antibióticos β-lactámicos según su estructura química y principales microorganismos productores.

ESTRUCTURA BÁSICA	ANTIBIÓTICO	PRINCIPALES MICROORGANISMOS PRODUCTORES
 **Penam**	Penicilinas	*Penicillium chrysogenum* *Aspergillus nidulans* *Acremonium chrysogenum* *Streptomyces clavuligerus*
 **Cef-3-em**	Cefalosporinas Cefamicinas	*Acremonium chrysogenum* *Nocardia lactamdurans* *Streptomyces clavuligerus*
 **Clavam**	Ácido clavulánico	*Streptomyces clavuligerus*
 **Carbapenem**	Tienamicinas Ácidos oliváncos Epitienamicinas	*Streptomyces cattleya* *Streptomyces olivaceus* *Streptomyces flavogriseus*
	Nocardicinas	*Nocardia uniformis* *sub sp. tsuyamenensis*
 **Monobactam**	Monobactamas	*Gluconobacter* sp. *Achromobacter violaceum* *Agrobacterium radiobacter* *Pseudomonas acidophila* *Pseudomonas mesoacidophila* *Acetobacter* sp.

R, R1 y R2 simbolizan los distintos sustituyentes posibles.

Algunas penicilinas (penicilina F y K) son producidas de forma natural por los microorganismos que las sintetizan en ausencia de precursores específicos de la cadena lateral. Si se añade al medio de cultivo un precursor determinado de la cadena lateral se producirá de forma mayoritaria un tipo de penicilina, las cuales se denominan biosintéticas, siendo las más importantes la penicilina G (ácido fenilacético como cadena lateral) y penicilina V (ácido fenoxiacético como cadena lateral). A partir de estas penicilinas biosintéticas se obtiene de forma química o enzimática el núcleo 6-APA escindiendo la cadena lateral e incorporando mediante procesos químicos otras cadenas laterales obteniéndose las penicilinas semisintéticas, las cuales se pueden agrupar en 5 categorías: penicilinas antiestafilocócicas (nafcilina), aminopenicilinas (ampicilina, amoxicilina), carboxipenicilinas (carbenicilina), ureidopenicilinas (piperacilina) y mezcla de penicilinas con inhibidor de β-lactamasas (p. ej. amoxicilina-ácido clavulánico) (Oshiro, 1999).

1.3. Mecanismo de acción de los antibióticos β-lactámicos

El mecanismo de acción de los antibióticos β-lactámicos está relacionado con su estructura química. Estos antibióticos son agentes bactericidas que bloquean el entrecruzamiento de las distintas cadenas del peptidoglicano, que es un componente indispensable de la pared celular bacteriana.

Los antibióticos β-lactámicos se unen de manera covalente al centro activo de las proteínas llamadas PBPs (Penicillin Binding Proteins o proteínas de unión a penicilina), principalmente DD-transpeptidasa (PBP3) y DD-carboxipeptidasas (PBP4, PBP5 y PBP6), las cuales son responsables de la última etapa de la biosíntesis de la pared celular bacteriana. Este mecanismo de acción es debido a la estructura de los antibióticos β-lactámicos, la cual es similar a los dos últimos aminoácidos acil-D-alanina-D-alanina (sustrato natural de las PBPs) del pentapéptido que une las moléculas de peptidoglicano. Como consecuencia de esta unión, las PBPs son inhibidas irreversiblemente, lo que provoca que la pared celular se vuelva osmóticamente sensible, con la consiguiente autolisis bacteriana (Giesbrecht y col.,

1991; Frère y col., 1993). Además, estos antibióticos desencadenan la activación de hidrolasas de la pared celular y de autolisinas, lo que conduce a la lisis celular (Kong y col., 2010).

De acuerdo al modelo de acción de los antibióticos β-lactámicos, la actividad de estos antibióticos es mayor frente a bacterias Gram positivas, ya que el peptidoglicano es el componente mayoritario de su pared celular y las PBPs están localizadas en la membrana citoplasmática expuesta al medio. Sin embargo, las PBPs en bacterias Gram negativas están presentes en el espacio perisplasmático protegido por la membrana externa, la cual actúa como una barrera para los antibióticos β-lactámicos. Este inconveniente ha sido superado con la incorporación de nuevas cadenas laterales al núcleo penam y cephem en las penicilinas y cefalosporinas semisintéticas.

1.4. Ruta de biosíntesis de los antibióticos β-lactámicos

La biosíntesis de penicilinas, cefalosporinas y cefamicinas comienza con la condensación no ribosómica de los aminoácidos precursores (ácido L-α-aminoadípico, L-cisteína y L-valina) dando lugar al tripéptido δ-(L-α-aminoadipil)-L-cisteinil-D-valina (LLD-ACV). Aunque inicialmente se postuló la necesidad de dos enzimas para llevar a cabo la condensación, se ha demostrado que las reacciones necesarias son catalizadas por la proteína multimodular y multidominio ACV sintetasa (ACVS), la cual lleva a cabo la activación con ATP de los tres aminoácidos y la isomerización de la L-valina a D-valina antes de formar el tripéptido y liberarlo gracias a su actividad tioesterasa (Wu y col., 2012). El tripéptido lineal (LLD-ACV) es posteriormente ciclado para formar isopenicilina N (IPN) mediante la enzima isopenicilina N sintasa (ciclasa). La IPN es el primer compuesto bioactivo de la ruta biosintética (Ramos y col., 1985). En la última etapa de la biosíntesis de penicilina se reemplaza la cadena lateral del ácido L-α-aminoadípico, por ácido fenilacético o ácido fenoxiacético, para dar lugar respectivamente, a la penicilina G o a la penicilina V. Esta reacción la lleva a cabo la acil-CoA:IPN aciltransferasa (IAT) (Alvarez y col., 1993) (Figura 2).

La biosíntesis de cefalosporinas a partir de la IPN se inicia con la isomerización de este compuesto por acción del sistema de dos proteínas, IPN sintetasa/epimerasa, pasando la cadena lateral de L-α-aminoadipil a su forma isomérica D y constituyendo la PenN. Posteriormente, el anillo tiazolidínico de cinco átomos de la PenN, es expandido para formar el anillo dihidrotiazínico de seis átomos de la desacetoxicefalosporina C (DAOC). A continuación, la DAOC es hidroxilada para dar lugar a la desacetilcefalosporina C (DAC). Mientras que estas dos reacciones de expansión e hidroxilación son llevadas a cabo en hongos (como *A. chrysogenum*) por la misma enzima bifuncional (DAOC sintasa/hidroxilasa), en procariotas ambas actividades están catalizadas por dos enzimas diferentes: una expandasa y una hidroxilasa (Samson y col., 1987). Finalmente, la formación de cefalosporina C tiene lugar por acetilación de la DAC, catalizada por la DAC acetiltransferasa (DAC-AT) (Figura 2).

En el caso de los actinomicetos productores de cefamicina, la DAC es modificada con la introducción de un grupo carbamoilo en la posición C-3′ y de un grupo metoxilo en la posición C-7 (Liras, 1999) (Figura 2).

1.4.1. Etapas enzimáticas iniciales comunes para la biosíntesis de penicilinas y cefalosporinas

Los microorganismos productores de antibióticos β-lactámicos tienen dos etapas enzimáticas en común, las cuales llevan a la biosíntesis de IPN, el primer compuesto activo de la ruta. Dependiendo de la presencia de enzimas específicas que llevan a cabo las etapas intermedias y finales, sintetizan diferentes antibióticos β-lactámicos.

La biosíntesis comienza en el citosol con la condensación no ribosómica de los aminoácidos ácido L-α-aminoadípico, L-cisteína y L-valina para dar lugar al tripéptido ACV. Esta reacción es catalizada por la ACVS (426 kDa), la cual posee un módulo para cada aminoácido. Cada módulo presenta las diferentes actividades catalíticas necesarias para el reconocimiento, activación del aminoácido precursor y formación de los enlaces peptídicos. Además de estos tres módulos, la ACVS contiene dos dominios adicionales, uno para la isomerización de la L-valina a

D-valina (dominio epimerasa) y otro para liberar el tripéptido formado (dominio tioesterasa) (Díez y col., 1990; Baldwin y col., 1991; Gutiérrez y col., 1991; De Crécy-Lagard y col., 1995; Stachelhaus y Marahiel, 1995; Martín, 2000a; Wu y col., 2012). La ACVS es sintetizada como una apoproteína inactiva cuya activación se logra mediante la adición de un radical de 4`-fosfopanteteína al dominio de tiolación de cada módulo. Esta reacción está catalizada por la enzima 4`-fosfopanteteinil transferasa (PPTasa). Por tanto, aunque la enzima PPTasa no participa directamente en la biosíntesis de ACV, sí es necesaria para la biosíntesis de penicilina tanto en *A. nidulans* (Lambalot y col., 1996; Keszenman-Pereyra y col., 2003; Márquez-Fernández y col., 2007) como en *P. chrysogenum* (García-Estrada y col., 2008a).

La enzima ACVS está codificada por el gen *pcbAB* (*acvA*) de 11 kpb, el cual no está interrumpido por ningún intrón (Díez y col., 1990; Smith y col., 1990) y se encuentra presente en la agrupación génica de penicilina, tanto de hongos como de bacterias, y de cefalosporina (Liras y col., 1998).

Durante la segunda etapa de la biosíntesis de penicilinas y cefalosporinas, cuatro átomos de hidrogeno del tripéptido ACV son eliminados consumiendo una molécula de oxígeno para formar la estructura bicíclica (núcleo penam) de la IPN, compuesto que constituye el punto de ramificación de la biosíntesis de penicilina y cefalosporina. Esta reacción está catalizada en el citosol por la IPN sintasa o ciclasa de 38 kDa (codificada por el gen sin intrones *pcbC*/*ipnA*), la cual es una dioxigenasa que requiere Fe^{2+} y oxígeno molecular como cofactores y ascorbato como donador de electrones (Perry y col., 1988; Bainbridge y col., 1992; Cooper, 1993).

Debido a que la IPN sintasa sólo acepta el tripéptido ACV monomérico reducido como sustrato (una vez que dicho tripéptido es sintetizado, las condiciones de aireación presentes en el medio de cultivo rápidamente oxidan el monómero a la forma disulfuro, formando el dímero bis-ACV) el bis-ACV oxidado tiene que ser previamente reducido antes de que pueda ser ciclado. La reducción de bis-ACV se lleva a cabo por otro sistema auxiliar (tiorredoxina disulfuro reductasa dependiente de NADPH (TrxAB)) (Cohen y col., 1994).

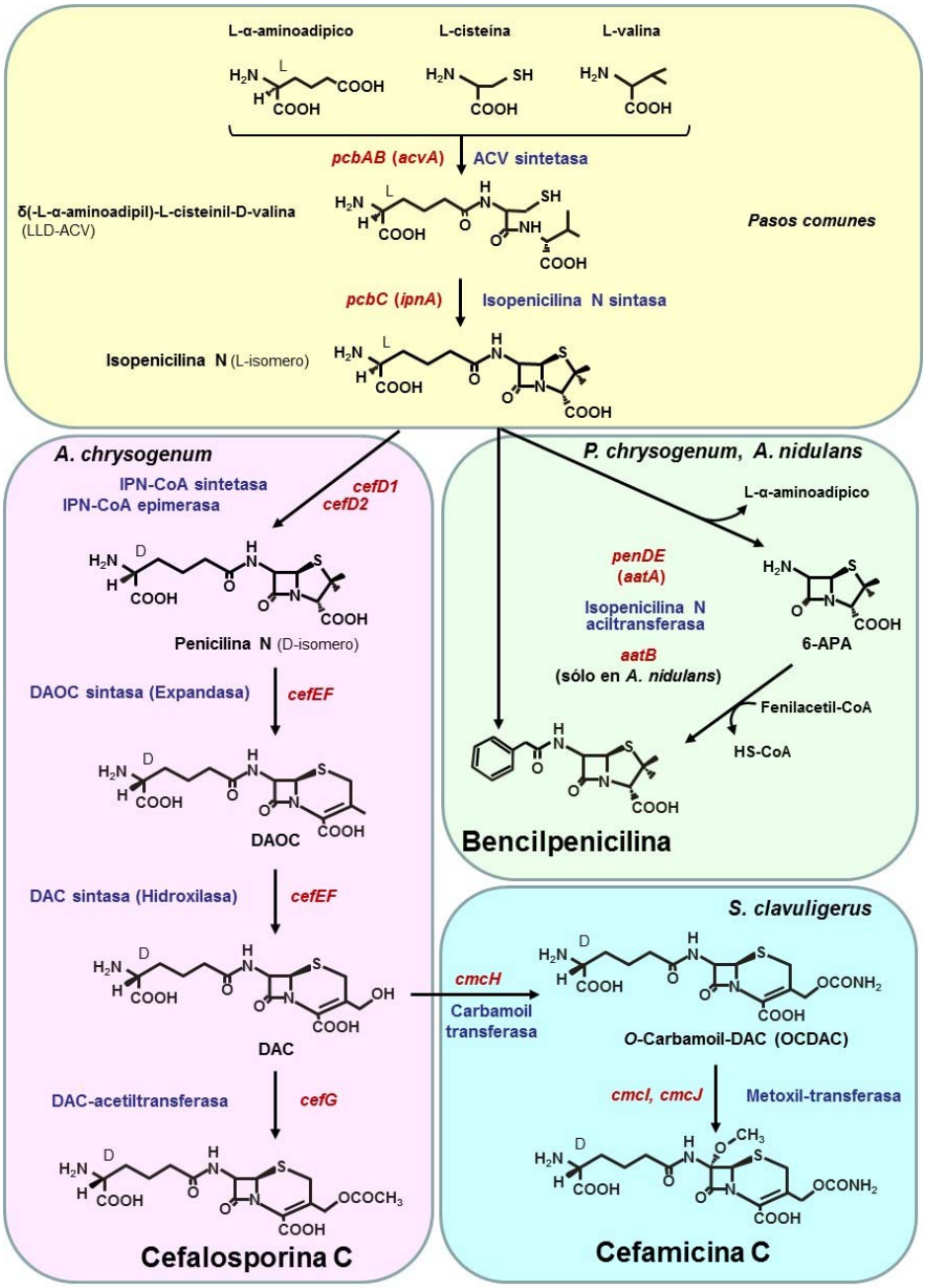


Figura 2. Ruta de biosíntesis de penicilina, cefalosporina C y cefamicina.

1.4.2. Etapa final para la biosíntesis de penicilinas

En aquellos hongos filamentosos que producen penicilinas hidrofóbicas (*P. chrysogenum* o *A. nidulans*), la IPN entra en el peroxisoma, lo cual implica la presencia de transportadores específicos en la membrana peroxisomal. En la matriz peroxisomal, se produce el intercambio de la cadena lateral de L-α-aminoadípico de la IPN por una molécula hidrofóbica activada en forma de acil-CoA. El reemplazamiento de la cadena lateral es catalizado por la IAT peroxisomal, la cual es codificada por el gen *penDE* (*aatA*). El gen *penDE*, a diferencia de los otros genes de la biosíntesis de penicilina (*pcbAB* y *pcbC*), contiene tres intrones y se traduce como una preproteína de 40 kDa, llamada proaciltransferasa o proIAT. La proaciltransferasa se procesa autocatalíticamente resultando un heterodímero activo que contiene dos subunidades (subunidad α de 11 kDa, correspondiente al fragmento N-terminal y subunidad β de 29 kDa, correspondiente a la región C-terminal) (Barredo y col., 1989; Whiteman y col., 1990; Tobin y col., 1990; 1993; García-Estrada y col., 2008b). Se ha propuesto un proceso de dos pasos enzimáticos para explicar la reacción catalizada por la IAT (Queener y Neuss, 1982; Alvarez y col., 1993). En primer lugar, una actividad amidohidrolasa elimina la cadena lateral de L-α-aminoadípico de la IPN generando 6-APA, y a continuación, una actividad acil-CoA:6-APA-aciltransferasa incorpora la nueva cadena lateral activada (Alvarez y col., 1993).

La activación de los precursores de la cadena lateral en sus correspondientes tioésteres es un prerrequisito para la incorporación de la cadena lateral acilo durante la biosíntesis de penicilinas hidrofóbicas. Por tanto, se requiere una actividad auxiliar acil-CoA ligasa en la matriz peroxisomal. De hecho, se identificó una actividad fenilacetil-CoA ligasa (PCL) en peroxisomas (Gledhill y col., 1997) y algunos años después se clonó el gen *phlA* en *P. chrysogenum*, el cual codifica una proteína con actividad PCL (Lamas-Maceiras y col., 2006). Un segundo gen *phlB*, implicado en la activación de ácido fenilacético, se ha clonado en *P. chrysogenum* (Wang y col., 2007). Sin embargo, estudios posteriores (Koetsier y col., 2009; 2010) han puesto en evidencia que la proteína codificada por este gen no interviene en la activación del ácido fenilacético (y por tanto en la biosíntesis de penicilina), ya que

codifica una acil-CoA ligasa de amplio espectro que activa ácido adípico. Otro estudio más reciente ha identificado en *P. chrysogenum* un tercer gen *phlC*, el cual codifica un proteína peroxisomal con actividad PCL (Yu y col., 2011).

A pesar de que a finales de los años 90 del siglo XX se tenía un concepto claro de las proteínas y genes implicados en la biosíntesis de penicilina en *P. chrysogenum* y *A. nidulans*, la secuenciación de los genomas de estos hongos filamentosos proporcionó la base para el descubrimiento de nuevos genes biosintéticos. En *A. nidulans* se identificó el gen *aatB*, un gen homólogo al gen *aatA* (que codifica la IAT), el cual no estaba localizado junto al resto de los genes de penicilina. La proteína codificada por este gen posee actividad aciltransferasa e interviene en la biosíntesis de penicilina en *A. nidulans* (Spröte y col., 2008). Por su parte, el análisis del genoma de *P. chrysogenum* reveló la presencia de un gen homólogo de *aatB* denominado *ial*, debido a que codifica una proteína con cierta similitud (IAL por IAT-like) a la IAT (García-Estrada y col., 2009). A diferencia del producto del gen *aatB* de *A. nidulans*, la IAL no tiene actividad relacionada con la biosíntesis de penicilina, lo que sugiere que los genes *ial* y *penDE* de *P. chrysogenum* podrían no haberse formado a partir de un mismo gen ancestral por duplicación génica. Por lo tanto, estos genes podrían no tener una función similar, sino distintas funciones dentro del organismo (García-Estrada y col., 2009).

Como se ha indicado anteriormente, el hecho de que la biosíntesis de penicilina se encuentre compartimentalizada entre el citosol y los peroxisomas, implica la presencia de transportadores para los precursores e intermediarios (Figura 3). En *P. chrysogenum*, se ha identificado y caracterizado una proteína de membrana vacuolar, denominada PenV, que pertenece a la familia de transportadores MFS (Major Facilitator Superfamily). PenV juega un papel importante en la formación del tripéptido ACV, probablemente debido a su función como una aminoácido permeasa, suministrando precursores desde el pool de aminoácidos de la vacuola (Fernández-Aguado y col., 2013a). Teniendo en cuenta que la IPN se sintetiza en el citosol y que el intercambio de la cadena lateral de L-α-aminoadípico de la IPN por una molécula hidrofóbica activada en forma de acil-CoA se produce en la matriz peroxisomal, debe existir un sistema transportador

específico para IPN a través de la membrana peroxisomal en *P. chrysogenum*. Esta función la lleva a cabo la proteína PenM, la cual pertenece al grupo de proteínas transportadoras de la superfamilia MFS (Fernández-Aguado y col., 2014).

También existe un sistema transportador de los precursores de la cadena lateral (ácido fenilacético y ácido fenoxiacético) desde el citosol a través de la membrana peroxisomal de *P. chrysogenum* (Figura 3). Este sistema está integrado por la proteína codificada por el gen *paaT*, la cual está implicada en la internalización del fenilacético en los peroxisomas (Fernández-Aguado y col., 2013b).

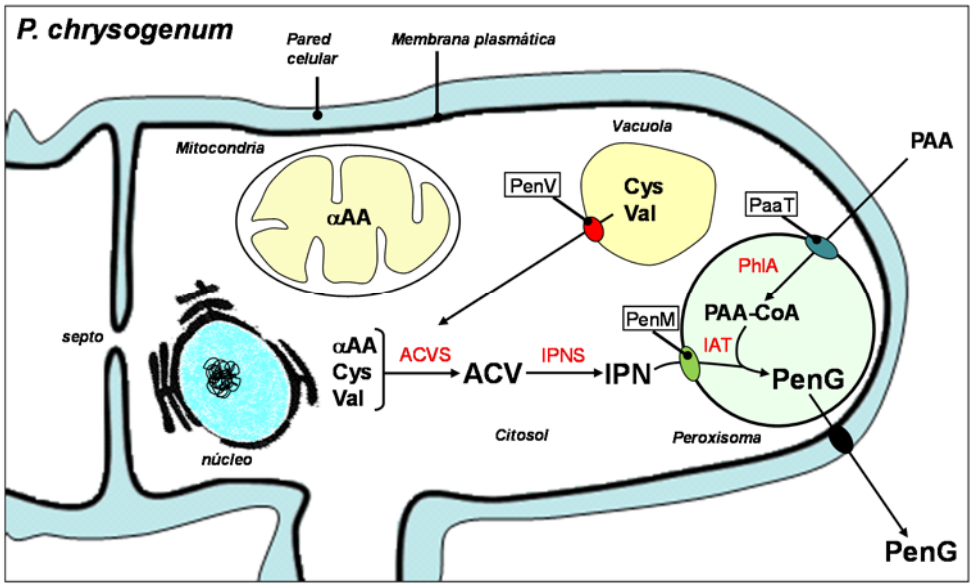


Figura 3. Compartimentalización de la ruta biosintética de penicilina. Representación esquemática mostrando las etapas enzimáticas y los orgánulos involucrados en la ruta biosintética de penicilina. ACV, δ-(L-α-aminoadipil)-L-cisteinil-D-valina. ACVS, ACV sinteta IPN, Isopenicilina N. IPNS, IPN sintasa. IAT, Isopenicilina N aciltransferasa. PhlA, fenilacetil-CoA ligasa. PAA-CoA, fenilacetil-CoA. αAA, L-α-aminoadípico. Cys, L-cisteína. Val, L-valina. PAA, ácido fenilacético. PenG, bencilpenicina.

1.4.3. Etapas enzimáticas intermedias para la biosíntesis de cefalosporinas

La etapa central de la ruta de biosíntesis de cefalosporinas consiste en la conversión de IPN a su D-isómero PenN (epimerización de la cadena lateral del ácido L-α-aminoadípico a D-α-aminoadípico), precursor de los antibióticos con núcleo cephem (p. ej. cephalosporinas y cefamicinas). En bacterias, esta reacción es llevada a cabo en un único paso por una epimerasa dependiente de piridoxal fosfato codificada por el gen *cefD*, localizado en la agrupación génica de cefamicina (Liras, 1999). En *A. chrysogenum*, la conversión de IPN en PenN está catalizada por un sistema de dos componentes, el cual está formado por las enzimas isopenicilinil N-CoA sintetasa (IPN-ACS) codificada por el gen *cefD1* (4 intrones), e isopenicilinil N-CoA epimerasa (IPN-ACE) codificada por el gen *cefD2* (1 intrón). Una tioesteresa, probablemente inespecífica, es también necesaria para liberar la PenN del CoA (Ullán y col., 2002a). El sistema CefD1-CefD2 parece encontrarse en peroxisomas, ya que ambas proteínas contienen una señal de localización peroxisomal. Esto podría implicar que, como fue indicado en *P. chrysogenum*, un sistema de transporte activo debe estar presente en la membrana peroxisomal para asegurar un flujo adecuado de IPN hacia el interior de los peroxisomas.

Después de la isomerización, la PenN debe ser transportada fuera de los peroxisomas para ser el sustrato de las siguientes enzimas de la ruta de biosíntesis de cefalosporinas. Una vez en el citosol, tiene lugar la expansión oxidativa del anillo tiazolidínico a un anillo dihidrotiazínico. Esta reacción es catalizada por la DAOC sintasa (expandasa), la cual es codificada por el gen *cefE* en bacterias y por el gen *cefEF* en *A. chrysogenum* (Liras, 1999). En la siguiente etapa, el grupo metilo del C-3′ (exocíclico) del DAOC es hidroxilado, formando DAC. Esta reacción es llevada a cabo por una DAC sintasa (hidroxilasa), codificada por el gen *cefF* en *S. clavuligerus* y por el propio gen *cefEF* en *A. chrysogenum*. Por tanto, en *A. chrysogenum*, a diferencia de lo que sucede en bacterias, ambas reacciones son catalizadas por la misma enzima DAOC sintasa (expandasa)/DAC sintasa (hidroxilasa) codificada por un único gen *cefEF* (Samson y col., 1987).

Teniendo en cuenta que: la IPN se sintetiza en el citosol, la epimerización ocurre en la matriz peroxisomal y la expansión-hidroxilación parece tener lugar en el citosol (Martín y col., 2010), debe existir un sistema transportador específico para ambos intermediarios biosintéticos (IPN y PenN) a través de la membrana peroxisomal. De hecho, dos transportadores de membrana peroxisomal (CefP y CefM) han sido identificados en *A. chrysogenum* (Teijeira y col., 2009; Ullán y col., 2010). CefP, codificado por el gen *cefP*, es una proteína de la membrana peroxisomal de 866 aminoácidos y con un peso molecular deducido de 99,2 kDa, probablemente implicada en el transporte de IPN al interior de los peroxisomas (Ullán y col., 2010). El gen *cefM* codifica el transportador CefM, el cual es una proteína de 482 aminoácidos (con un peso molecular deducido de 52,2 kDa) probablemente implicado en el transporte de PenN desde los peroxisomas al citosol, donde tiene lugar la conversión en cefalosporina C (Teijeira y col., 2009) (Figura 4).

1.4.4. Etapa enzimática final de la biosíntesis de cefalosporina C

La última etapa de la biosíntesis de cefalosporina C es la conversión de DAC en cefalosporina C. Esta reacción es catalizada por la DAC acetiltransferasa, la cual transfiere un grupo acetilo activado como acetil-CoA a la molécula de DAC. Esta enzima es codificada por el gen *cefG* (2 intrones). Al finalizar la biosíntesis, la cefalosporina C tiene que ser transportada al exterior celular. Se ha demostrado que otro transportador (CefT) puede estar implicado en este proceso. Aunque este transportador participa en la secreción de cefalosporina C (Ullán y col., 2002b), no es el principal transportador de este antibiótico y además está implicado en la secreción de β-lactamas hidrofílicas que contienen como cadena lateral el ácido α-aminoadípico (IPN y PenN) (Ullán y col., 2008; Nijland y col., 2008) (Figura 4).

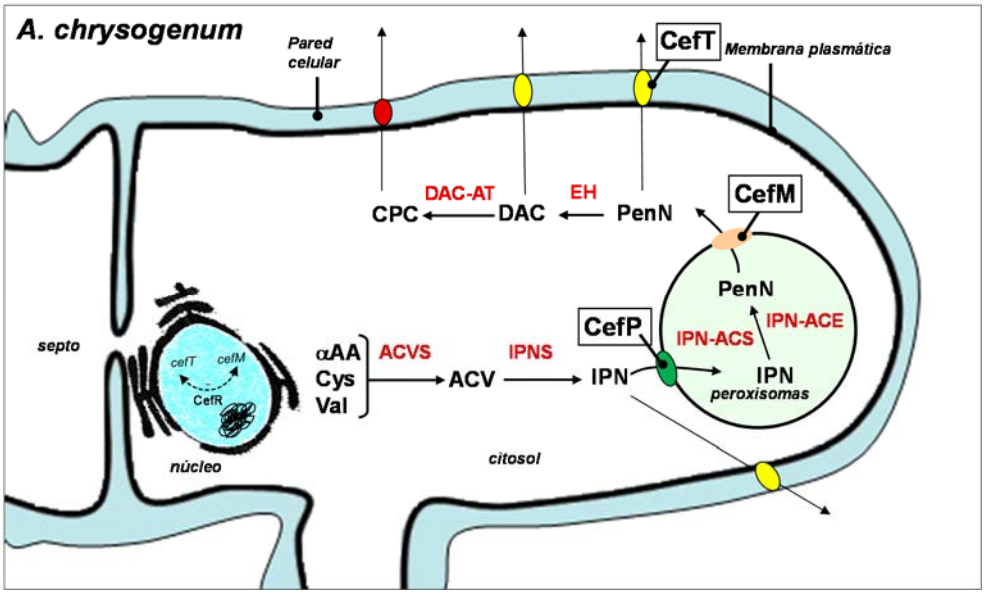


Figura 4. Modelo propuesto de la compartimentalización de la ruta biosintética de cefalosporinas en A. chrysogenum. ACV, δ-(L-α-aminoadipil)-L-cisteinil-D-valina. ACVS, ACV sintetasa. IPN, Isopenicilina N. IPNS, IPN sintasa. IPN-ACS, Isopenicilina N-CoA sintetasa IPN-ACE, Isopenicilina N-CoA epimerasa. PenN, Penicilina N. EH, desacetoxicefalosporina sintasa (expandasa/hidroxilasa). DAC, desacetilcefalosporina C. DAC-AT, desacetilcefalosporina acetiltransferasa. CPC, cefalosporina.

1.5. Organización de los genes implicados en la biosíntesis de penicilina y cefalosporina C

Los genes biosintéticos de penicilina y cefalosporina C están organizados en agrupaciones génicas. Esta organización génica es típica, tanto en hongos, como en bacterias.

En *P. chrysogenum* y *A. nidulans*, los genes *pcbAB-pcbC* (*acvA-ipnA*) están organizados en orientación divergente y comparten un promotor bidireccional, mientras el gen *penDE* (*aatA*) está localizado "corriente abajo" del gen *pcbC* (*ipnA*) (Liras y Martín, 2006). El *"cluster"* génico de penicilina se encuentra en el cromosoma I en *P. chrysogenum* (Fierro y col., 1993) y en el cromosoma VI en *A. nidulans* (Montenegro y col., 1992) (Figura 6). En el caso de la cepas de alta producción de penicilina en *P. chrysogenum*, la región que contiene los genes biosintéticos sufre una

amplificación en tándem, la cual contribuye a un incremento de la productividad (Fierro y col., 1995).

Como se ha mencionado con anterioridad, el genoma de *A. nidulans* contiene otro gen (*aatB*) que codifica una enzima que es capaz, del mismo modo que la IAT, de biosintetizar penicilina a partir de IPN. Sin embargo, dicho gen no está agrupado con el resto de los genes de penicilina (Brakhage y col., 2009).

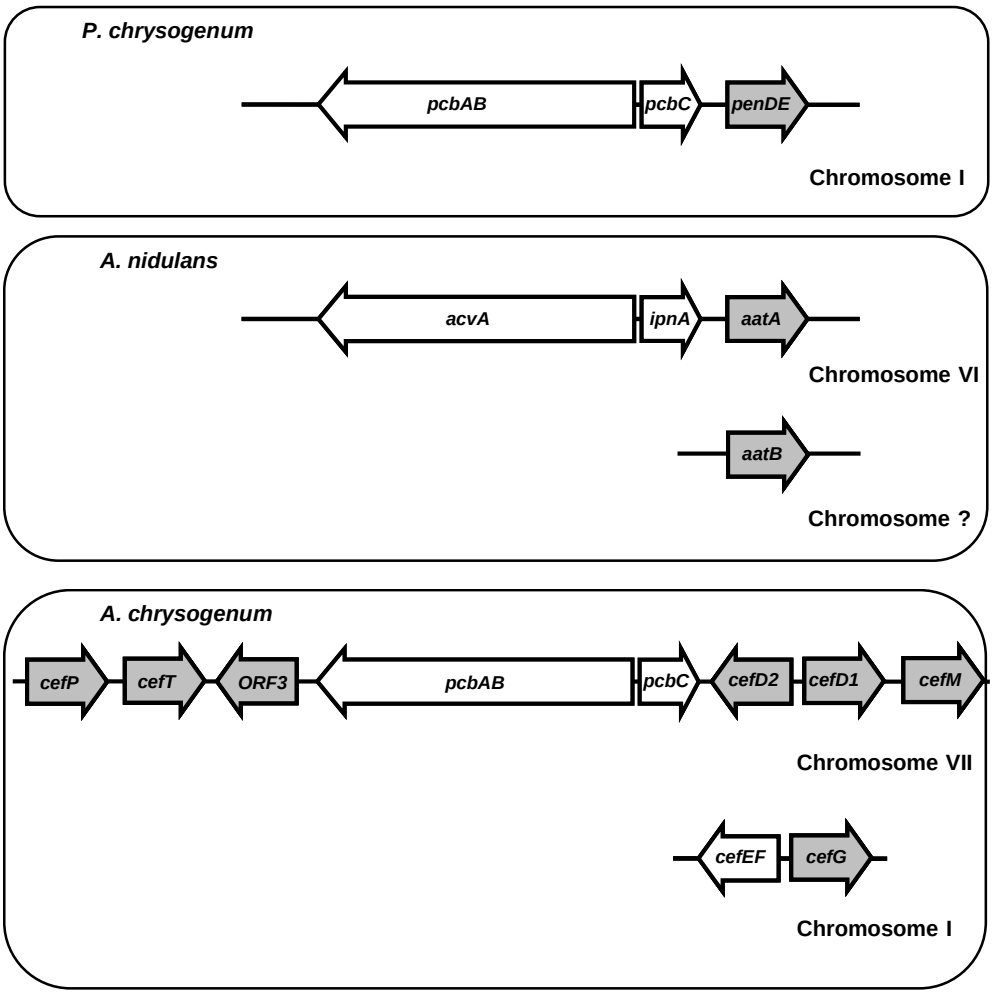


Figura 1. "*Clusters*" génicos para la biosíntesis de penicilina y cefalosporina C en hongos filamentosos. Genes con origen procariota representados en blanco, mientras que los genes con origen eucariota están en gris.

En *A. chrysogenum*, los genes de biosíntesis de cefalosporina C se organizan junto con los genes transportadores en dos "*clusters*" localizados en dos cromosomas diferentes (Figura 6). Los genes *pcbAB*, *pcbC*, *cefD1* y *cefD2*, junto con los genes de secreción/transporte *cefT*, *cefM* y *cefP* están agrupados en el llamado "*cluster*" temprano, ya que contiene toda la información genética para la biosíntesis y secreción de IPN y PenN (Martín y col., 2012). El resto de genes implicados en la biosíntesis de cefalosporinas, *cefEF* y *cefG*, están incluidos en el "*cluster*" tardío. En una cepa industrial de *A. chrysogenum* (394-4, Ely Lilly, USA), el "*cluster*" temprano está localizado en el cromosoma VI y el "*cluster*" tardío en el cromosoma II (Skatrud y Queener, 1989). Sin embargo, en *A. chrysogenum* C10 (ATCC 48272) existe una diferente localización para ambos "*clusters*": el "*cluster*" temprano se encuentra en el cromosoma VII, mientras que el "*cluster*" tardío está localizado en el cromosoma I (Gutiérrez y col., 1999a). Esta diferente localización cromosómica de las agrupaciones génicas de cefalosporinas indica que ha ocurrido un reordenamiento significativo de los cromosomas durante la mejora de las cepas.

1.6. Control y regulación de la biosíntesis de penicilinas y cefalosporinas en *P. chrysogenum* y *A. chrysogenum*

Aparte del interés científico, existe un gran interés desde el punto de vista industrial en dilucidar la regulación molecular de la biosíntesis de antibióticos β-lactámicos, ya que estos compuestos siguen siendo de los fármacos más importantes en términos de uso terapéutico y de volumen de producción.

Las cepas de *P. chrysogenum* de alta producción de penicilina derivan de la serie Wisconsin (procedente a su vez de la cepa NRRL 1951), desarrollada gracias al esfuerzo de varias instituciones americanas tales como las universidades de Wisconsin-Madison, Stanford, Minnesota y el Carnegie Institution en Cold Spring Harbor. A pesar de que las cepas han sufrido durante varias décadas mutaciones diversas con el fin de incrementar su producción (al menos en tres órdenes de magnitud desde el aislamiento de la cepa NRRL 1951), se conoce poco de los fundamentos moleculares que han convertido a este microorganismo en un gran

productor de antibióticos. Entre los datos publicados hasta la fecha, se sabe que la región en la que se hallan los tres genes biosintéticos de penicilina (*pcbAB*, *pcbC* y *penDE*) se encuentra amplificada en tándem en las cepas de gran producción de penicilina (Fierro y col., 1995; Fierro y col., 2006). Sin embargo, no ha sido hasta hace unos años cuando se han conocido en profundidad algunos de los mecanismos por los que *P. chrysogenum* se ha adaptado tan bien a las condiciones de producción industrial de penicilina. La secuenciación del genoma de la cepa Wisconsin 54-1255, junto con datos de transcriptómica, han revelado que los genes que codifican proteínas implicadas en la biosíntesis de aminoácidos precursores de la biosíntesis de penicilina, además de aquellos que codifican proteínas de los peroxisomas, tienen aumentada su tasa de expresión en las cepas de alta producción de penicilina (van den Berg y col., 2008).

Los datos de proteómica obtenidos en nuestro laboratorio corroboran estos resultados e indican que durante el proceso de mejora industrial de cepas ha ocurrido una compleja reorganización de distintos procesos metabólicos, dando lugar a una disminución de la biosíntesis de otros metabolitos secundarios diferentes a la penicilina y a un incremento de la biosíntesis de NADPH y del metabolismo redox (Jami y col., 2010).

A pesar de toda la información proporcionada por las nuevas técnicas "ómicas", la regulación de los genes *pcbAB*, *pcbC* y *penDE* sigue constituyendo un reto importante para la obtención de mayores niveles de producción, más aún, cuando no se ha identificado ningún regulador específico de ruta en la región amplificada que contiene los tres genes de biosíntesis de penicilina en *P. chrysogenum* (Fierro y col., 2006). Esto indica que la biosíntesis de penicilina está controlada directamente por reguladores globales, más que por reguladores específicos de ruta.

En *A. chrysogenum*, el "*cluster*" temprano de cefalosporina contiene un ORF (ORF3 en la figura 6) que codifica una proteína nuclear reguladora (CefR). Esta proteína contiene un dominio "*fungal-trans*" (dominio característico de múltiples reguladores fúngicos), la cual es el primer modulador de la secreción de los intermediarios y del producto final de la ruta de biosíntesis de cefalosporina C en *A. chrysogenum*. La interrupción del gen *cefR* disminuye y retrasa la producción de

cefalosporinas e incrementa la secreción de PenN. Por otro lado, la sobreexpresión del gen *cefR* disminuye la secreción de PenN, evitando la pérdida de este intermediario y por tanto, incrementando la producción de cefalosporina C. El análisis transcripcional de los genes biosintéticos y de los transportadores de la ruta biosintética de cefalosporina C en los transformantes de *cefR* (interrumpido y sobreexpresado) reveló que la proteína CefR actúa como un represor de los genes *cefT* y *cefM*, evitando así la pérdida de intermediarios y favoreciendo la síntesis de cefalosporina C, teniendo también un pequeño efecto estimulador sobre la expresión del gen *cefEF*. CefR constituye el primer ejemplo de modulador de transportadores de β-lactámicos en hongos filamentosos (Teijeira y col., 2011).

A continuación, se indican los principales mecanismos y factores que están implicados en la regulación de la biosíntesis de penicilina y cefalosporina.

1.6.1. Condiciones ambientales y nutricionales que controlan la biosíntesis de antibióticos β-lactámicos

- ***Regulación por el pH externo***

Muchos hongos filamentosos son capaces de sobrevivir y crecer en un amplio intervalo de pH ambiental, el cual podría ser tan ácido como pH=2 o tan alcalino como pH=10. Aparte de su capacidad de homeostasis, los hongos adaptan la secreción de enzimas y metabolitos secundarios en respuesta al pH ambiental.

La producción de antibióticos β-lactámicos está regulada por el pH externo del medio (Espeso y col., 1993). Dicha regulación está mediada en hongos filamentosos por el activador transcripcional PacC (Tilburn y col., 1995).

PacC es una proteína con tres dedos de zinc del tipo Cys2His2 (Espeso y col., 1997) que activa la transcripción por unión a la secuencia 5`-GCCARG-3`(Tilburn y col., 1995). Las penicilinas y cefalosporinas son producidas en mayor medida a pH alcalino, ya que bajo estas condiciones, un procesamiento proteolítico activa el factor transcripcional PacC. La activación de PacC ocurre a pH alcalino en respuesta a una ruta de transducción de señales procedente de seis proteínas Pal (Orejas y col., 1995). Esta activación requiere de dos pasos proteolíticos secuenciales. La proteína PacC

"cerrada" se encuentra en una conformación que impide la proteólisis gracias a la interacción entre una zona próxima al extremo C-terminal y una región que está corriente abajo del dominio de unión al ADN. A pH alcalino, la ruta Pal modifica dicha conformación "cerrada" dando lugar a la conformación "abierta", impidiendo que se den las interacciones intramoleculares, y haciéndola susceptible a la proteólisis, eliminando la zona C-terminal de la proteína. La proteína PacC una vez activada, funciona como activador o represor. PacC activa la transcripción de los genes expresados a pH alcalino, reprime la transcripción de los genes expresados a pH ácido y probablemente ejerza una fuerte autorregulación de su propio gen, debido a la existencia de supuestos sitios de unión PacC en el promotor del gen *pacC* de *A. chrysogenum*, *P. chrysogenum* y *A. niger* (MacCabe y col., 1996; Schmitt y col., 2001; Suárez y Peñalva, 1996).

El uso de fuentes de carbono represoras, como glucosa o sacarosa, causan acidificación del medio de cultivo, mientras que las fuentes de carbono no represoras, como lactosa, producen alcalinización. Por tanto, aunque la fuente de carbono y el pH tienen sus propios mecanismos específicos reguladores, estas dos formas independientes de regulación normalmente actúan en sintonía (Espeso y col., 1993). Mientras que en *A. nidulans* el pH alcalino es capaz de anular la represión catabólica ejercida por la sacarosa sobre la expresión del gen *ipnA*, en *P. chrysogenum* la represión catabólica por glucosa parece ser más fuerte, ya que aún se mantiene activa a pH alcalino (Suárez y Peñalva, 1996).

En *P. chrysogenum*, están presentes siete supuestos sitios de unión a PacC en la región intergénica *pcbAB-pcbC* y ocho en la región promotora del gen *penDE* (Suárez y Peñalva, 1996). En *A. chrysogenum*, están presentes dos supuestos sitios de unión a PacC en cada una de las regiones intergénicas entre *pcbAB-pcbC* y entre *cefEF-cefG* (Schmitt y col., 2001). Estos datos sugieren una regulación completa de los genes biosintéticos de penicilina y parcial de los genes de biosíntesis de cefalosporina según el pH ambiental.

- ***Regulación por fuente de Carbono***

La regulación por fuente de carbono es un mecanismo que opera en bacterias y hongos impidiendo la síntesis y/o la actividad de las enzimas necesarias para la asimilación de una amplia variedad de fuentes de carbono cuando una fuente de carbono, más fácilmente asimilable, está disponible (Ronne, 1995). Este tipo de regulación es beneficiosa para el microorganismo, ya que emplea la fuente de carbono más energética y no desperdicia energía en la síntesis de sistemas catabólicos para fuentes de carbono alternativas. Los genes sujetos a la represión por fuente de carbono pueden dividirse en tres grupos: (i) los que codifican enzimas implicadas en el metabolismo de fuentes de carbono menos favorables; (ii) los que codifican enzimas implicadas en la gluconeogénesis y en el ciclo del glioxilato; y (iii) los relacionados con el metabolismo secundario. El mejor ejemplo de este último grupo es la regulación de la biosíntesis de antibióticos β-lactámicos (Sánchez y col., 2010).

La biosíntesis de penicilina en *P. chrysogenum* está fuertemente regulada por glucosa, sacarosa y en menor grado, por maltosa, fructosa y galactosa, pero no por lactosa. Por lo tanto, el efecto negativo que la glucosa ejerce sobre la producción de penicilina puede ser parcialmente solventado usando lactosa como fuente de carbono o utilizando glucosa en dosis no represoras. Esto indica que la producción de penicilina parece estar favorecida por condiciones subóptimas de crecimiento, ya que los hongos crecen mejor con glucosa que con lactosa. La lactosa no ejerce el efecto represor, debido probablemente a una lenta hidrólisis de este azúcar como resultado de la muy baja actividad β-galactosidasa de este hongo. La regulación por carbono se ejerce a diferentes niveles de la biosíntesis de penicilina: (i) flujo del ácido L-α-aminoadípico; (ii) toma y activación de los precursores de la cadena lateral; (iii) y a nivel de la regulación transcripcional y post-transcripcional de los genes biosintéticos de penicilina.

La glucosa reduce la concentración intracelular de ácido L-α-aminoadípico, probablemente reduciendo también el flujo a ACV, un fenómeno que no se observa con lactosa. La formación de ACV e IPN están también reprimidas por altas concentraciones de glucosa (Revilla y col., 1986) en la cepa AS-P-78 de

P. chrysogenum (cepa de alta producción de penicilina). De acuerdo con este descubrimiento, está la observación realizada por Feng y colaboradores (1994) de que la expresión de ambos genes *pcbAB* y *pcbC* está reprimida por glucosa en otra cepa de *P. chrysogenum* (Q176). Sin embargo, Renno y colaboradores (1992) afirmaron que los niveles de ARNm maduros de los tres genes de biosíntesis de penicilina en *P. chrysogenum* eran mayores durante el crecimiento rápido con niveles elevados de glucosa. Esto indica que la magnitud de la regulación por carbono depende, al menos en parte, de la estrategia experimental utilizada.

En *A. nidulans*, la regulación por glucosa del gen *ipnA* tiene lugar, en parte, a nivel transcripcional, con una reducción en la actividad de la IPN sintasa cuando el micelio crece en presencia de glucosa. El efecto de la glucosa sobre el gen *aatA* está mediado post-transcripcionalmente y la actividad específica de la IAT en *A. nidulans* y la cepa silvestre de *P. chrysogenum* (NRRL 1951) está reducida en micelios crecidos en presencia de glucosa (Brakhage y col., 1992), a diferencia de los resultados obtenidos por Revilla y colaboradores (1986) en la cepa de alta producción AS-P-78 de *P. chrysogenum*. Esto indica que en las cepas de alta producción de penicilina, la regulación por glucosa puede haber sufrido modificaciones parciales, al menos en el control de la actividad de la IAT.

También se considera que la glucosa tiene un efecto negativo sobre la acetil-CoA sintetasa de *P. chrysogenum*, la cual es diferente a las PhlA, PhlB y PhlC CoA-ligasas y es capaz de catalizar la activación de algunos de los precursores de las cadenas laterales requeridos para la producción de algunas penicilinas *in vitro* (Martínez-Blanco y col., 1992). En *P. chrysogenum*, la captación de ácido fenilacético está reprimida por diferentes fuentes de carbono y ciertos aminoácidos y es inducida por fenilacetato (Ozcengiz y Demain, 2013).

La señal nutricional (alta o baja concentración de glucosa) es transmitida a la agrupación génica de biosíntesis de penicilina por una proteína reguladora, la cual parece formarse inmediatamente después de la inoculación de la fermentación de penicilina (el efecto represor es claramente menor cuando la glucosa se añade de 12 a 24 horas después de la incubación). En *A. nidulans*, la regulación por carbono del metabolismo primario está mediada por una proteína reguladora codificada por el

gen *creA* (Arst y MacDonald, 1975; Bailey y Arst, 1975; Dowzer y Kelly, 1991; Kulmburg y col., 1993). CreA es un factor transcripcional que contiene dos dedos de zinc del tipo Cys2His2, una región rica en alanina y motivos S(T)PXX frecuentes (Dowzer y Kelly, 1991). Esta proteína reconoce la secuencia consenso de unión SYGGRG (Cubero y Scazzocchio, 1994; Cubero y col., 2000; Kulmburg y col., 1993).

Sin embargo, en *A. nidulans* los niveles de los transcriptos del gen *ipnA* de todos los mutantes defectivos en *creA* ensayados, siguen mostrando represión mediada por glucosa (Espeso y Peñalva, 1992). Además, la deleción de una secuencia de 29 pb protegida por CreA no causó ningún tipo de efecto en la represión por sacarosa (Espeso y col., 1993), aunque existen varias posibles secuencias de unión de CreA. Al igual, las mutaciones en *creB* (codifica un miembro de la familia de enzimas de desubiquitinación (Lockington y Kelly, 2001)), y *creC* (codifica una proteína con una región rica en prolinas, una supuesta región de localización nuclear y cinco motivos repetidos WD-40 (Todd y col., 2000)), tuvieron muy poco efecto en la regulación por fuente de carbono de la biosíntesis de penicilina. Todos estos resultados sugieren que en *A. nidulans* existe un segundo mecanismo independiente de CreA implicado en el control de la biosíntesis de penicilina.

En *A. chrysogenum*, la producción de cefalosporina C depende también de la fuente de carbono utilizada (Demain, 1963). Aquellas fuentes de carbono que llevan a un crecimiento rápido (glucosa o glicerol), tienen un efecto negativo en la producción de antibióticos β-lactámicos. La glucosa tiene un efecto negativo mayor sobre la producción de cefalosporina C que sobre la producción de PenN, indicando que la represión es más intensa durante las últimas etapas de la ruta de biosíntesis (Behmer y Demain, 1983; Martín-Zanca y Martín, 1983). Las actividades enzimáticas de los productos de los genes *pbcAB*, *pcbC* y *cefEF* están reducidas en presencia de glucosa y por tanto, esta fuente de carbono reduce la producción de cefalosporina C. El efecto de la glucosa sobre la ACVS es ejercido a nivel postraduccional, ya que la actividad específica de la ACVS está seriamente inhibida por glucosa y glicerol, a diferencia de los niveles de la proteína ACVS, los cuales permanecen inalterados. Este fenómeno es debido a la disminución del cofactor ATP a través del metabolismo del azúcar (Zhang y col., 1989). Por otro lado, el efecto de la glucosa sobre los genes

pcbC y *cefEF* es ejercido a nivel transcripcional. Los promotores de los genes *pcbC* y *cefEF* contienen cuatro supuestos sitios de unión Cre1, y el producto del gen *cre1* controla la expresión de los genes *pcbC* y *cefEF* (Jekosch y Kück, 2000a). Este mecanismo control fue desregulado durante el programa de mejora de cepas (Jekosch y Kück, 2000b).

En el promotor bidireccional *pcbAB-pcbC* de *P. chrysogenum* hay seis secuencias consenso de unión 5′-SYGGRG-3′, mientras que han sido identificados siete supuestos sitios de unión de CreA en la región promotora del gen *penDE* (van den Berg y col., 2008). Sin embargo, hasta ahora no está claro si *P. chrysogenum* tiene un mecanismo similar de represión catabólica por glucosa mediada por CreA como en *A. chrysogenum*. Por tanto, es de gran interés el estudio de la posible implicación de CreA en la regulación de la biosíntesis de penicilina en *P. chrysogenum*, ya que la regulación por fuente de carbono es una posible diana para la optimización de los procesos de producción y en consecuencia, es uno de los objetivos de la presente Tesis Doctoral.

- ***Regulación por fuente de Nitrógeno***

La represión por fuentes de nitrógeno en hongos es un ejemplo de un mecanismo regulador global necesario para la coordinación de la expresión de los genes que aseguren el aporte de nitrógeno adecuado para el crecimiento en respuesta a cambios ambientales. El ión amonio y la glutamina o el glutamato son las fuentes de nitrógeno preferiblemente utilizadas por los hongos. En ausencia de fuentes de nitrógeno favorables, se inicia una síntesis *de novo* de permeasas y enzimas catabólicas, cuya expresión es altamente regulada por el circuito regulador que permite el uso de fuentes de nitrógeno secundarias, como pueden ser nitratos, purinas, proteínas y amidas (Caddick, 1994; Marzluf, 1993).

En *P. chrysogenum* y *A. chrysogenum*, la fuente de nitrógeno tiene una gran influencia sobre la producción de antibióticos β-lactámicos. En *A. chrysogenum*, una concentración de amonio superior a 100 mM interfiere considerablemente en la producción de cefalosporina C. La adición de amonio causa represión de expandasa/hidroxilasa, pero no de la IPN sintasa. Shen y colaboradores (1984)

probaron que la L-arginina y la L-asparagina son las mejores fuentes de nitrógeno para la producción de antibióticos β-lactámicos. En *P. chrysogenum*, la adición de 40 mM de amonio a micelios crecidos con lactosa causa represión de la expresión del gen reportero *uidA* fusionado a los promotores de los genes *pcbAB* y *pcbC* (Feng y col., 1994), con lo que se demuestra la influencia directa del ión amonio sobre la expresión de los genes biosintéticos de penicilina.

La regulación por nitrógeno en hongos está mediada por AreA en *A. nidulans* y por su homólogo NRE en *P. chrysogenum*. Estos genes codifican un factor transcripcional con un dedo de zinc tipo Cys-X2-Cys-X27-Cys-X2-Cys y una región adyacente básica que constituyen el dominio de unión al ADN. Las secuencias de aminoácidos de estas proteínas presentan sólo un 30% de homología entre ellas. En cambio, sus dominios de unión al ADN tienen una homología del 97%. Estos factores reguladores reconocen la secuencia consenso 5′-GATA-3′ (Marzluf, 1997). A veces, la interacción ocurre con dos elementos GATA separados por un número variable de 3 a 30 nt (Chiang y Marzluf, 1994). En *P. chrysogenum*, la región promotora bidireccional *pcbAB-pcbC* contiene un total de seis secuencias GATA, aunque sólo NRE interacciona fuertemente *in vitro* con un sitio que contiene dos de estas secuencias. En este sitio de unión, las dos secuencias GATA están dispuestas en configuración cabeza-cabeza y separadas por 27 nt (Haas y Marzluf, 1995). Por lo tanto, parece muy probable que la regulación por nitrógeno de los genes de biosíntesis de penicilina esté mediada a través de NRE. Esto sugiere que la disponibilidad de fuentes de nitrógeno favorables y por tanto, unas buenas condiciones de crecimiento, llevan a una disminución de la síntesis de penicilina en *P. chrysogenum*.

Hasta ahora, en *A. nidulans*, no hay evidencia de una posible regulación de la biosíntesis de penicilina en función de la fuente de nitrógeno. Esto concuerda con que hay únicamente una secuencia GATA en la región promotora bidireccional *pcbAB-pcbC*. Sin embargo, cuando el promotor del gen *pcbC* de *P. chrysogenum* es introducido en *A. nidulans*, la expresión del mismo es sensible a la regulación por nitrógeno (Kolar y col., 1991). Esto indica que la misma maquinaria que media la

represión por nitrógeno de la expresión del gen *pcbC* en *P. chrysogenum*, está también presente en *A. nidulans*.

En *A. chrysogenum*, la región intergénica de los genes *pcbAB-pcbC* contiene 15 motivos GATA, lo cual sugiere que la represión por nitrógeno de la producción de cefalosporina puede estar también regulada por un factor de unión a la secuencia GATA (Menne y col., 1994).

- ***Regulación por fuente de fosfato***

Un exceso de fosfato aumenta la represión por glucosa sobre la biosíntesis de penicilina. En un medio complejo de fermentación con la fuente de fosfato limitante (conteniendo Pharmamedia como única fuente de fosfato y nitrógeno), el efecto represor de la glucosa es sólo de un 13% cuando el azúcar es añadido en el momento de inoculación, mientras que se incrementa hasta un 59% cuando el medio es suplementado con una concentración de 100 mM de fosfato inorgánico (Martín y col., 1999). El fosfato inorgánico no tiene efecto *per se* en la producción de penicilina en condiciones no represoras de carbono.

En *A. chrysogenum*, una elevada concentración de fosfato ejerce un efecto negativo sobre la producción de cefalosporina. En ausencia de glucosa, el fosfato en sí disminuye el flujo global de formación de cefalosporina C. Hay un efecto negativo directo sobre la formación de ACVS, IPN sintasa y DAOC sintasa (expandasa)/DAC sintasa (hidroxilasa) (Zhang y col., 1988). Probablemente el fosfato actúa sobre la IPN sintasa y DAOC sintasa (expandasa)/DAC sintasa (hidroxilasa) a través de la formación de un complejo con el hierro. El hierro es requerido para la actividad de estas dos últimas enzimas y la inhibición por fosfato puede ser revertida por la adición de más hierro como sal ferrosa (Lübbe y col., 1984). Sin embargo, este mecanismo no está completamente claro, ya que la inhibición de la ACVS, la cual no requiere hierro como sal ferrosa para su actividad, es revertida por Fe^{2+} (Zhang y col., 1989).

- ***Regulación por condiciones de aireación***

La disponibilidad de oxígeno es importante para la producción de penicilina. Una buena aireación del micelio con oxígeno es una de las condiciones imprescindibles para la producción de grandes cantidades de antibióticos β-lactámicos (Hilgendorf y col., 1987). Ya que algunas enzimas, tales como la IPN sintasa y DAOC sintasa (expandasa)/DAC sintasa (hidroxilasa), requieren oxígeno para su actividad. La posibilidad de incrementar la producción de cefalosporina por ingeniería genética mediante la introducción directa de una proteína bacteriana de unión a oxígeno en *A. chrysogenum,* también apoya la gran importancia que el oxígeno puede tener en la producción de estos compuestos (DeModena y col., 1993). Sin embargo, Renno y colaboradores (1992) indicaron que la expresión de los genes *pcbAB* y *pcbC* en *P. chrysogenum* puede también ser inducida en respuesta al estrés por la reducción de los niveles de oxígeno.

- ***Aminoácidos como mediadores de la regulación***

Ya que la penicilina y la cefalosporina son sintetizadas a partir de los aminoácidos precursores L-α-aminoadípico, L-cisteína y L-valina, es lógico pensar que los aminoácidos juegan un papel en la regulación de su biosíntesis.

En *A. chrysogenum*, la producción de PenN y cefalosporina C está estimulada por metionina, particularmente por el isómero D (Komatsu y col., 1975; Zhang y col., 1987). El efecto estimulador de la metionina durante la producción de cefalosporina C en *A. chrysogenum* podría ser debido a un incremento en los niveles de ARNm de los genes *pcbAB*, *pcbC*, *cefEF* (y en menor grado en los transcriptos de *cefG*) o al suministro del azufre de la cisteína a la cefalosporina C (Velasco y col., 1994). La presencia de varias secuencias consenso 5′-CANNTG-3′ en la región promotora bidireccional *pcbAB-pcbC* en *A. chrysogenum*, sugiere que la regulación por metionina puede estar mediada por un miembro de la familia de proteínas con una región básica hélice-vuelta-hélice (bHLH). Algunos de estos factores de transcripción están implicados en el control transcripcional de los genes implicados en el metabolismo del azufre en *Saccharomyces cerevisiae* (Thomas y col., 1992).

La adición de L-lisina al medio de fermentación en *A. nidulans* y *P. chrysogenum* reduce la producción de penicilina (Demain, 1957; Brakhage y Turner, 1992). En *A. chrysogenum*, elevados niveles de L-lisina también interfieren en la producción de cefalosporina C (Mehta y col., 1979). El precursor de los antibióticos β-lactámicos, ácido L-α-aminoadípico, es un intermediario de la ruta que lleva a la síntesis del aminoácido L-lisina. La primera enzima de la ruta de la biosíntesis de L-lisina, la homocitrato sintasa, es sensible a la retroregulación negativa por la L-lisina en *P. chrysogenum* (Demain y Masurekar, 1974; Friedrich y Demain, 1977; Luengo y col., 1980). La L-lisina también inhibe a la α-aminoadipato reductasa a concentraciones fisiológicas (Affenzeller y col., 1989). Por tanto, el efecto de la L-lisina en la producción de penicilina es probablemente ejercido a través de la reducción de la reserva de L-α-aminoadípico, a través de la retroregulación negativa y a través de la represión de varios genes y enzimas de la biosíntesis de L-lisina. En *A. nidulans*, la L-lisina reprime la expresión de los genes reporteros *uidA* y *lacZ* fusionados, respectivamente, a los promotores génicos de *acvA* e *ipnA*, sugiriendo un control más directo de la expresión de los genes de biosíntesis de penicilina (Brakhage y Turner, 1992). En este hongo, se midieron los efectos diferenciales sobre la expresión de los genes de biosíntesis de penicilina *acvA* e *ipnA* y sobre la producción de penicilina debido a la presencia de varios aminoácidos en el medio (Litzka y col., 1998). La L-treonina, el L-aspartato, el L-glutamato y la L-cisteína produjeron un incremento en la expresión del gen reportero *uidA* (controlado por el promotor del gen *acvA*), pero no tuvieron efecto sobre la expresión del gen *lacZ* (controlado por el promotor del gen *ipnA*). La L-metionina (a concentraciones mayores de 10 mM), la L-leucina, la L-isoleucina, la L-fenilalanina, la L-valina, la L-histidina y la L-lisina llevaron a una represión de la expresión de ambos genes reporteros, la cual fue dependiente de la concentración de los aminoácidos. La L-tirosina, el L-triptófano, la L-prolina y el ácido L-α-aminoadípico no tuvieron mayores efectos en la expresión del gen reportero controlado por el promotor del gen *acvA*, pero llevaron a la represión de la expresión del gen *lacZ* controlado por el promotor del gen *ipnA*. La L-serina y L-arginina no mostraron ningún efecto en la expresión de ninguno de estos genes reporteros a ninguna concentración. Los efectos negativos de la L-histidina y la L-valina fueron debidos a la activación reducida de

PacC bajo las condiciones ácidas causadas por estos aminoácidos. Sin embargo, los efectos represivos de la L-lisina y L-metionina actuaron independientemente de PacC por mecanismos desconocidos hasta ahora.

- ***Regulación por poliaminas***

La biosíntesis de bencilpenicilina está estimulada por el 1, 3-diaminopropano (1,3-DAP) y la espermidina en *P. chrysogenum*. A diferencia de la putrescina, ambos compuestos aminados producen un incremento drástico en la expresión de los genes biosintéticos *pcbAB*, *pcbC* y *penDE* (Martín y col., 2011). El efecto estimulador de estas poliaminas no es debido a un cambio en el mecanismo que controla el pH, ya que estas no afectan a la expresión de *pacC*. El efecto del 1,3-diaminopropano y de la espermidina es ejercido, al menos en parte, a través de un incremento en la expresión del gen *laeA* (J. Martín y col., 2012), el cual codifica un regulador global que actúa epigenéticamente sobre la expresión de genes del metabolismo secundario por reorganización de la heterocromatina (ver más adelante). El análisis proteómico del efecto del 1,3-DAP y espermidina ha puesto de manifiesto que ambos compuestos promueven una profunda reorganización del proteoma, además de aumentar el contenido intracelular de vesículas que derivan a vacuolas en las etapas finales (García-Estrada y col., 2013). Curiosamente, el 1,3-DAP y la espermidina inducen a la formación de una modificación post-traduccional de la IAT (probablemente mejorando la actividad de esta enzima y aumentando la producción de penicilina) y reducen la síntesis de las enzimas tardías de la ruta del homogentisato (ruta catabólica del ácido fenilacético). Además, estas poliaminas estimulan la síntesis de enzimas implicadas en la biosíntesis de valina y otros precursores de la penicilina, como la coenzima A (García-Estrada y col., 2013).

- ***Regulación por la adición de líquido de maceración del maíz***

El líquido de maceración del maíz (Corn steep liquid o CSL) es un subproducto del proceso de elaboración de almidón de maíz y ha sido usado como componente habitual en los medios de cultivo microbiológicos.

La adición de CSL al medio de producción incrementa notablemente los rendimientos de penicilina en *P. chrysogenum* (Liggett y Koffler, 1948). En *A. nidulans*, la producción de penicilina es indetectable, a menos que el CSL sea añadido al medio de producción (Cove, 1966). Una posible explicación de las propiedades básicas del CSL es su composición, ya que hay un alto contenido de aminoácidos (incluyendo el ácido α-aminoadípico), polipéptidos, minerales y ácido láctico que favorecen la biosíntesis de penicilina.

El resultado más importante obtenido después de la adición de CSL en *A. nidulans,* es un gran incremento en la expresión de los genes biosintéticos de penicilina. El efecto estimulador incluso se produce en presencia de fuentes de carbono represoras de la biosíntesis de penicilina (MacCabe y col., 1990; Peñalva y col., 1991a, 1991b). Sin embargo, los mecanismos moleculares que conectan la presencia de CSL y el incremento de la expresión génica aún son desconocidos.

- ***Regulación mediada por Pga1***

Las proteínas G heterotriméricas median varios procesos celulares en organismos eucariotas en respuesta a estímulos nutricionales y ambientales. Las proteínas G constan de tres subunidades, α, β y γ, que permanecen inactivas en el estado heterotrimérico cuando el GDP está unido a la subunidad α (denominada Pga1 en *P. chrysogenum*). La activación ocurre cuando el GTP se une a la subunidad α, la cual se disocia del dímero $\beta\gamma$ promoviendo la interacción independiente con los efectores "corriente abajo" (Hamm, 1998).

Pga1 regula la biosíntesis de penicilina controlando la expresión de los genes del "*cluster*" biosintético de penicilina. Mutantes en el alelo pga1G42R (constitutivamente activado) muestran un incremento en la producción de penicilina y en los niveles de ARNm de los genes biosintéticos (*pcbAB*, *pcbC* y *penDE*) (García-Rico y col., 2008b).

El AMPc es un mensajero secundario en la ruta de transducción de señales mediada por Pga1 y sus niveles intracelulares están claramente regulados por Pga1 (García-Rico y col., 2008a). En *P. chrysogenum*, los niveles de AMPc son altos durante el crecimiento con lactosa y descienden considerablemente cuando se añade glucosa o fructosa al medio (Kozma y col., 1993). Sin embargo, la regulación de la biosíntesis de penicilina por Pga1 puede no ser mediada por el AMPc (García-Rico y col., 2008b).

1.6.2. Factores reguladores globales

- ***LaeA***

LaeA es un regulador global del metabolismo secundario formado por una proteína nuclear con un dominio metiltransferasa (Bok y Keller, 2004). Este regulador controla la expresión de las agrupaciones génicas a través de la remodelación de la cromatina. Concretamente, el mecanismo regulador de LaeA es a través de la represión de la heterocromatina, quizás por la interacción con metilasas o desacetilasas que están asociadas con la misma (Keller y col., 2005; Shwab y col., 2007). LaeA forma parte de un complejo, denominado complejo velvet, el cual contiene al menos 10 proteínas diferentes que coordinan el metabolismo secundario con el desarrollo (Hoff y col., 2010).

En *Aspergillus spp*., la deleción de *laeA* bloquea la expresión del "*cluster*" génico de biosíntesis de penicilina, mientras que la sobreexpresión de *laeA* desencadena un aumento en la transcripción de estos genes con el consiguiente aumento en la producción de antibiótico (Bok y Keller, 2004). En *P. chrysogenum* se ha identificado el gen *laeA* que codifica el ortólogo de LaeA (denominado PcLaeA). La sobreexpresión de PcLaeA da lugar a un incremento del 25% en la producción de penicilina. Mutantes silenciados en el gen *laeA* de *P. chrysogenum* muestran un descenso drástico en la expresión de todos los genes biosintéticos de penicilina, indicando que PcLaeA actúa como un activador de la biosíntesis de penicilina (Kosalková y col., 2009).

- ***Complejo velvet***

VeA, componente del complejo velvet, está codificado por el primer gen que se aisló inicialmente en *A. nidulans* como implicado en el desarrollo y se definió como un regulador de la morfogénesis en hongos. Más tarde, se observó que afecta a la biosíntesis de diferentes metabolitos, tales como la penicilina. VeA reprime la transcripción de *ipnA* y es a la vez necesario para la expresión de *acvA* (Kato y col., 2003). Sin embargo, Spröte y Brakhage (2007) demostraron más tarde que este componente del complejo velvet, principalmente reprime la transcripción de *acvA*. Estas diferencias podían ser debidas a la composición del complejo velvet, donde VeA interacciona con otros factores en un delicado equilibrio estequiométrico para controlar la producción de metabolitos secundarios. Por tanto, la producción de penicilina puede verse modificada a través de la alteración en la síntesis de VeA, hipótesis que está apoyada por varias investigaciones que caracterizan homólogos de velvet como activadores, además de represores del metabolismo secundario en hongos filamentosos (Hoff y col., 2010).

Por el contrario, la interrupción del gen *AcveA* de *A. chrysogenum* reprime la expresión de los genes de la biosíntesis de cefalosporina C *pcbAB*, *pcbC*, *cefD1*, *cefD2*, *cefEF* y *cefG* (el efecto más drástico se observó en *cefEF*), con la siguiente modificación en la producción de cefalosporina C (Dreyer y col., 2007). Esto también se ha descrito en *P. chrysogenum*, donde PcVelA actúa como un activador transcripcional de los genes de biosíntesis de penicilina *pcbC* y *penDE* (Hoff y col., 2010).

Recientemente, se han caracterizadas en *P. chrysogenum* nuevas subunidades del complejo velvet (PcVelB, PcVelC y PcVosA) (Kopke y col., 2013). PcVelC es un fuerte activador de la biosíntesis de penicilina y por tanto, actúa en *P. chrysogenum* junto con PcVelA y PcLaeA controlando la producción de metabolitos secundarios. PcVelB actúa como represor de PcVelA y PcVelC y por consiguiente, es un represor de este proceso (Kopke y col., 2013). Sin embargo, en *A. nidulans* el homólogo de PcVelB tiene un papel activador (Bayram y col., 2008).

- ***CPCR1-AcFKH1***

CPCR1 es otro de los factores transcripcionales que han sido identificados en el hongo productor de cefalosporina C *A. chrysogenum*. Este factor muestra gran similitud con reguladores humanos de la familia de factores de regulación X (RFX) (Schmitt y Kück, 2000). Las proteínas de la clase RFX constituyen un tipo de factores transcripcionales de tipo "*winged helix*" (hélice alada) y se caracterizan por un modo no convencional de reconocimiento del ADN. La estructura cocristalizada del dominio de unión al ADN del factor RFX1 humano a su ADN diana reveló diferencias con otras proteínas del tipo "*winged helix*". En la estructura de la proteína RFX, la mayoría de los contactos con el surco mayor del ADN se realizan por el ala 1, mientras que en otras proteínas "*winged helix*" bien caracterizadas, todos los contactos con el surco mayor se realizan a través de la hélice de reconocimiento 3 (Gajiwala y Burley, 2000).

El miembro más importante de la familia "*winged helix*" es el factor nuclear 3 de hepatocito humano (HNF-3) y las proteínas homeóticas "*forkhead*" de *Drosophila*. Se han identificado miembros de la clase de factores de transcripción RFX en eucariotas inferiores y en mamíferos, y sus funciones adscritas son diversas (Gajiwala y Burley, 2000).

CPCR1, además de poseer el dominio N-terminal de unión al ADN, contiene un dominio de dimerización en la región C-terminal. Se ha demostrado que CPCR1 se dimeriza y se une al ADN únicamente en estado multimérico, y por tanto la proteína completa es necesaria para su función como factor de transcripción (Schmitt y Kück, 2000). CPCR1 actúa como un regulador de la biosíntesis de cefalosporina C al unirse, al menos, a dos secuencias de la región intergénica entre los genes *pcbAB-pcbC* de *A. chrysogenum,* ya que mutantes defectivos en este factor mostraron un descenso en la expresión del gen *pcbC*, así como una disminución en la producción del intermediario de la biosíntesis PenN, pero no en los niveles de cefalosporina C. Por tanto, CPCR1 no está probablemente implicado en la regulación de los genes tardíos del "*cluster*" de biosíntesis de cefalosporina C (Schmitt y Kück, 2000; Schmitt y col., 2004a).

Se han encontrado homólogos de CPCR1 en hongos no productores de antibióticos β-lactámicos, tales como *Neurospora crassa* y *Fusarium graminearum*, lo que indica que este factor puede tener funciones reguladoras diferentes no restringidas a la biosíntesis de antibióticos. De hecho, se ha demostrado que CPCR1 también está implicado en el control del desarrollo morfológico y es necesario para la fragmentación de la hifa y por tanto, para la formación de artrosporas en *A. chrysogenum* (Hoff y col., 2005). También en *Penicillium marneffei*, hongo patógeno oportunista, CPCR1 (RfxA) tiene un papel de conexión entre la división celular y la morfogenésis, principalmente durante la conidiación y el crecimiento en forma de levadura, en los que el estado mononucleado de este tipo celular necesita una mayor conexión entre la división celular y nuclear que durante el crecimiento de la hifa (Bugeja y col., 2010).

También se ha visto que AcFKH1, un miembro de la familia de factores transcripcionales de tipo "*forkhead*" que también pertenece a la subclase de factores de transcripción "*winged helix*", se asocia con CPCR1 en *A. chrysogenum*. En seres humanos y levaduras, los miembros de la familia de factores de transcripción "*forkhead*" están implicados en procesos tales como la regulación del ciclo celular, el control de la muerte celular, el procesamiento del pre-mARN o la morfogénesis (Burgering y Kops, 2002; Carlsson y Mahlapuu, 2002; Morillon y col., 2003). AcFKH1 se caracteriza por poseer dos dominios conservados; (i) el dominio asociado a la región "*forkhead*" (FHA), que podría estar implicada en la interacción fosfo-proteína, y (ii) el dominio C-terminal "*forkhead*" (FKH) de unión al ADN. AcFKH1 reconoce dos sitios de unión consenso dentro de la región promotora bidireccional de los genes *pcbAB-pcbC* (Schmitt y col., 2004b). Este factor, sin embargo, no está directamente implicado en la fragmentación de las hifas, sino que su presencia parece ser necesaria para la función de CPCR1 en la morfogénesis de *A. chrysogenum*. Esto se deduce de los resultados obtenidos en una cepa carente del gen que codifica el factor AcFKH1 y tras la sobreexpresión de un gen funcional *cpcR1*, la cual no tuvo efecto sobre la formación de artrosporas. Además, las cepas que carecían de factor AcFKH1 mostraron defectos en la separación celular, indicando una implicación de este factor en el crecimiento micelial (Hoff y col., 2005). Por tanto, CPCR1, probablemente en la forma de heterodímero, junto con AcFKH1 (a través de una

interacción específica y directa entre CPCR1 y la región C-terminal de AcFKH1), se comporta como un eslabón molecular entre el metabolismo secundario (producción de antibiótico) y la morfogénesis. En consecuencia, estos factores son de suma importancia en el control del crecimiento fúngico en los procesos de producción.

Es de gran interés, por tanto, comprobar la existencia de ortólogos de CPCR1 y AcFKH1 en *P. chrysogenum* con el fin de valorar la existencia de mecanismos reguladores parecidos a los que operan en *A. chrysogenum* y analizar su posible implicación en la regulación de la biosíntesis de penicilina. En consecuencia, se ha planteado el estudio en profundidad de los factores ortólogos de CPCR1 y AcFKH1 en *P. chrysogenum* como otro de los objetivos de la presente Tesis Doctoral.

1.6.3. Otros factores de transcripción

- ***Complejo AnCF de unión a CCAAT***

El primer factor de unión a 5′-CCAAT-3′ fue descubierto en *S. cerevisiae* (denominado complejo HAP) y consta al menos, de cuatro subunidades: Hap2, Hap3 y Hap5 que forman un complejo heterotrimérico que es esencial para la unión al ADN, y Hap4 que es una proteína ácida que actúa como el dominio de activación transcripcional (McNabb y col., 1995).

En *A. nidulans*, AnCF (*A. nidulans* CCAAT-binding factor), anteriormente denominado PENR1 (Penicillin regulator 1), está formado por las subunidades HapB, HapC y HapE, las cuales son todas necesarias para la unión al ADN (Steidl y col., 1999). Considerando que AnCF se une a secuencias que contienen CCAAT, las cuales están presentes en las regiones promotoras de un gran número de genes eucariotas, se ha estimado que AnCF regula más de 200 genes (Brakhage y col., 1999). Sin embargo, la deleción de los genes que codifican las subunidades de AnCF no es letal (Papagiannopoulos y col., 1996), indicando que este complejo regulador no es esencial para la supervivencia directa de las células y está únicamente implicado en la regulación de ciertos subconjuntos de genes, incluyendo algunos de los genes de biosíntesis de penicilina o genes requeridos para superar la limitación de hierro como los sideróforos.

La proteína AnCF se une a una caja CCAAT (caja I) en la región bidireccional entre los genes *acvA* e *ipnA* (caja I localizada 409 pb "corriente arriba" del inicio de transcripción del gen *acvA*) (Bergh y col., 1996). Este factor también se une a otra caja CCAAT (caja II) en el promotor del gen *aatA* de *A. nidulans* (caja II localizada a unos 250 pb "corriente arriba" del inicio de transcripción del gen *aatA*) (Litzka y col., 1996).

La deleción de 4 nt en la caja I produjo un incremento en la expresión del gen *acvA* de hasta diez veces y simultáneamente, una reducción del 30% en la expresión del gen *ipnA* (Bergh y col., 1996). Además, la sustitución de la secuencia CCAAT por GATCC llevó a una reducción de cuatro veces en la expresión de una fusión génica *aatA-lacZ* (Litzka y col., 1996). AnCF también se une al promotor del gen *aatB* (Spröte y col., 2008).

De acuerdo con los datos obtenidos después de la deleción de las cajas CCAAT, la expresión de ambos genes *ipnA* y *aatA* se vio reducida en el mutante Δ*hapC*. Sin embargo, en el mutante Δ*hapC*, la expresión de *acvA* se vio sólo ligeramente afectada (Bergh y col., 1996). Por tanto, parece probable que, además de AnCF, una proteína represora se una cerca o solape el sitio de unión de AnCF. Esto podría explicar que el sitio de unión de AnCF muestre un efecto represivo sobre la expresión de *acvA* en la cepa silvestre. De acuerdo con este punto de vista, la falta de unión de AnCF en el mutante Δ*hapC* no impidió la unión de esta supuesta proteína represora y por consiguiente, la expresión de la fusión génica *acvA-uidA* no se vio incrementada.

Weidner y colaboradores (1997) mostraron que el gen lysF implicado en la biosíntesis de lisina de *A. nidulans* estaba regulado negativamente por AnCF. Además, AnCF es autoregulado negativamente por la represión del gen *hapB* (Steidl y col., 2001). Estos resultados indican que AnCF es capaz de actuar o como un activador o un represor de la transcripción dependiendo específicamente del gen.

En el promotor bidireccional *pcbAB-pcbC* de *P. chrysogenum* y *A. chrysogenum*, además de en el promotor del gen *penDE* de *P. chrysogenum*, están presentes varias cajas de unión CCAAT con alto grado de similitud a las secuencias consenso de unión de AnCF. Por otra parte, la proteína AnCF de *A. nidulans* es capaz de unirse a

estos fragmentos (Bergh y col., 1996; Litzka y col., 1996), lo que sugiere que complejos homólogos pueden existir en *P. chrysogenum* y en *A. chrysogenum*. Esto es coherente con el hecho de que supuestas subunidades ortólogas de HapB (Pc12g01590), HapC (Pc14g01630) y HapE (Pc12g04670) hayan sido identificadas en el genoma de *P. chrysogenum* (van den Berg y col., 2008), aunque aún no hayan sido caracterizadas.

- ***AnBH1***

La proteína AnBH1 (PENR2) pertenece a la familia de factores de transcripción con una región básica hélice-vuelta-hélice (bHLH) y ha sido identificada en *A. nidulans*. El correspondiente gen *anbH1* está localizado en el cromosoma IV. AnBH1 se une *in vitro* como un homodímero a una caja E asimétrica en el promotor del gen *aatA*, la cual solapa con la caja II CCAAT para AnCF. AnBH1 actúa como un represor de la expresión del gen *aatA* y por tanto, contrarresta el efecto positivo de AnCF (Caruso y col., 2002). AnBH1 también se une a la caja E en el promotor del gen *aatB*, la cual no está solapada con la caja unión CCAAT para para AnCF (Spröte y col., 2008). Hasta ahora, sólo se han descritos tres genes regulados por AnBH1 (*aatA*, *aatB* y el propio gen *anbH1*) (Caruso y col., 2002; Spröte y col., 2008). Sin embargo, ya que la deleción del gen *anbH1* parece ser letal para el hongo (Caruso y col., 2002), cabe esperar que más genes estén regulados por AnBH1. Curiosamente, a nivel postraduccional, AnBH1 parece estar controlada por otro regulador global, la proteína quinasa C PkcA (Herrmann y col., 2006), cuyos ortólogos en otros hongos están involucrados en la ruta de integridad de la pared celular (Schmitz y Heinisch, 2003).

En el genoma de *P. chrysogenum* se ha identificado un supuesto homólogo de AnBH1 (Pc22g15870) (van den Berg y col., 2008) que aún no ha sido caracterizado.

- ***PTA1***

En *P. chrysogenum*, la expresión de los dos primeros genes de la ruta biosintética de penicilina, *pcbAB* y *pcbC*, se deben expresar de manera coordinada, ya que ambos genes muestran un modelo regulador y de expresión similar.

La deleción secuencial de la región promotora del gen *pcbAB* permitió la identificación de tres regiones, caja A, B y C, las cuales estaban implicadas en la expresión de este gen. Cuando la más distal de estas cajas (cajaA) se incuba con extractos crudos de micelio crecido con glucosa como fuente de carbono, se forma un complejo de retraso específico y definido. La proteína que forma este complejo de retraso fue denominada *penicillin transcriptional activator 1* (PTA1). Sin embargo, un complejo de retraso distorsionado y menos específico era visible cuando se utilizaban extractos crudos proteicos de micelios crecidos con lactosa como fuente de carbono, quizás debido a la modificación de la proteína PTA1 (Kosalková y col., 2000).

A través de un ensayo de interferencia por uracilo, se determinó la existencia de una secuencia heptamérica (TTAGTAA) en la caja A, la cual es el sitio de unión para PTA1. La deleción de la secuencia heptamérica provoca una drástica reducción de la expresión del gen *pcbAB*, por lo que se requiere este factor regulador para conseguir un alto nivel de expresión del gen *pcbAB* (Kosalková y col., 2000). La secuencia heptamérica se parece a la secuencia del factor BAS2 (PHO2) (TTAGTTAA), el cual es un factor de transcripción con dos dedos de zinc necesario para la expresión de diversos genes en *S. cerevisiae* (Tice-Baldwin y col., 1989).

1.7. Objetivos

Hasta la fecha, no se ha encontrado ningún regulador específico de la ruta de biosíntesis de penicilina en *P. chrysogenum*. Por tanto, la producción de penicilina en este hongo filamentoso está en principio controlada, como se ha indicado ya en este trabajo, por diferentes reguladores globales. Así, la finalidad principal de este trabajo ha sido aportar mayor conocimiento sobre algunos de los elementos reguladores que controlan la producción de penicilina en *P. chrysogenum*.

Además, los mecanismos reguladores de la ruta de biosíntesis de penicilina son posibles dianas para la optimización de la producción y la mejora de cepas de *P. chrysogenum*.

En concreto, los objetivos propuestos para este trabajo son los siguientes:

- Estudiar los factores ortólogos de CPCR1 y AcFKH1 en *P. chrysogenum* con el fin de valorar si existen mecanismos reguladores parecidos a los que operan en *A. chrysogenum* y analizar su posible implicación en la regulación de la biosíntesis de penicilina.
- Estudiar la posible implicación del factor transcripcional CreA en la regulación de la biosíntesis de penicilina en *P. chrysogenum*.

2. Publicaciones

Artículo 1

The regulatory factor PcRFX1 controls the expression of the three genes of β-lactam biosynthesis in *Penicillium chrysogenum*

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The regulatory factor PcRFX1 controls the expression of the three genes of β-lactam biosynthesis in *Penicillium chrysogenum*

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ABSTRACT

Penicillin biosynthesis is subjected to a complex regulatory network of signalling molecules that may serve as model for other secondary metabolites. The information provided by the new "omics" era about *Penicillium chrysogenum* and the advances in the knowledge of molecular mechanisms responsible for improved productivity, make this fungus an excellent model to decipher the mechanisms controlling the penicillin biosynthetic pathway. In this work, we have characterized a novel transcription factor PcRFX1, which is an ortholog of the *Acremonium chrysogenum* CPCR1 and *Penicillium marneffei* RfxA regulatory proteins. PcRFX1 DNA binding sequences were found in the promoter region of the *pcbAB*, *pcbC* and *penDE* genes. We show in this article that these motifs control the expression of the β-galactosidase *lacZ* reporter gene, indicating that they may direct the PcRFX1-mediated regulation of the penicillin biosynthetic genes. By means of P*crfx1* gene knock-down and overexpression techniques we confirmed that PcRFX1 controls penicillin biosynthesis through the regulation of the *pcbAB*, *pcbC* and *penDE* transcription. Morphology and development seemed not to be controlled by this transcription factor under the conditions studied and only sporulation was slightly reduced after the silencing of the P*crfx1* gene. A genome-wide analysis of processes putatively regulated by this transcription factor was carried out in *P. chrysogenum*. Results suggested that PcRFX1, in addition to regulate penicillin biosynthesis, is also involved in the control of several pathways of primary metabolism.

1. Introduction

Antibiotics from the β-lactam group are among the most commonly prescribed medications in modern medicine. During the industrial strain improvement programs applied to the wild type strain *Penicillium chrysogenum* NRRL 1951, which was isolated from an infected cantaloupe in Peoria, IL (Raper et al., 1944), several modifications responsible for increased penicillin productivity occurred. It is well-known that the region including the penicillin biosynthetic genes *pcbAB*, *pcbC* and *penDE*, underwent tandem amplification (56.8-kb amplifiable unit) in high-producing strains (Fierro et al., 1995). In addition, the number of peroxisomes increased in high-penicillin producing strains (van den Berg et al., 2008). Transcriptomics analysis also revealed that those genes encoding enzymes responsible for the biosynthesis of the amino acid precursors as well as those gene encoding microbody proteins were overexpressed in high-producing strains (van den Berg et al., 2008). More recently, proteomics studies also revealed that the increase in penicillin titers observed in high-producing strains was the consequence of a complex rebalancing of global metabolism, including redox reactions, production of energy, biosynthesis of amino acid precursors, virulence or secondary metabolism (Jami et al., 2010).

Taking into account the information provided by the new "omics", regulation of penicillin biosynthetic genes represents an interesting challenge in order to increase productivity. The penicillin biosynthetic pathway is compartmentalized between the cytosol and peroxisomes and has been largely described from the molecular and biochemical point of views (revised by Martín et al., 2010).

Penicillin biosynthesis starts with the non-ribosomal condensation of L-α-aminoadipic acid, L-cysteine and L-valine, giving rise to the tripeptide δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). This reaction is catalyzed by the multienzyme ACV synthetase (encoded by the *pcbAB* gene). Then, ACV undergoes internal cyclization in a reaction catalyzed by the isopenicillin N (IPN) synthase (encoded by the *pcbC* gene), thus forming IPN, the first bioactive compound

Abbreviations: ACV, δ(L-α-aminoadipyl)-L-cysteinyl-D-valine; IAT, IPN acyltransferase; IPN, isopenicillin N.

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of the pathway (Aharonowitz et al., 1992). In the last step, the IPN acyltransferase (IAT, encoded by the *penDE* gene) synthesizes hydrophobic penicillins by substitution of the L-α-aminoadipyl side chain of IPN by aromatic acyl side chains, which have to be previously activated by specific aryl-CoA ligases. Easily utilizable carbon, nitrogen and phosphorous sources dramatically affect the production of this antibiotic (Martín et al., 1999). The control of penicillin production is exerted by transcription factors that bind *cis* elements in the biosynthetic gene promoter regions (Haas et al., 1995; Kosalková et al., 2000). Since no penicillin pathway-specific regulators have been found in the genome region that contains the penicillin gene cluster (Fierro et al., 2006; van den Berg et al., 2007), penicillin biosynthesis seems to be controlled by global factors that bind the promoter region of the biosynthetic genes (Espeso et al., 1993; Chu et al., 1995; Feng et al., 1995; Haas and Marzluf, 1995; Suárez and Peñalba, 1996; Kosalková et al., 2000) through complex regulatory processes (Chang et al., 1990; Feng et al., 1994; Martín, 2000; Brakhage et al., 2004). One of these global regulators is LaeA, a nuclear protein with a methyltransferase domain controlling the expression of the penicillin genes and other secondary metabolites in *P. chrysogenum* (Kosalková et al., 2009). LaeA is part of a protein complex of at least ten different proteins (the velvet complex). Recently, one of the components of this complex, Velvet A, was identified in *P. chrysogenum* (Hoff et al., 2010). LaeA and Velvet A play an essential role in the biosynthesis of penicillin and in different developmental processes (Hoff et al., 2010). However, other general regulators controlling penicillin biosynthesis in *P. chrysogenum* are still unknown.

Control of gene expression through global regulators is also present in other β-lactam producers, such as *Aspergillus nidulans* and *Acremonium chrysogenum*. In *A. nidulans* LaeA controls the synthesis of penicillin and other secondary metabolites (Bok and Keller, 2004), whereas Velvet A regulates the biosynthesis of penicillin via repression of the *pcbAB* (*acvA*) gene (Spröte and Brakhage, 2007). In *A. chrysogenum*, Velvet A positively controls the expression of the cephalosporin biosynthetic genes *pcbAB*, *pcbC*, *cefD1*, *cefD2*, *cefEF* and *cefG* (specially the *cefEF* gene encoding the DAOC synthetase/hydroxylase) and also regulates hyphal fragmentation (Dreyer et al., 2007). Another transcription factor that has been recently identified in *A. chrysogenum* is CPCR1 (encoded by the *cpcR1* gene), which is related to human transcription factors of the RFX family (a small class of winged-helix factors characterized by a nonconventional mode of DNA recognition (Gajiwala et al., 2000)). CPCR1 is associated with AcFKH1 (Schmitt et al., 2004a; Hoff and Kück, 2005), which is another subclass of winged-helix transcription factor and a member of the "forkead" family of regulators (Gajiwala et al., 2000). It has been reported that in *A. chrysogenum*, CPCR1 only binds DNA in a dimeric state (Schmitt and Kück, 2000), positively regulating the cephalosporin C biosynthesis by binding at least two sequences at the *pcbAB-pcbC* intergenic region. Knock-out mutants defective in this transcription factor showed reduced levels in the expression of the *pcbC* gene together with a reduction in the biosynthesis of the cephalosporin C intermediate penicillin N (Schmitt and Kück, 2000; Schmitt et al., 2004b). CPCR1 homologs have also been found in β-lactam non-producer fungi, such as *Neurospora crassa* or *Fusarium graminearum*, pointing to this transcription factor as a regulator of other functions different from antibiotic production.

In this work we characterize the *P. chrysogenum* CPCR1 homolog (PcRFX1), providing evidence that this regulator controls the expression of the three penicillin biosynthetic genes and penicillin production. In addition, we also provide a global map of processes putatively regulated by this transcription factor in *P. chrysogenum*, which contributes to the understanding of the complex regulatory network underlying the process of penicillin biosynthesis.

2. Materials and methods

2.1. Strains, media and culture conditions

P. chrysogenum NRRL 1951 (wild type strain), *P. chrysogenum* Wisconsin 54-1255 (reference strain for the genome sequencing project) and *P. chrysogenum* Wisconsin 54-1255 PyrG- (an uridine auxotroph derived from the Wisconsin 54-1255 strain) were used in this work. These strains contain a single copy of the penicillin gene cluster (Fierro et al., 1995). *P. chrysogenum* Wisconsin 54-1255 npe10 (Δ*pen*) pPyrG-, which is derived from the Wisconsin 54-1255 strain and lacks the penicillin biosynthetic genes (Cantoral et al., 1993), was also used.

Strains were grown in solid Power sporulation medium (Casqueiro et al., 1999) for 5–7 days at 28 °C. *P. chrysogenum* liquid cultures were initiated inoculating fresh spores in 100 ml of complex medium CIM (García-Estrada et al., 2008a) without phenylacetate. After incubation at 25 °C for 24 h in an orbital shaker (250 rpm), aliquots (5%) were inoculated in CP complex penicillin production medium (García-Estrada et al., 2008a) with 0.4% (v/v) potassium phenylacetate. Uridine auxotrophs were grown in the presence of 140 μg/ml uridine. For genomic DNA extraction and transformation experiments, spores from *P. chrysogenum* were inoculated into MPPY medium (40 g/l glucose, 3 g/l $NaNO_3$, 2 g/l yeast extract, 0.5 g/l KCl, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.01 g/l $FeSO_4{\cdot}7H_2O$, pH = 6.0), supplemented with 140 μg/ml uridine if necessary.

In general, 24 h, 48 h and 72 h were selected as sampling times since they represent characteristic time-points of the *P. chrysogenum* growth phase. Both 24 h and 48 h represent the early and late linear growth phase, respectively, whereas 72 h represents the stationary phase. Time-points shorter than 24 h (low biomass and low penicillin production) and longer than 72 h (the beginning of cellular lytic events) were considered inappropriate for sampling.

Escherichia coli DH5α cells, used for plasmid amplification, were grown in Luria–Bertani medium (LB) with ampicillin (100 μg/ml).

2.2. Plasmid constructions

Plasmid pJL43-RNAi-rfx1 was constructed for Pc*rfx1* gene silencing. It is a derivative of pJL43-RNAi (Ullán et al., 2008), which contains the *ble* gene marker (for phleomycin resistance). Oligonucleotides 2RFXFNcoI and 2RFXRNcoI (see Supplementary Table S1) were used to amplify a 479-bp exonic fragment from Pc*rfx1*. The PCR product was digested with *Nco*I and cloned into pJL43-RNAi (previously digested with *Nco*I) to yield pJL43-RNAi-rfx1. This plasmid was used to transform the *P. chrysogenum* Wisconsin 54-1255 strain.

Plasmid pIBRC43-Pcrfx1 was constructed for the overexpression of the *P. chrysogenum* Pc*rfx1* gene. Oligonucleotides RFXBglIIF and RFXStuIR (See Supplementary Table S1) were used to amplify the 2782-bp Pc*rfx1* gene. The PCR product was digested with *Bgl*II and *Stu*I and cloned between the strong *Aspergillus awamori gdh* gene promoter and the *Saccharomyces cerevisiae cyc*1 transcriptional terminator into pIBRC43BglII (Kosalková et al., 2009), which was previously digested with *Bgl*II-*Stu*I, thus generating plasmid pIBRC43-Pcrfx1. This plasmid was used to cotransform the *P. chrysogenum* Wisconsin 54-1255 PyrG- strain with plasmid pBG, which includes the *P. chrysogenum pyrG* gene.

Plasmids pZ2bAB, pZ2bC and pZ3bDE contain respectively, the promoters of the *pcbAB*, *pcbC* and *penDE* genes coupled to the *lacZ* gene (Gutiérrez et al., 1999). Deletion or replacement of the putative PcRFX1 DNA binding sequences that are present in the promoter region of the penicillin biosynthetic genes were carried out in these plasmids using the QuikChange® II XL site-directed mutagenesis kit (Stratagene), following manufacturer's instructions.

With this purpose, several primers were designed (Supplementary Table S1). Deletions and substitutions were confirmed by sequencing.

2.3. Transformation of P. chrysogenum protoplasts

Protoplasts were obtained and transformed as previously described (Cantoral et al., 1987). Briefly, spores from *P. chrysogenum* were inoculated into MPPY medium supplemented with 140 μg/ml uridine (for auxotrophs) and incubated in an orbital shaker at 250 rpm for 20 h at 25 °C. Mycelia were collected by filtration through Nytal filters and washed twice with 100 ml NaCl 0.9% (w/v). Up to 2 g of mycelia were lysed at 28 °C and 80 rpm using fungal lytic enzymes (Sigma) until the release of protoplasts was completed. Protoplasts were collected and transformed using polyethylene glycol 6000. After transformation, protoplasts were grown in Czapeck minimal medium (García-Estrada et al., 2008a). Selection of transformant clones was achieved by the complementation of uridine auxotrophy or by resistance to phleomycin (final concentration 30 μg/ml).

2.4. Genomic DNA extraction and Southern blotting

Spores from *P. chrysogenum* were inoculated into MPPY medium supplemented with 140 μg/ml uridine (if necessary), and incubated in an orbital shaker at 250 rpm for 24 h at 25 °C. The mycelium was collected by filtration through Nytal filters, washed twice with 100 ml NaCl 0.9% (w/v) and dried. Samples were frozen in liquid nitrogen and ground with a mortar until a fine powder was obtained. One hundred mg of this powder were treated with 0.5 ml 0.18 M Tris/HCl pH 8.2; 10 mM EDTA; 1% (w/v) SDS and 0.5 ml phenol/chloroform/isoamyl alcohol (25:24:1, by vol.; phenol-CIA) and incubated for 30 min at 50 °C. Then, phenol-CIA treatment was repeated until the interface was clear. DNA was precipitated with ethanol and resuspended in Milli-Q H_2O.

A total of 4 μg genomic DNA from *P. chrysogenum* were digested with appropriate endonucleases for Southern hybridizations. Genomic DNA was electrophoresed in 0.8% agarose gels and blotted onto nylon membranes (Hybond-N; GE Healthcare) using a vacuum system (Pharmacia VacuGene). Hybridizations were done at 65 °C using 5× SSC (1× SSC: 0.15 M NaCl/0.015 M sodium citrate); 0.1% lauroyl sarcosine; 0.02% (w/v) SDS and 2% (w/v) blocking reagent. Digoxigenin labelling of DNA fragments was performed by random-priming with the DIG-High Prime kit (Roche) according to the manufacturer's instructions. Detection was carried out by chemiluminescence using the CDP-Star kit (Roche), following manufacturer's instructions.

2.5. RNA extraction

RNA was extracted from cultures of *P. chrysogenum* at 24 h, 48 h and 72 h using "RNeasy Mini Kit" columns (Qiagen, Hilden, Germany), following the manufacturer's instructions. 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.

2.6. Semiquantitative RT-PCR assays

RT-PCR was performed using 200 ng of total RNA and the "SuperScript One-Step RT-PCR with Platinum Taq" system (Invitrogen Corporation, Carlsbad, CA, USA), following the manufacturer's instructions. Several primers were designed with this purpose (see Supplementary Table S1). The signals provided by the RT-PCR assays were quantified by densitometry using the "GelPro Analizer" software (Media Cybernetics). The transcript levels were normalized comparing the intensity of each mRNA signal to the β-actin mRNA signal. Expression levels were considered as significantly different according to the standard deviation and when the *p*-value provided by the Student's *T* test was $p < 0.01$.

2.7. Complementary DNA synthesis and qPCR experiments

Total RNA was extracted and treated as indicated before. Retrotranscription was carried out with the Superscript III Reverse Transcriptase (Invitrogen) using 2 μg of total RNA as template and random primers, following manufacturer's instructions. The reaction volume (20 μl) was twofold diluted in RNase-free H_2O and 2 μl of that dilution were used for qPCR (see below).

Gene expression was analyzed by qPCR following the recommendations and conditions described by Applied Biosystems. For this purpose, several primers were designed (Supplementary Table S2). Annealing temperatures and primer concentrations were optimized to obtain the highest efficiency (close to 100%) with no more than 10% differences between each set of primers. All reactions were performed in a volume of 20 μl, which contained 10 μl of SYBR Green PCR master mix (Applied Biosystems), 6.8 μl of H_2O, 0.6 μl of each primer (final concentrations ranging from 0.3 to 0.5 μM, each) and 2 μl of cDNA. Thermo cycling conditions were as follows: 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at annealing temperatures ranging from 56 °C to 64 °C. Quantitation was carried out with a StepOnePlus™ Real-Time PCR system (Applied Biosystems). Appropriate negative controls containing no template DNA (negative control) or total RNA (RT-negative control) were performed. At the end of each reaction a melting curve was performed to check the specificity of real-time PCR reactions and the absence of primer dimer formation by holding the samples at 60 °C for 60 s followed by slow ramping of temperature to 95 °C. SYBR Green fluorescence was normalized by ROX fluorescence and baseline and threshold values were determined by StepOnePlus™ software. The *actA* gene (encoding the β-actin) was used as internal control for normalization. Since the optimal conditions (temperature and concentration) were established for each set of primers, the relative expression of each gene was calculated following the mathematical model based on the $2^{-\Delta\Delta CT}$ Method (Livak and Schmittgen, 2001). Results were expressed as the mean and standard deviation of five biological replicates and three technical replicates each. Expression levels were considered as significantly different according to the standard deviation and when the *p*-value provided by the Student's *T* test was $p < 0.01$.

2.8. Cell Extracts and β-galactosidase Assays

Mycelia were grown in CP medium at 48 h, harvested by filtration through Nytal filters, washed with 0.9% (w/v) NaCl, cooled rapidly in liquid nitrogen and stored at -20 °C. The extraction of cellular proteins for the β-galactosidase assay and the measurement of β-galactosidase activity were previously described (Kosalková et al., 2000). Briefly, the mycelial cake was ground to a fine powder with liquid nitrogen in a mortar. The powder was resuspended in 1 ml of extraction buffer (50 mM sodium phosphate buffer, pH 7.0; 1 mM EDTA supplemented with tablets (one tablet/20 ml of buffer) of the protease inhibitor mixture Complete™ (Roche Applied Science)), and kept on ice for 30 min. Extracts were centrifuged at 4000 rpm for 10 min at 4 °C and the supernatants were transferred to clean tubes, centrifuged at 13,200 rpm for 10 min at 4 °C, and stored on ice. Protein concentration was determined by the Bradford method (Bio-Rad). O-nitrophenyl-β-D-galactopyranoside was used as substrate for the β-galactosidase assays.

2.9. Western blotting

Protein extraction and immunological detection of IAT was performed using protein extracts obtained from *P. chrysogenum* Wisconsin 54-1255 (control strain) and Pc*rfx1* knock-down transformants (see Section 3.3). *P. chrysogenum* mycelia were harvested by filtration through Nytal filters, washed with 0.9% (w/v) NaCl and powdered in a grinder using liquid nitrogen. The disrupted cells were resuspended in TD buffer (50 mM Tris–HCl pH 8.0, 5 mM DTT). Extracts were centrifuged at 4000 rpm for 10 min at 4 °C and the supernatants were transferred to clean tubes, centrifuged at 13,200 rpm for 10 min at 4 °C, and stored at −80 °C. Protein concentration was determined by the Bradford method (Bio-Rad). A total of 5 μg of protein was mixed with loading buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 100 mM DTT, 10% glycerol and 0.1% bromophenol blue), boiled for 5 min, and run in 12% SDS–PAGE. The "Precision Plus Protein All Blue Standards" (Bio-Rad, Hercules, CA, USA) was used as molecular weight marker. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore) using a wet transfer procedure and a MiniProtean II system (Bio-Rad), following manufacturer's instructions. The membrane was incubated with the primary polyclonal antibody raised against *P. chrysogenum* IAT (Fernández et al., 2003) and with the alkaline phosphatase-conjugated secondary antibody. Detection was achieved using NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3́-indolylphosphate p-toluidine salt) (Roche), following manufacturer's instructions. Immunological detection of α-tubulin was used as control. The experiment was carried out as indicated above, but using a monoclonal antibody raised against the human α-tubulin (Sigma–Aldrich) and the specific secondary antibody conjugated with alkaline phosphatase.

Signals provided by western blot assays were quantified by densitometry using the "Gel-Pro Analizer" software (Media Cybernetics). Protein levels were normalized comparing the intensity of each IAT signal to the corresponding α-tubulin signal. Differences were considered as significant according to the standard deviation and when the *p*-value provided by the Student's *T* test was $p < 0.01$.

2.10. Penicillin bioassays and HPLC analysis

Production of total penicillins was quantified by bioassay as previously described (García-Estrada et al., 2008b). Briefly, *Micrococcus luteus* (test microorganism) was grown at OD600 = 2 in TSB (Difco) and inoculated in TSA (Difco) medium at a final OD600 = 0.01. This inoculum was poured in a Petri dish until it was solidified. Filtrates (50 μl) obtained from *P. chrysogenum* cultures were added to wells performed in the solidified *M. luteus* inoculum. The assay was left for 2 h at 4 °C (antibiotic diffusion into the agar) and then placed at 30 °C for 16 h. Inhibition halos were measured. Penicillin production was assessed using a calibration curve performed with increased concentrations of potassium benzylpenicillin.

Analysis and quantitation of benzylpenicillin and IPN were carried out by HPLC as previously described (García-Estrada et al., 2007). An Agilent 1100 HPLC system (Santa Clara, CA, USA) with an analytical 4.6 × 250 mm (5 μm) RPC18 Lichrospher® 100 (Merck, Darmstadt, Germany) column, a flow rate of 1 ml/min, a detector wavelength of 214 nm and an isocratic method were used for the analysis of benzylpenicillin. For the analysis of IPN, samples were derivatizated with fluoronylmethyl chloroformate (FMOC) (Sigma–Aldrich) and analyzed in the same HPLC system as described above, but using an analytical 4.6 × 150 mm (3.5 μm) Eclipse XDB-C18 (Agilent) column and an elution gradient with a flow rate of 1 ml/min. Detection was performed at 254 nm.

2.11. Electrophoretic mobility shift assay (EMSA)

P. chrysogenum Wisconsin 54-1255 was grown for 48 h in CP medium. The mycelium was harvested by filtration through Nytal filters, washed with 0.9% (w/v) NaCl, frozen in liquid nitrogen, and stored at −80 °C. The mycelium was ground to a fine powder in a mortar with liquid nitrogen and resuspended in 1 volume of extraction buffer: 20 mM HEPES, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 2 mM dithiothreitol, pH 7.9, one tablet/20 ml of the protease inhibitor mixture Complete™ (Roche Applied Science). The mixture was kept on ice for 30 min. The suspension was centrifuged at 4000 rpm for 5 min at 4 °C. The supernatant was transferred to clean tubes and centrifuged at 14,000 rpm for 10 min at 4 °C. The clean supernatant (crude extract) was used for the binding reaction.

The putative PcRFX1 DNA binding box included in the promoter region of the *penDE* gene (see Section 3.2) was analyzed in this experiment. For this purpose, a probe consisting of annealed oligonucleotides boxXF and boxXR was used. These primers were 25 nt long and included the 13-nt putative PcRFX1 DNA binding box of the *penDE* gene promoter in the middle of the sequence (see Supplementary Table S1). Primer boxXF was designed to be labelled with Cy3 at the 5′ end. DNA binding assay using crude extracts of *P. chrysogenum* Wisconsin 54-1255 (2.5–10 μg) was performed by standard EMSA as follows: a 30 μl reaction mixture contained 1.5 μl of binding buffer (1 M NaCl, 200 mM Tris, 50 mM $MgCl_2$, 50% glycerol, pH 7.5) and poly-(dI-dC) (1 μg). The DNA probe (1 pmol) was added, and the mixtures were incubated at 25 °C for 20 min. A native 5% polyacrylamide (29:1) gel was pre-run in 0.5X TBE buffer for 30 min at 100 V. The electrophoresis was performed for l h at 100 V using a Bio-Rad Mini Protean III apparatus. After electrophoresis, the gel was scanned in an Ettan DIGE Imager charge-coupled device (CCD) camera (GE Healthcare) and analyzed using the software ImageQuant TL (GE Healthcare).

3. Results

3.1. Characterization and expression of the P. chrysogenum CPCR1 ortholog (PcRFX1)

In silico analysis of the *P. chrysogenum* genome (van den Berg et al., 2008) revealed the presence of a gene encoding a putative CPCR1 ortholog (Pc20g01690). The whole sequence of the gene encoding the CPCR1 ortholog, hereafter referred to as Pc*rfx1* (2782 bp, four introns), codes a protein of 859 amino acids (PcRFX1) with strong similarity to several RFX proteins from different filamentous fungi (i.e. 80% similarity and 67% identity to a DNA damage and replication checkpoint protein RFX1 from *Aspergillus clavatus*, 74% homology and 61% identity to the *P. marneffei* RfxA protein and 47% homology and 30% identity to the *A. chrysogenum* CPCR1 protein). The DNA-binding domains of the *P. chrysogenum* and *A. chrysogenum* RFX orthologs shared 78% similarity and 60% identity, whereas the DNA-binding domains of the *P. chrysogenum* and *P. marneffei* RFX orthologs shared 97% similarity and 93% identity (Fig. 1A). The Pc*rfx1* gene was amplified from the wild-type strain *P. chrysogenum* NRRL 1951 using primers listed in Supplementary Table S1. After sequencing, it was confirmed that the Pc*rfx1* genes from strains NRRL 1951 and Wisconsin 54-1255 are 100% identical with each other (data not shown).

The expression of the Pc*rfx1* gene in the Wisconsin 54-1255 strain was tested by RT-PCR using RNA extracted at 24 h, 48 h and 72 h from cultures grown in CP medium, which supports high penicillin production. One band was visible after retrotranscription and amplification of the Pc*rfx1* gene transcript (Fig. 1B). Quantitation of the band intensity revealed that the higher expression

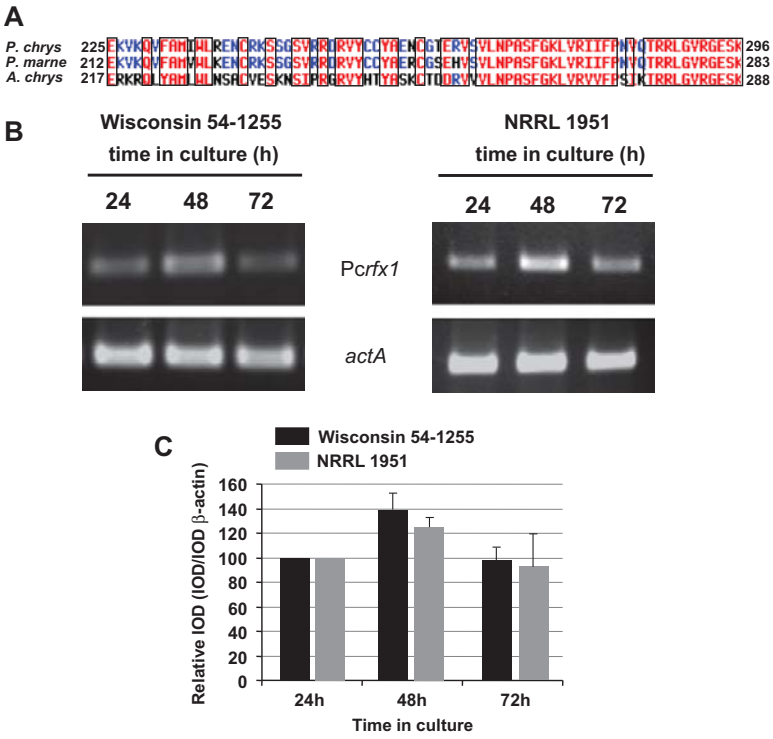

Fig. 1. Characterization and expression of the *P. chrysogenum* CPCR1 ortholog (PcRFX1). (A) Alignment of the PcRFX1 (*P. chrysogenum*), RfxA (*P. marneffei*) and CPCR1 (*A. chrysogenum*) DNA binding domains. Common motifs inside the DNA binding domain of these proteins are included in a frame. (B) Semiquantitative RT-PCR showing the expression profiles of Pc*rfx1* in *P. chrysogenum* Wisconsin 54-1255 and NRRL 1951 at 24 h, 48 h and 72 h. The signal provided by the β-actin gene (*actA*) was used as control. (C) The intensity of the bands (IOD) obtained in panel B was determined by densitometry. Those values corresponding to the expression of the Pc*rfx1* gene at 24 h were set to 100. Results correspond to the mean plus standard deviation of three independent measurements. Note that there are no significant differences between these two strains.

levels were reached at 48 h (Fig. 1C), coinciding with the phase of intense penicillin production. Similar results were obtained when the expression of this gene was analyzed in the wild-type strain of *P. chrysogenum* (NRRL 1951) (Fig. 1B and C).

3.2. Identification of putative DNA binding sites in the penicillin biosynthetic gene promoters

Due to the regulatory role played by CPCR1 after binding the *pcbAB-pcbC* promoter of *A. chrysogenum* (Schmitt and Kück, 2000), we analyzed the promoter region of the penicillin biosynthetic genes in search for putative PcRFX1 DNA binding sites. On the basis of the DNA binding sequences previously reported for RFX (Emery et al., 1996), no evident putative DNA binding sites for PcRFX1 were found in the promoter regions of the three biosynthetic genes. Similarly, when we used the two sequences reported for CPCR1 (Schmitt et al., 2004b), no putative DNA binding sites for PcRFX1 were found in the promoter regions of the three biosynthetic genes. However, combination of the DNA binding sequences for RFX and CPCR1 allowed the identification of a putative DNA binding site for PcRFX1 in the promoter region of the *pcbC* gene. The last five nucleotides of this binding site also include the CCAAT box (see Section 4). When we considered the DNA binding sequence proposed for RfxA in *P. marneffei* (Bugeja et al., 2010), one putative DNA binding site was found in the promoter region of the *pcbAB* gene. In the promoter region of the *penDE* gene, initially we did not find a clear PcRFX1 binding site using those consensus sequences, but modification of one nucleotide (corresponding to the first nucleotide of the above-mentioned consensus sequences) gave rise to the identification of a putative PcRFX1 binding site in the promoter region of the *penDE* gene. In light of these results, a new consensus sequence based on the above-mentioned sequences and taking into account the sequence of the penicillin biosynthetic gene promoters was established. With this sequence (NNRCCNNRSHWAY), one putative binding sequence was found in both *pcbC* and *penDE* promoter regions and two putative PcRFX1 binding sites, which overlap each other in four nucleotides, were found in the promoter region of the *pcbAB* gene (Fig. 2A). It is noteworthy that a GATA box is present in the convergence of the two putative PcRFX1 binding boxes of the *pcbAB* promoter (see Section 4).

In order to test the functionality of the putative PcRFX1 DNA binding sequences that were found in the penicillin biosynthetic gene promoters, reporter assays were carried out using plasmids bearing either *pcbAB*, *pcbC* or *penDE* promoter regions coupled as an in-frame translational unit with the *lacZ* gene encoding β-galactosidase. This promoter-reporter gene fusion was introduced in a vector designed for targeted integration at the pyrG locus in *P. chrysogenum* Wisconsin 54-1255 npe10 PyrG- following the approach described by Gutiérrez et al. (1999). *P. chrysogenum* Wisconsin 54-1255 npe10 PyrG- is an uridine auxotroph that lacks the whole penicillin gene cluster, thus avoiding interference by the expression of the endogenous copy of this cluster (Cantoral et al., 1993). The resultant plasmids were mutated in order to either delete or replace the putative PcRFX1 DNA binding sequences (see Section 2.2).

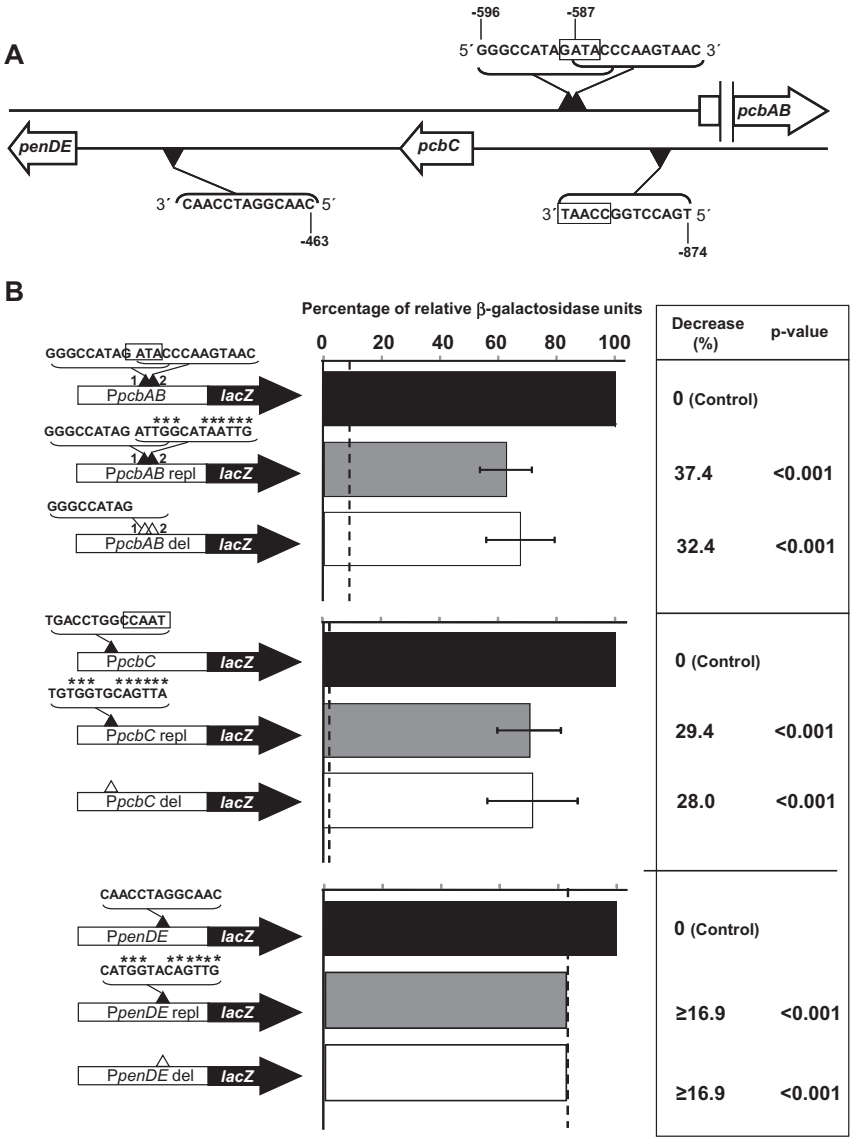

Fig. 2. Functional analysis of putative DNA binding sites in the penicillin biosynthetic gene promoters. (A) Schematic representation of the penicillin gene cluster. Putative PcRFX1 DNA binding sequences are indicated and represented as black triangles in the sense DNA strand for each biosynthetic gene. The position of the first nucleotide of each PcRFX1 DNA binding sequence (regarding the start codon of the corresponding biosynthetic gene) is also indicated. CCAAT and GATA boxes inside two of the putative PcRFX1 DNA binding sequences are included in a frame. (B) β-galactosidase reporter activity after replacement (indicated as an asterisk over the corresponding nucleotide) or deletion of the putative PcRFX1 DNA binding sequences present in the *pcbAB*, *pcbC* and *penDE* promoters. A scheme of each reporter plasmid, containing the *lacZ* gene (black arrows) is represented. Putative PcRFX1 DNA binding sequences are indicated and represented as black triangles, whereas deletion of these boxes is represented as white triangles. Those β-galactosidase values provided by the unmodified *pcbAB*, *pcbC* and *penDE* gene promoters were set to 100%. Results correspond to the mean plus standard deviation of three independent experiments carried out in triplicate. The cut-off line (β-galactosidase levels provided by the untransformed control strain), is represented as a dotted line.

P. chrysogenum Wisconsin 54-1255 npe10 transformants that had incorporated at the *pyrG* locus a single copy of the vectors carrying the penicillin biosynthetic gene promoters fused to the *lacZ* gene were selected by their restriction pattern after Southern blot analysis (data not shown). Reporter gene expression from these promoters was tested by assaying the β-galactosidase activity in mycelia collected from cultures at 48 h. Untransformed controls showed a very low endogenous β-galactosidase activity that was set as the cut-off line. Another control, which consisted of a plasmid with the *lacZ* gene without promoter regions, was also transformed, providing β-galactosidase levels similar to those provided by untransformed controls. Transformants including the *pcbAB* and *pcbC* promoters fused to the *lacZ* gene provided high β-galactosidase activity, unlike the *penDE* promoter, which gave rise to low levels of β-galactosidase (Fig. 2B), confirming an observation previously made on the low level of expression of this promoter

(Gutiérrez et al., 1999). Replacement or full deletion of the second putative PcRFX1 DNA binding sequence in the *pcbAB* promoter also led to the modification or deletion of the first putative PcRFX1 DNA binding box (Fig. 2B) and to the modification or deletion of the GATA motif. In the same way, replacement or full deletion of the putative PcRFX1 DNA binding sequence in the *pcbC* promoter also led to the modification or deletion of the CCAAT motif (Fig. 2B). In general, replacement or full deletion of any of the putative PcRFX1 DNA binding sequences that are present in the biosynthetic gene promoters led to a significant reduction in β-galactosidase levels (around 30%). Since β-galactosidase levels obtained with the *penDE* promoter are slightly above the cut-off line (provided by the untransformed control), it was difficult to estimate the real effect of deletion/replacement of the targeted sequences in this promoter, although these modifications always led to a reduction in the reporter activity (Fig. 2B). These results indicate that the putative PcRFX1 DNA binding sequences located on the penicillin biosynthetic gene promoters are functional, although the presence of other motifs within the PcRFX1 DNA binding sequences of the *pcbAB* and *pcbC* promoters may interfere with the interpretation of these results (see Section 4).

In order to confirm the ability of these regions to bind proteins, we decided to carry out EMSA experiments with the putative PcRFX1 DNA binding region of the *penDE* gene promoter, since this region did not include other known binding motifs (such as GATA or CCAAT) that may interfere with the experiment. Two primers, one of them labelled at the 5′ end with Cy3 were annealed and used as probe (25 nt including the 13-nt putative PcRFX1 DNA binding region of the *penDE* gene promoter (see Section 2.11)). Three different amounts (2.5 μg, 5 μg and 10 μg) of crude protein extracts obtained from 48-h cultures of *P. chrysogenum* Wisconsin 54-1255 were tested with this probe (Fig. 3). A single retardation complex was formed and the intensity of this band was dependent on the protein amount present in the assay. The specificity of the binding process was tested in the presence of BSA (20 μg), which failed to produce an electromobility shift of the same DNA probe. This indicates that at least the putative PcRFX1 DNA binding region of the *penDE* gene promoter is able to bind proteins from *P. chrysogenum*.

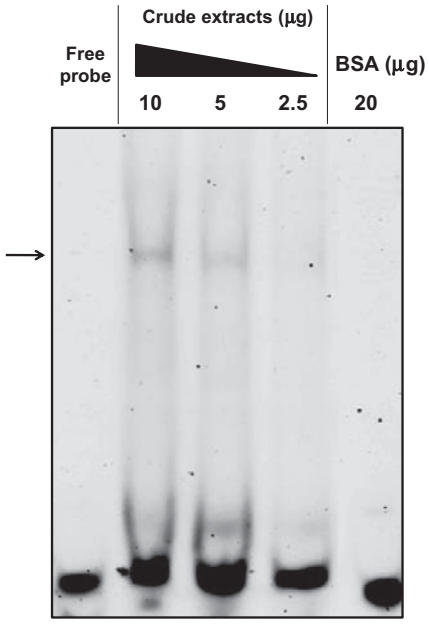

Fig. 3. DNA binding assay with the putative PcRFX1 DNA binding box included in the promoter region of the *penDE* gene. The 25-nt Cy3-labelled probe including the putative PcRFX1 DNA binding region of the *penDE* gene promoter (13 nt) was incubated with different amounts (2.5 μg, 5 μg and 10 μg) of crude protein extracts obtained from 48-h cultures of *P. chrysogenum* Wisconsin 54-1255. The retardation complex is indicated with an arrow. The specificity of the assay was tested with BSA.

3.3. PcRFX1 controls the expression of the penicillin biosynthetic genes pcbAB, pcbC and penDE

Since the above-mentioned results suggested the functionality of the putative PcRFX1 DNA binding sequences present in the penicillin biosynthetic gene promoters, we decided to test whether PcRFX1 regulates the expression of *pcbAB*, *pcbC* and *penDE* genes. For this purpose, gene silencing of Pc*rfx1* was carried out using plasmid pJL43-RNAi-*rfx*, which includes a 479-bp Pc*rfx1* exonic fragment (see Section 2.2). After transformation of *P. chrysogenum* protoplasts, several phleomycin-resistant transformants were obtained. Several transformants were randomly selected and were analyzed by PCR to confirm the presence of the silencing cassette (data not shown). Seven positive transformants were further analyzed by Southern blotting using as probe the digoxigenin-labelled 479-bp Pc*rfx1* exonic fragment described above. These transformants showed a correct amplification of the silencing cassette (Fig. 4A). Transformants T1r and T7r provided only one band (2.1 kbp) corresponding to the silencing cassette, whereas transformant T2r showed several bands, probably as a consequence of the ectopic integration of partial silencing fragments in the genome. Transformants T3r, T4r, T5 and T6r showed intermediate situations. In all the cases, the 6.6-kbp band corresponding to the DNA fragment containing the internal Pc*rfx1* gene is visible (Fig. 4A). Pc*rfx1* gene-silencing was confirmed by qPCR (Fig. 4B) using RNA samples taken from cultures grown in CP medium at 48 h, which is the time point when the higher expression levels of Pc*rfx1* are achieved as indicated before. Transformants T1r, T2r, T6r and T7r showed a significant reduction in Pc*rfx1* gene expression, with decreases between 3.39-fold (for T2r) and 2.67-fold (for T6r), whereas the rest of transformants seemed not to modify the expression of the Pc*rfx1* gene.

The expression of the penicillin biosynthetic genes *pcbAB*, *pcbC* and *penDE* was assessed by qPCR in the seven transformants mentioned above. For this purpose, RNA samples were taken at 48 h from cultures grown in CP medium. Fig. 4C shows that gene silencing of Pc*rfx1* leads to a reduction in the expression of *pcbAB* (fold decreases between 2.76 for T1r and 1.85 for T7r), *pcbC* (fold decreases between 2.59 for T2r and 1.83 for T6r) and *penDE* (fold decreases between 3.46 for T2r and 1.94 for T6r) in transformants T1r, T2r, T6r and T7r. This result is concordant with the reduction in the expression of the Pc*rfx1* gene shown by the same transformants (Fig. 4B).

In order to confirm whether the decrease in the steady-state levels of the *penDE* gene transcripts shown by transformants T1r, T2r, T6r and T7r was translated in a decrease in the levels of the *penDE*-encoded IAT, western blot analysis was carried out. Protein extracts taken at 48 h from cultures of those transformants were hybridized with antibodies raised against IAT. Results confirmed a reduction in the IAT levels in all transformants (Fig. 4D).

To further characterize the role of Pc*rfx1*, overexpression of this gene was also carried out. The *P. chrysogenum* Wis54-1255 PyrG- strain was cotransformed together with the pIBRC43-Pcrfx1 vector (see Section 2.2), which contains the Pc*rfx1* gene under the control of the strong *A. awamori gdh* gene promoter (Fig. 5A), and plasmid pBG (which contains the *pyrG* gene). Those transformants complementing the uridine auxotrophy were selected and analyzed by

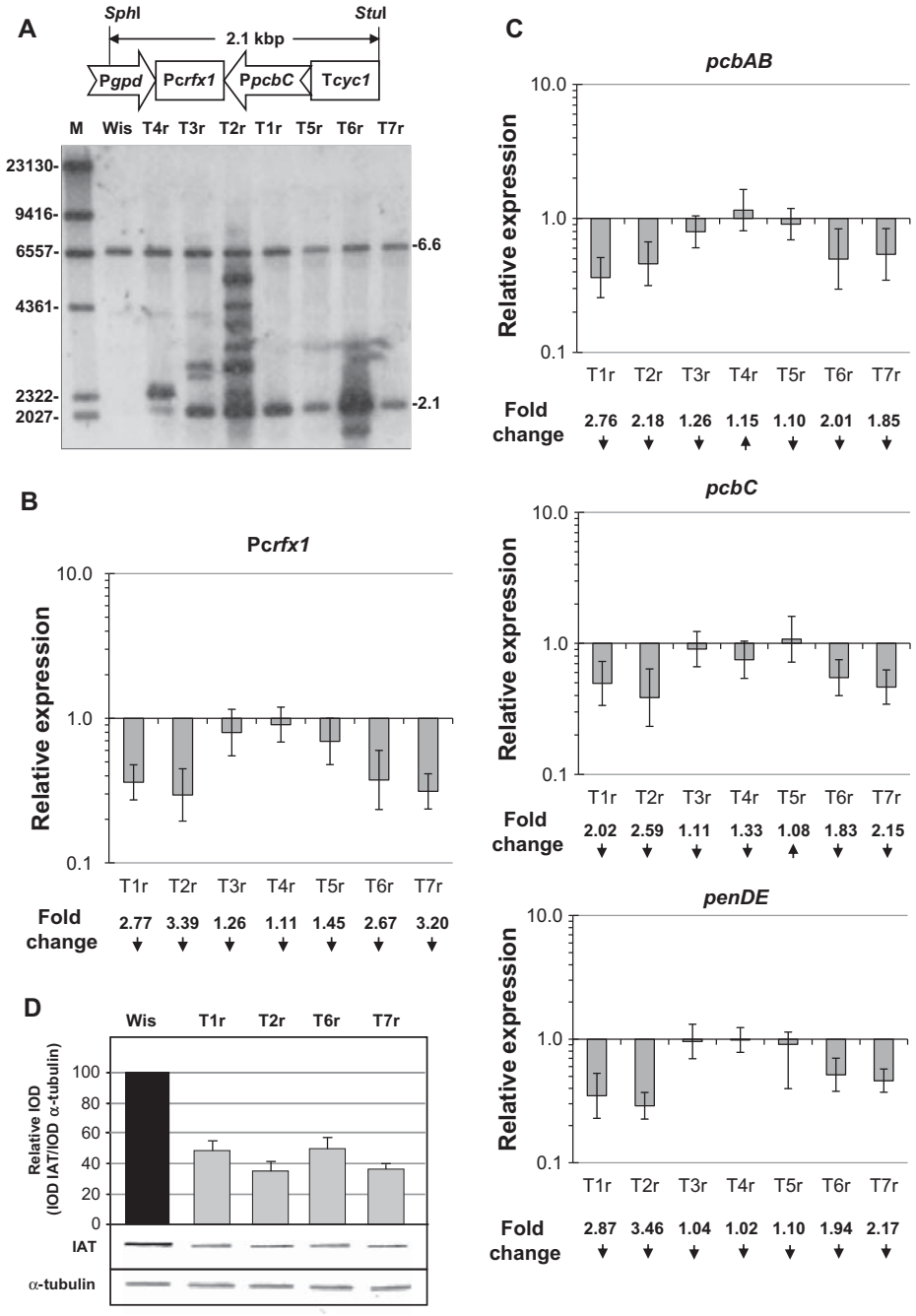

Fig. 4. Gene silencing of Pcrfx1. (A) Southern blot analysis of transformants and the parental *P. chrysogenum* Wisconsin 54-1255 strain (Wis) showing integration of the full silencing cassette (2.1 kbp). (B) Relative expression (quantified by real-time qPCR) of P*crfx1* in transformants T1r-T7r compared to the Wisconsin 54-1255 strain. Values correspond to the mean plus standard deviation of five independent experiments. Fold change is indicated for each transformant. The increase or decrease in relative expression is represented by arrows. (C) Relative expression (quantified by real-time qPCR) of *pcbAB*, *pcbC* and *penDE* in transformants T1r-T7r compared to the Wisconsin 54-1255 strain. Values correspond to the mean plus standard deviation of five independent experiments. Fold change is indicated for each transformant. The increase or decrease in relative expression is represented by arrows. (D) Translational analysis of IAT in *P. chrysogenum* Wisconsin 54-1255 strain (Wis) and transformants T1r, T2r, T6r and T7r. The immunoreactive band of α-tubulin was used as control. The intensity of the signals (IOD) provided by western blot was determined by densitometry. IAT values were normalized to those provided by α-tubulin. Values corresponding to the IAT immunoreactive band for the Wisconsin 54-1255 strain (Wis) were set to 100. Values correspond to the mean plus standard deviation of three independent measurements.

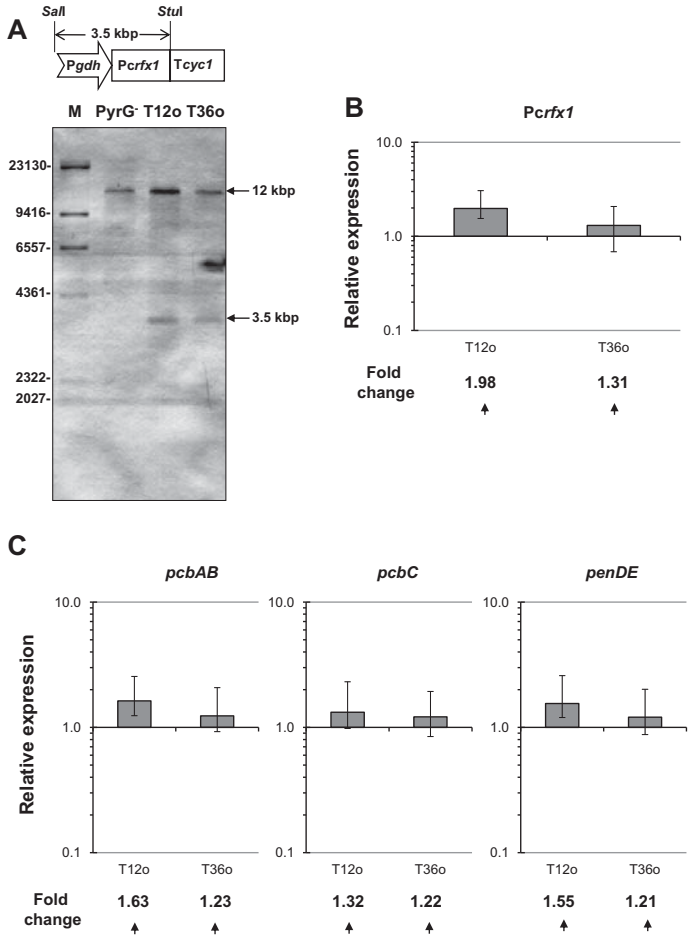

Fig. 5. Overexpression of the Pc*rfx1* gene. (A) Southern blot analysis of transformants and the parental *P. chrysogenum* Wisconsin 54-1255 PyrG- strain (PyrG-) showing integration of the overexpression cassette (3.5 kbp). (B) Relative expression (quantified by real-time qPCR) of Pc*rfx1* in transformants T12o and T36o compared to the Wisconsin 54-1255 PyrG- strain. Values correspond to the mean plus standard deviation of five independent experiments. Fold change is indicated for each transformant. The increase in relative expression is represented by arrows. (C) Relative expression (quantified by real-time qPCR) of *pcbAB*, *pcbC* and *penDE* in transformants T12o and T36o compared to the Wisconsin 54-1255 PyrG- strain. Values correspond to the mean plus standard deviation of five independent experiments. Fold change is indicated for each transformant. The increase in relative expression is represented by arrows.

PCR to confirm the presence of the full overexpression cassette (data not shown). Two transformants that met this requirement were tested by Southern blot (Fig. 5A) to confirm the presence of the overexpression cassette in the *P. chrysogenum* genome. The 479-bp Pc*rfx1* exonic fragment described above was labelled with digoxigenin and used as probe. These transformants (T12o and T36o) showed a 3.5-kbp band, which contains the Pgdh–Pc*rfx1* integrative cassette, in addition to the band containing the endogenous gene (12 kbp).

Pc*rfx1* gene overexpression was assessed in these transformants by qPCR using RNA extracted from 48-h cultures grown in CP medium (Fig. 5B). Transformant T12o showed a significant 1.98-fold increase in the Pc*rfx1* mRNAs at 48 h, whereas transformant T36o did not significantly overexpressed the Pc*rfx1* gene. The effect of Pc*rfx1* overexpression on the transcription of the penicillin biosynthetic genes was tested by qPCR. RNA samples were taken from cultures of transformants T12o and T36o grown in CP medium for 48 h. Overexpression of Pc*rfx1* in transformant T12o leads to a slight increase in the *pcbAB* (1.63-fold) and *penDE* (1.55-fold) mRNA levels (Fig. 5C). The steady-state levels of the *pcbC* gene transcript seemed not to be affected by the overexpression of Pc*rfx1* in this transformant. As expected, transformant T36o showed no significant overexpression of the penicillin biosynthetic genes (Fig. 5C).

Taken together, these results indicate that although the effect of Pc*rfx1* overexpression on the penicillin biosynthetic genes expression was less marked than that of gene silencing, PcRFX1 plays a role in the control of penicillin biosynthetic genes.

3.4. PcRFX1 is a regulator of penicillin biosynthesis in P. chrysogenum

In order to confirm the effect of PcRFX1 in the process of penicillin biosynthesis in *P. chrysogenum* Wisconsin 54-1255, penicillin production was assessed in the knock-down transformants (T1r, T2r, T3r, T4r, T5r, T6r and T7r) as well as in the overexpression transformants (T12o and T36o). Samples were taken from three biological replicates cultured in CP medium for 24 h, 48 h and 72 h and analyzed by bioassay.

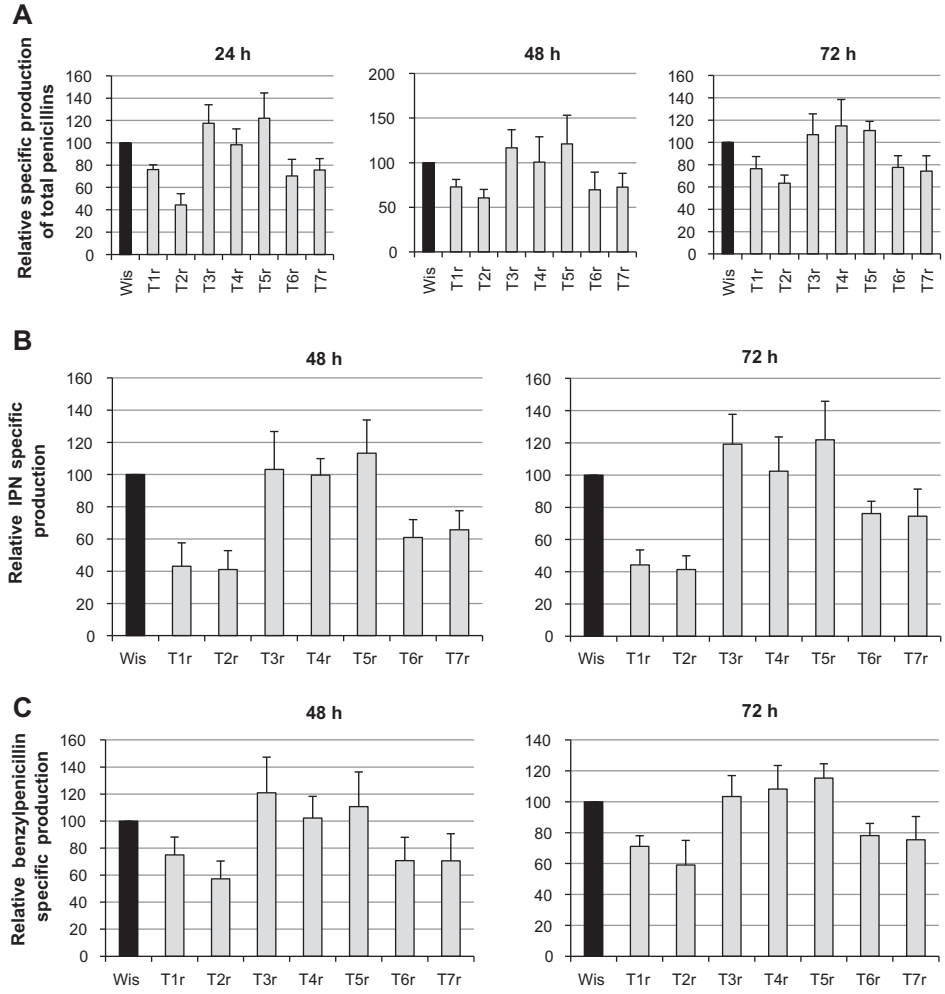

Fig. 6. Production of penicillins in the P*crfx1* knock-down transformants. Relative specific production of (A) total penicillins, (B) IPN and (C) benzylpenicillin in the Wisconsin 54-1255 strain (Wis) and in the P*crfx1* knock-down mutants (T1r-T7r) at different time points. Those values provided by the Wisconsin 54-1255 strain were set to 100. Results correspond to the mean plus standard deviation of three independent experiments carried out in triplicate.

As it is shown in Fig. 6A, the relative specific production of total penicillins was significantly reduced in the knock-down transformants T1r, T2r, T6r and T7r along the culture time, whereas transformants T3r, T4r and T5r showed non-significant increases in penicillin titers. Transformant T2r produced the lowest levels of penicillin (between 50% and 60% compared to the parental Wisconsin 54-1255 strain). These results are in agreement with the lower expression of the P*crfx1* gene observed for these transformants (Fig. 3B). In order to test whether the decrease in total penicillin levels observed for the knock-down transformants T1r, T2r, T3r and T4r was due to a decrease in benzylpenicillin or IPN levels, culture supernatants were taken at 48 h and 72 h, and analyzed by HPLC. The specific production of these antibiotics provided by this method revealed that both IPN and benzylpenicillin levels were reduced in these transformants. IPN production underwent a reduction between 59% (for T2r) and 35% (for T7r) at 48 h and between 59% (for T2r) and 24% (for T6r) at 72 h (Fig. 6B). Benzylpenicillin production was decreased between 43% (for T2r) and 25% (for T1r) at 48 h and between 41% (for T2r) and 22% (for T6r) at 72 h (Fig. 6C).

Interestingly, at 48 h and 72 h the overexpression transformant T12o produced about 60% more total penicillins than the control strain, whereas the overexpression transformant T36o produced about 40% more total penicillins at 48 h and showed an increase of about 20% at 72 h (Fig. 7A). Benzylpenicillin and IPN specific production was determined by HPLC using culture supernatants taken at 48 h and 72 h. IPN levels increased at 48 h (91% for T12o and 71% for T36o) and 72 h (72% for T12o and 116% for T36o), whereas benzylpenicillin specific production increased at 48 h (26% for T12o and 12% for T36o) and 72 h (67% for T12o and 18% for T36o) (Fig. 7B). The high increase in IPN levels suggests that some step in the transport of IPN to the peroxisomes may be limiting in the overexpression transformants.

These results indicate that PcRFX1 controls the initial and late steps of the penicillin biosynthetic process.

3.5. Other biological processes potentially controlled by PcRFX1

In order to test whether PcRFX1 is involved in controlling hyphal morphology in *P. chrysogenum*, the parental strain and the

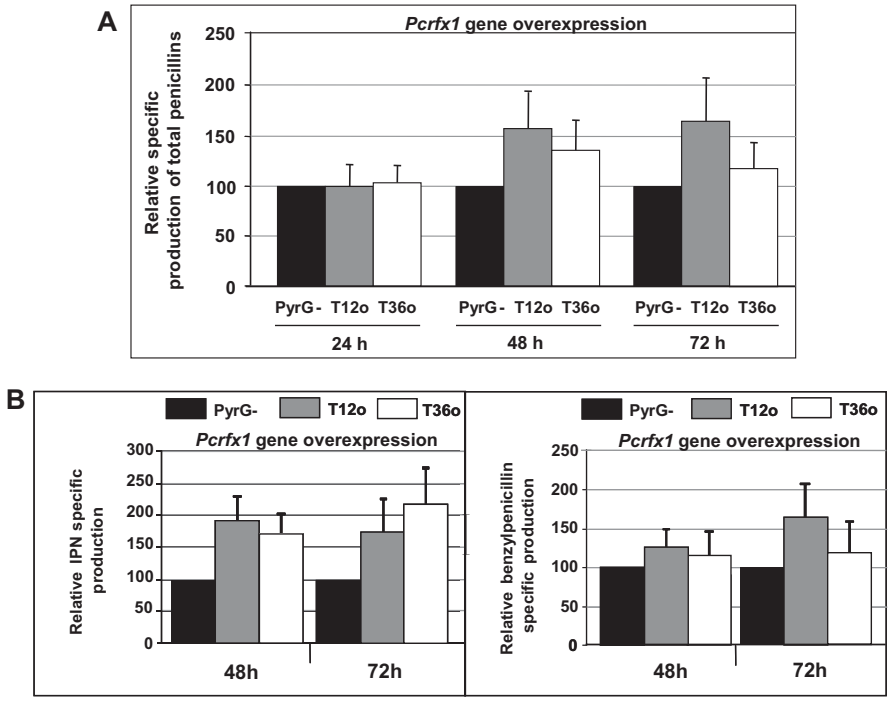

Fig. 7. Production of penicillins in the P*crfx1* overexpression transformants. Relative specific production of (A) total penicillins, (B) IPN and benzylpenicillin in the Wisconsin 54-1255 PyrG- strain (PyrG-) and in the P*crfx1* overexpression transformants (T12o and T36o) at different time points. Those values provided by the Wisconsin 54-1255 PyrG- strain were set to 100. Results correspond to the mean plus standard deviation of three independent experiments carried out in triplicate.

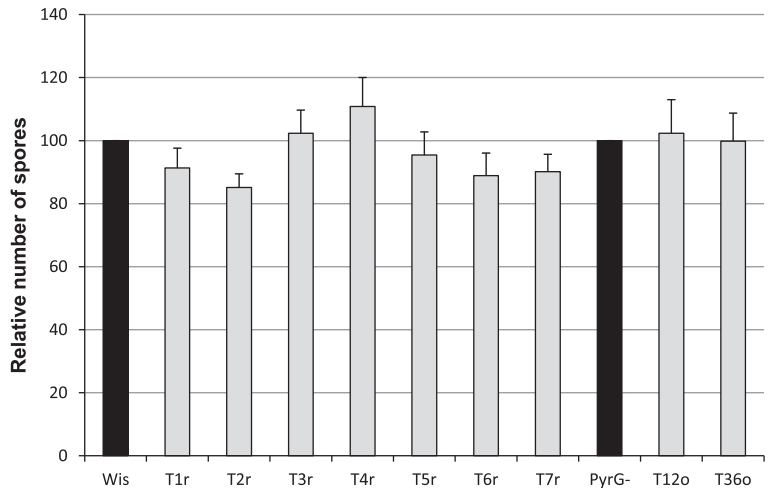

Fig. 8. Sporulation in the P*crfx1* knock-down and overexpression transformants. Spores were collected from Petri dishes from the parental strains (Wis and PyrG-), the knock-down (T1r-T7r) and the overexpression (T12o and T36o) transformants after six days of incubation, and counted. Those values provided by the parental strains were set to 100. Results correspond to the mean plus standard deviation of three independent experiments carried out in triplicate.

knock-down and overexpression transformants were analyzed by microscopy. Hyphal morphology and conidiophore formation were not modified after P*crfx1* gene silencing or overexpression (data not shown). After counting the spores present in Petri dishes sed with similar amount of spores (1×10^8) from the parental strain, the knock-down and overexpression transformants, a slight reduction was observed in the number of spores in the P*crfx1*-knock-down transformants T1r, T2r, T6r and T7r (Fig. 8). This reduction reached values of 14.87% (p-value < 0.01) in transformant T2r. On the contrary, the overexpression transformants did not show

Table 1
P. chrysogenum homologous genes to those putative genes of *P. marneffei* involved in cell division that have putative PcRFX1 consensus binding sites.[a]

P. chrysogenum ORF	Strand	Description	Putative function[a]	PcRFXI putative binding sites in *P. chrysogenum* (using the consensus sequences reported in this article)	PcRFXI putative binding sites in *P. chrysogenum* (using the consensus sequences proposed for *P. marneffei*[a]
Pc20g01370	+	strong similarity to calmodulin 6 CaM6 – *Arabidopsis thaliana*	Cytokinesis EF hand protein (Cdc4)	Not found	−262 (−)
Pc13g10210	−	strong similarity to cdc15p – *Schizosaccharomyces pombe*	Similarity to protein kinase (Cdc15) regulating the mitotic exit network	Not found	−194 (+)
Pc18g04320	+	Strong similarity to kinesin-related protein Kip3 – *Saccharomyces cerevisiae*	Related to kinesin protein (KipB) involved in mitotic-spindle dynamics	Not found	−264 (−)(+)
Pc22g09820	+	Strong similarity to spindle assembly checkpoint protein sldA – *Aspergillus nidulans*	Similarity to protein kinase (SldA/Bub1) regulating the spindle assembly checkpoint	−91 (+)	−149 (+)
Pc12g05830	−	Strong similarity to spindle pole body component bimB – *Aspergillus nidulans*	Related to separin protein (BimB) involved in chromatid segregation and DNA repair	Not found	−634 (−)(+), −112 (+)
Pc20g14730	−	Strong similarity to protein required for completion of mitosis and maintenance of ploidy like protein An02g04510 – *Aspergillus niger*	Maintenance of ploidy protein kinase (Mob1)	−331 (+)	−331 (−)
Pc20g06510	−	Strong similarity to serine/threonine protein kinase Rpk1 – *Saccharomyces cerevisiae*	Spindle checkpoint kinase (Mph1)	−146 (−)	−146 (−)
Pc22g24040	−	Strong similarity to serine/threonine protein kinase nimA – *Aspergillus nidulans*	G2-specific protein kinase (NimA)	Not found	−548 (+)(−)
Pc12g11470	−	Similarity to the protein involved in sister chromatid segregation Src1 – *Saccharomyces cerevisiae*	Sister chromatid separation protein (Src1)	−262 (+), −261 (−)	−262 (+)

[a] As it has been previously reported by Bugeja et al. (2010). Searches for the putative PcRFXI DNA binding sites have been carried out with a limit of 900 bp upstream of the gene of interest.

significant differences in the number of spores (Fig. 8). These results indicate that sporulation was slightly affected after Pc*rfx1* gene silencing.

It has been reported that nine genes putatively regulated by RfxA (PcRFX1 homolog) in *P. marneffei* are involved in cell division (Bugeja et al., 2010) (Table 1). We only found a putative PcRFX1 DNA binding site in four of these genes (*sldA*, *src1*, *mob1* and *mph1*) using the consensus sequences reported in this work (see Section 4). However, when the consensus sequence provided for *P. marneffei* (Bugeja et al., 2010) was used, putative PcRFX1 DNA binding sites appeard in the promoter region of the nine genes analyzed (Table 1). In an attempt to clarify whether all these genes or only some of them are controlled by PcRFX1 in *P. chrysogenum* we carried out experiments to assess the expression of those genes in the knock-down and overexpression transformants. RNA was extracted at 24 h, 48 h and 72 h from T2r (knock-down) and T12o (overexpression) transformants and the expression level of *src1*, *cdc4*, *sldA*, *bimB*, *cdc15*, *kipB*, *mph1*, *nimA* and *mob1* genes was analyzed by RT-PCR. All genes provided retrotranscription bands, with the exception of *mob1*, which was not able to be amplified under different conditions (data not shown). No consistent changes in the expression level of the above-mentioned genes were detected when Pc*rfx1* was either silenced or overexpressed (Fig. 9), indicating that they are not targets of this regulator under the conditions examined.

In an attempt to provide putative targets for PcRFX1 and to understand the cellular processes potentially controlled by this regulator, we did an *in silico* analysis of the *P. chrysogenum* genes that contain a putative PcRFX1 DNA binding sequence. For this study we used the consensus sequence NNRCCNNRSHWAY (see Section 4). Firstly, sites with a regulation pattern were found in the whole *P. chrysogenum* genome using the program Patmatch (Yan et al., 2005). These sites were filtered in order to obtain only those present in intergenic regions and within 900 bp upstream of the corresponding ORF. At the end of this process, 2502 genes putatively regulated by the PcRFX1 protein were found, of which 1176 have Gene Ontology annotation. These genes were analyzed in order to find a common functional signal in the group. Biological Process annotations of Gene Ontology were retrieved from UniProt (Dimmer et al., 2012) and a *p*-value for each functional annotation category in the gene group was calculated by means of a hypergeometric distribution test. The results were filtered in order to avoid the functional categories in the group with less than three genes and with *p*-values over 0.01. In this way, the characteristic functions of the putative regulated genes were statistically measured and identified. Table 2 shows the Gene Ontology terms of biological processes which are over-represented in the group of putative regulated genes. The functional analysis revealed that this transcription factor has a certain tendency to regulate genes involved in processes of primary metabolism (i.e. carbohydrate catabolism, chitin catabolism, polysaccharide catabolism, histidine biosynthesis, lipid metabolism, one-carbon metabolism, phospholipid biosynthesis).

4. Discussion

Penicillin is one of the most important secondary metabolites produced by microorganisms. During the last decades, the unprecedented joint effort between industry and academia has not only contributed to strain improvement and optimization of the industrial production of this β-lactam antibiotic, but to the understanding of some of the mechanisms responsible for the increased penicillin productivity as well (van den Berg et al., 2008; van den Berg, 2011; Barreiro et al., 2012). However, many of the global regulatory mechanisms still remain to be elucidated.

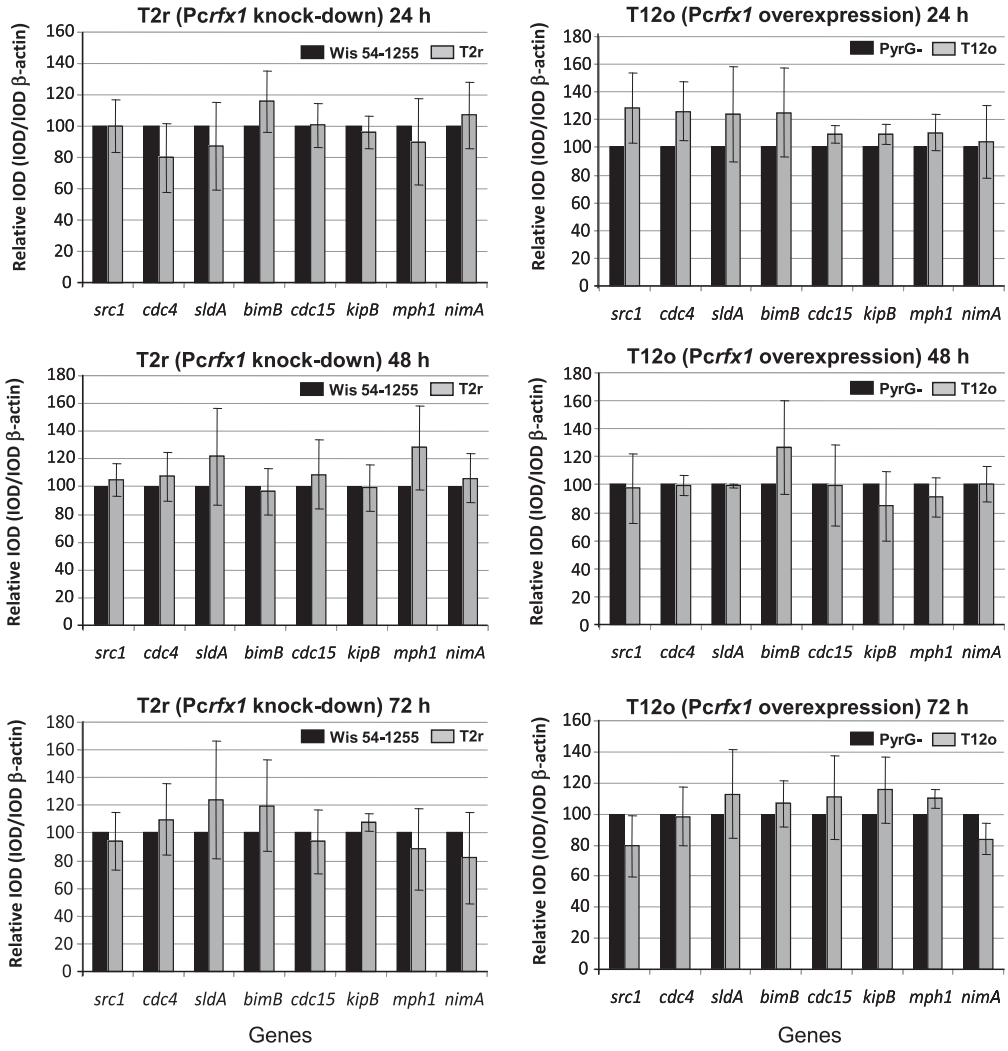

Fig. 9. Transcriptional analysis of eight genes involved in cell division putatively regulated by PcRFX1. Densitometry (IOD) graphs showing the expression profiles (normalized to the β-actin expression levels) at 24 h, 48 h and 72 h of *src1*, *cdc4*, *sldA*, *bimB*, *cdc15*, *kipB*, *mph1* and *nimA* after Pc*rfx1* gene silencing (transformant T2r) and overexpression (transformant T12o). Those values corresponding to the expression of each gene in *P. chrysogenum* Wisconsin 54-1255 (for knock-down) or Wisconsin 54-1255 strain PyrG- (for overexpression) were set to 100. Densitometry values correspond to the mean plus standard deviation of three independent measurements.

Table 2
Processes putatively controlled by PcRFX1.

GO ID	GO Description	N of putative regulated genes with this GO	N of putative regulated genes with GO annotation	N of genes with this GO	N of genes with GO annotation	*p*-Value
GO:06355	Regulation of transcription, DNA-dependent	106	1176	455	6288	0.0004
GO:06032	Chitin catabolic process	4	1176	6	6288	0.0011
GO:00272	Polysaccharide catabolic process	4	1176	6	6288	0.0011
GO:16052	Carbohydrate catabolic process	6	1176	11	6288	0.0013
GO:06730	One-carbon metabolic process	3	1176	5	6288	0.0052
GO:06629	Lipid metabolic process	13	1176	40	6288	0.0102
GO:00160	Two-component signal transduction system	8	1176	22	6288	0.0127
GO:06457	Protein folding	15	1176	49	6288	0.0127
GO:00105	Histidine biosynthetic process	3	1176	6	6288	0.0132
GO:08654	Phospholipid biosynthetic process	5	1176	12	6288	0.0138

Characterization of penicillin regulators is especially relevant for the optimization of the production process. It is well known that complex regulatory processes control growth and penicillin production in *P. chrysogenum* (Martín et al., 1999). No penicillin

pathway-specific regulators have been found so far, indicating that this process may be controlled by global regulators of secondary metabolism (Kosalková et al., 2009; Hoff et al., 2010). CPCR1 is a global regulator recently found in *A. chrysogenum* (Schmitt and Kück, 2000). The fact that CPCR1 positively regulates cephalosporin C biosynthesis by binding at least two sequences of the *pcbAB-pcbC* intergenic region (Schmitt and Kück, 2000; Schmitt et al., 2004b), suggested that this regulator may also be present in *P. chrysogenum* controlling the biosynthesis of penicillin. In fact, a partial incomplete sequence of the gene encoding the putative CPCR1 homolog was previously identified in *P. chrysogenum* (Schmitt and Kück, 2000). However, this sequence showed some nucleotide modifications compared to that reported later in the genome project for the gene encoding the transcription factor-like protein RFX (Pc20g01690) of *P. chrysogenum* Wisconsin 54-1255 (van den Berg et al., 2008). In this work we have found that the expression of this gene in *P. chrysogenum* is similar in both the wild type strain (NRRL 1951) and the laboratory reference strain Wisconsin 54-1255, which indicates that the sequence and expression of the Pc*rfx1* gene remained unmodified during the early phylogeny of penicillin-producing strains.

Combination of DNA binding sequences previously reported for RFX1 orthologs (Emery et al., 1996; Schmitt et al., 2004b; Bugeja et al., 2010), allowed us to identify putative PcRFX1 DNA binding sites in the promoter region of *pcbAB* and *pcbC* genes, although no PcRFX1 DNA binding site was identified in the promoter region of *penDE* gene with those consensus sequences. Nevertheless, results provided by expression analysis in the knock-down transformants indicated that the mRNA levels of the three penicillin biosynthetic genes were decreased when the expression of the Pc*rfx1* gene was silenced (Fig. 4), suggesting that the *penDE* gene was also regulated by PcRFX1. This was also supported by the fact that the putative PcRFX1 DNA binding site of the promoter region of this gene was able to bind protein extracts from *P. chrysogenum*, as it was shown in EMSA studies (Fig. 3). In light of these results, a new consensus sequence that was based on the above-mentioned sequences and taking into account the sequence of the penicillin biosynthetic gene promoters was established. With this sequence (NNRCCNNRSHWAY; where the first two "N" are included for comparative alignment with Rfx-binding sequences in other organisms), one putative binding sequence was found in both *pcbC* and *penDE* promoter regions and two putative DNA binding sites, which overlap each other in four nucleotides, were found in the promoter region of the *pcbAB* gene (Fig. 2A). It is noteworthy that a GATA box is present in the convergence of the two putative PcRFX1 binding boxes of the *pcbAB* gene and that the last five nucleotides of the putative PcRFX1 binding site of the *pcbC* promoter also include the CCAAT box (see below).

Gene reporter assays confirmed the functionality of the PcRFX1 DNA binding sequences. However, the GATA motif that is present in the convergence of the two putative PcRFX1 binding boxes in the *pcbAB* promoter region was removed after substitution/deletion of one of the putative PcRFX1 binding sequences. The GATA box represents the consensus sequence for the NRE transcription factor in *P. chrysogenum* (Haas and Marzluf, 1995). Six GATA sequences were found in the *pcbAB-pcbC* intergenic region in *P. chrysogenum* (four in the sense DNA strand for *pcbAB* and two in the sense DNA strand for *pcbC*). Two of these GATA motifs (one for each DNA strand, separated by 27 bp and arranged in a head-to-head fashion) have been reported to interact with NRE (Haas and Marzluf, 1995). Both NRE and PcRFX1 factors overlap and may be competing in the regulatory roles. The fact that the GATA motif removed in our experiment corresponds to the second of these two GATA boxes (the one in sense orientation for *pcbAB*) may be relevant for the interpretation of the β-galactosidase results obtained for the *pcbAB* gene. The same situation occurred with the replacement/deletion of the putative PcRFX1 DNA binding sequence in the *pcbC* promoter, which led to the modification/deletion of the CCAAT motif, which is the consensus sequence for binding of the PENR1 transcription factor. There are six CCAAT motifs in the *pcbAB-pcbC* intergenic region in *P. chrysogenum* (4 in the sense DNA strand for *pcbAB* and two in the sense DNA strand for *pcbC*). Although there are *in vitro* evidences of the binding of PENR1 to this region (Bergh et al., 1996), the functionality of the CCAAT box that has been modified/deleted in the gene reporter experiments has not been confirmed so far. Therefore, based only on this evidence, we cannot rule out that the decrease in β-galactosidase observed in the gene reporter assay is a direct consequence of the replacement/deletion of the putative PcRFX1 DNA binding sequence present in the *pcbC* gene promoter.

Supporting evidence was provided by Pc*rfx1* gene silencing and overexpression experiments, which confirmed the role of PcRFX1 in the control of penicillin biosynthesis. IPN and benzylpenicillin production were modified in the knock-down and overexpression transformants. In the cephalosporin producer *A. chrysogenum*, disruption of the *cpcR1* gene (encoding CPCR1, the ortholog of PcRFX1) reduced the levels of penicillin N and the activity of IPN synthase (IPN levels are likely also reduced), but cephalosporin levels remained unmodified (Schmitt et al., 2004b). This indicates that CPCR1 is not involved in the regulation of "late" cephalosporin biosynthetic genes in *A. chrysogenum*. In contrast, in *P. chrysogenum* the whole penicillin biosynthetic process seems to be controlled by PcRFX1.

Another difference between *A. chrysogenum* and *P. chrysogenum* was found when morphological development was analyzed. In the cephalosporin producer fungus, CPCR1 is also involved in the control of *A. chrysogenum* morphological development and is required for hyphal fragmentation and therefore, for the formation of arthrospores (Hoff et al., 2005). Hence, the winged-helix transcription factor CPCR1 seems to be the molecular link controlling cephalosporin C biosynthesis and morphogenesis. This linking role has also been suggested in the opportunistic human pathogen fungus *P. marneffei*. In this fungus, where arthroconidiation is analogous to the transition from a hyphal growth form to an unicellular spore form in *A. chrysogenum*, RfxA (the CPCR1 and PcRFX1 homolog) regulates cellular proliferation and differentiation and it has been suggested to link cellular division with morphogenesis, particularly during conidiation and yeast growth (Bugeja et al., 2010). Unlike what occurs in *P. marneffei*, which undergoes a clear differentiation, *P. chrysogenum* does not undergo a yeast-like growth and indeed, Pc*rfx1* knock-down and overexpression mutants showed a hyphal morphology and conidiophore formation similar to the control strain. Only sporulation seemed to be slightly affected after Pc*rfx1* gene silencing. In addition, transcription of eight genes putatively involved in cellular division (only three contained the consensus PcRFX1 DNA binding sequence proposed in this work), was not modified after Pc*frx1* gene silencing or overexpression. These results may be taken with caution, since although they suggest that PcRFX1 is not involved in development and differentiation in *P. chrysogenum*, we have to keep in mind that the Wisconsin 54-1255 strain (the reference laboratory strain) has been subjected to different rounds of classical mutagenesis during industrial strain improvement programs, which may have affected different genes. Therefore, we cannot rule out that morphological development in wild-type strains of *P. chrysogenum* can also be controlled by PcRFX1.

In conclusion, publication of the *P. chrysogenum* Wisconsin 54-1255 genome (van den Berg et al., 2008) has provided us with useful information and the possibility to carry out a global study of the genes putatively regulated by PcRFX1. In addition to the important role played by PcRFX1 in the regulation of the penicillin biosynthetic genes, this functional analysis paves the way for the study

of metabolic processes potentially regulated by this transcription factor and to investigate the link with the production of penicillin.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2012.08.002.

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SUPPLEMENTARY MATERIAL

Journal: FUNGAL GENETICS AND BIOLOGY

Article Title: "The regulatory factor PcRFX1 controls the expression of the three genes of beta-lactam biosynthesis in *Penicillium chrysogenum*"

Supplementary Table S1. List of primers used in this work.

Primer name	Sequence (5´- 3´)	Use
RFXBglIIF	CAACGAAGCAGAGATCTATGCCACCAGAAC [1]	Amplification of the 2782-bp Pc*rfx1* gene. Sequencing of the full gene and overexpression.
RFXStuIR	CAGAAATCAAAAAGGCCTCTAGATGACGAC [2]	
2RFXFNcoI	CTGCTTTCCATGGAACACTGACTGTTCC [3]	Amplification of a 479-bp Pc*rfx1* exonic fragment. Gene silencing.
2RFXRNcoI	CAATGGGGTCCATGGCGCACGAATGTCATG [3]	
FPpcbABboxX	GAAAACGGGGCCATAGATTGGCATAATTGACCGTCGAGTCAATC	Replacement of the putative PcRFX1 DNA binding sequences of the *pcbAB* gene promoter. Site-directed mutagenesis.
RPpcbABboxX	GATTGACTCGACGGTCAATTATGCCAATCTATGGCCCCGTTTTC	
FPpcbCboxX	CAGGGTATATATGGCTGTGGTGCAGTTACTCCATCGAGAATCTG	Replacement of the putative PcRFX1 DNA binding sequences of the *pcbC* gene promoter. Site-directed mutagenesis.
RPpcbCboxX	CAGATTCTCGATGGAGTAACTGCACCACAGCCATATATACCCTG	
FPpenDEboxX	GCCAAGCTTTCAGGCATGGTACAGTTGCCAATAGGAACCAAGTG	Replacement of the putative PcRFX1 DNA binding sequences of the *penDE* gene promoter. Site-directed mutagenesis.
RPpenDEboxX	CACTTGGTTCCTATTGGCAACTGTACCATGCCTGAAAGCTTGGC	
FPpcbABΔboxX	GAAAACGGGGCCATAGACCGTCGAGTCAATC	Deletion of the putative PcRFX1 DNA binding box of the *pcbAB* gene promoter. Site-directed mutagenesis.
RPpcbABΔboxX	GATTGACTCGACGGTCTATGGCCCCGTTTTC	
FPpcbCΔboxX	CAGGGTATATATGGCCTCCATCGAGAATCTG	Deletion of the putative PcRFX1 DNA binding box of the *pcbC* gene promoter. Site-directed mutagenesis.
RPpcbCΔboxX	CAGATTCTCGATGGAGGCCATATATACCCTG	
FPpenDEΔboxX	GCCAAGCTTTCAGGCCAATAGGAACCAAGTG	Deletion of the putative PcRFX1 DNA binding box of the *penDE* gene promoter. Site-directed mutagenesis.
RPpenDEΔboxX	CACTTGGTTCCTATTGGCCTGAAAGCTTGGC	
RTactAF	CTGGCCGTGATCTGACCGACTAC	Amplification of 457 bp from the *actA* -encoding β-actin gene. RT-PCR experiments.
RTactAR	GGGGGAGCGATGATCTTGACCT	
RFXFNcoI	CAGTGGCTCAGTACGTCGTG	Amplification of 568 bp from the Pc*rfx1* mRNA. RT-PCR experiments.
RFXRNcoI	GTGTTTGAAGGTAAGTAGGCAC	
RPc12g11470	GGGAGTCGATACGCGAGGAGAGC	Amplification of 414 bp from Pc12g11470 (Src1). RT-PCR experiments.
FPc12g11470	CACTTGCCCTCGGTGGCCTAGTG	
RPc20g06510	GAGCTGCCGCCTCTACCAATG	Amplification of 399 bp from Pc20g06510 (Mph1). RT-PCR experiments.
FPc20g06510	CTCGAAGGAACAACCACAACCC	
FPc20g01370	TCAGTTGGCGAGGATAGTACGGAC	Amplification of 435 bp from Pc20g01370 (Cdc4). RT-PCR experiments.
RPc20g01370	ATGGCTAATAACGACCAGGCCTC	
FPc13g10210	GCCTTTCTTTGTCATGGCGACTC	Amplification of 418 bp from Pc13g10210 (Cdc15). RT-PCR experiments.
RPc13g10210	CAGCAGCCTCCTATGCAACAGC	
FPc18g04320	CAAAGTGCATTGATGCAGCTTCC	Amplification of 411 bp from Pc18g04320 (KipB). RT-PCR experiments.
RPc18g04320	CGAAACCATCCGTGACTTGCTTG	
FPc22g09820	CCGTTAGGGGTTCTGCCATCATC	Amplification of 416 bp from Pc22g09820 (SldA/Bub1). RT-PCR experiments.
RPc22g09820	GCAGCTGATGTACCATTCAGTCC	
FPc12g05830	GAGATGTGAGCGAATGTCAGCAG	Amplification of 461 bp from Pc12g05830 (BimB). RT-PCR experiments.
RPc12g05830	GATCACGATGCGGTGGCATTGTC	
FPc20g14730	GCCTTCATCTCGGGACATGTCTG	Amplification of 464 bp from Pc20g14730 (Mob1). RT-PCR experiments.
RPc20g14730	ATGTCTGTCCCCGCTACCCCTC	
FPc22g24040	CTACCGCAGGTTGCGGATAACC	Amplification of 400 bp from Pc22g24040 (NimA). RT-PCR experiments.
RPc22g24040	GCGGTGCAAGTGAGAGCAGATC	
boxXF	TTCAGGCAACCTAGGCAACTCGAGC [4] (Cy3 labelled at the 5´ end)	Probe for the EMSA experiments
boxXR	GCTCGAGTTGCCTAGGTTGCCTGAA [4]	

[1] **The sequence corresponding to the *Bgl* II restriction site is underlined**

[2] **The sequence corresponding to the *Stu* I restriction site is underlined**

[3] **The sequence corresponding to the *Nco* I restriction site is underlined**

[4] **The sequence corresponding to the putative PcRFX1 binding site is underlined**

Supplementary Table S2. Primers used for qPCR. The primers for the genes of interest were designed using the Primer3 software (Rozen and Skaletsky 2000).

Pc21g21390 (*pcbAB*)

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	3323	20	59.93	50.00	5´-atggggacaatctcacttcg-3´
RIGHT PRIMER	3457	20	59.99	50.00	5´-cgttcatatcacaccgttcg-3´

Pc21g21380 (*pcbC*)

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	545	20	60.11	50.00	5´-cctcggttgttctgattcgt-3´
RIGHT PRIMER	668	20	60.14	55.00	5´-aggacggtaatgagcgacac-3´

Pc21g21370 (*penDE*)

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	118	20	59.99	50.00	5´-acgaagaagacggacgaaga-3´
RIGHT PRIMER	253	20	59.79	50.00	5´-tgacaatctcggagacatcg-3´

Pc20g01690 (Pc*rfx1*)

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	18	20	60.31	45.00	5´-ttatttggttcccggtgcta-3´
RIGHT PRIMER	158	20	59.99	50.00	5´-gagtggatgcttgctgttga-3´

Pc20g11630 (*actA*)

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	9	20	58.91	50.00	5´-agaagttgctgctctcgtca-3´
RIGHT PRIMER	106	20	60.86	45.00	5´-cgacaatggaagggaaaaca-3´

Rozen, S., and Skaletsky, H.J. (2000). Primer3 on the WWW for general users and for biologist programmers. In Bioinformatics Methods and Protocols: Methods in Molecular Biology, S. Krawetz, and S. Misener, eds. (Totowa, NJ: Humana Press), pp. 365–386.

Artículo 2

PcFKH1, a novel regulatory factor from the forkhead family, controls the biosynthesis of penicillin in *Penicillium chrysogenum*

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Research paper

PcFKH1, a novel regulatory factor from the forkhead family, controls the biosynthesis of penicillin in *Penicillium chrysogenum*

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ABSTRACT

Penicillin biosynthesis in *Penicillium chrysogenum* (re-identified as *Penicillium rubens*) is a good example of a biological process subjected to complex global regulatory networks and serves as a model to study fungal secondary metabolism. The winged-helix family of transcription factors recently described, which includes the forkhead type of proteins, is a key type of regulatory proteins involved in this process. In yeasts and humans, forkhead transcription factors are involved in different processes (cell cycle regulation, cell death control, pre-mRNA processing and morphogenesis); one member of this family of proteins has been identified in the *P. chrysogenum* genome (Pc18g00430). In this work, we have characterized this novel transcription factor (named PcFKH1) by generating knock-down mutants and overexpression strains. Results clearly indicate that PcFKH1 positively controls antibiotic biosynthesis through the specific interaction with the promoter region of the *penDE* gene, thus regulating *penDE* mRNA levels. PcFKH1 also binds to the *pcbC* promoter, but with low affinity. In addition, it also controls other ancillary genes of the penicillin biosynthetic process, such as *phlA* (encoding phenylacetyl CoA ligase) and *ppt* (encoding phosphopantetheinyl transferase). PcFKH1 also plays a role in conidiation and spore pigmentation, but it does not seem to be involved in hyphal morphology or cell division in the improved laboratory reference strain Wisconsin 54-1255. A genome-wide analysis of processes putatively coregulated by PcFKH1 and PcRFX1 (another winged-helix transcription factor) in *P. chrysogenum* provided evidence of the global effect of these transcription factors in *P. chrysogenum* metabolism.

1. Introduction

Penicillium chrysogenum is a filamentous fungus well known by its use in the industrial production of the beta-lactam antibiotic penicillin, which dramatically transformed the global health since Sir Alexander Fleming's discovery of the *Penicillium* that produces this antibiotic [1]. Recent studies have confirmed that the Fleming's original penicillin producing strain and the full genome sequenced strain of *P. chrysogenum* corresponds actually to *Penicillium rubens* [2].

Since the isolation of the original natural fungal isolate, selection and mutagenesis applied during industrial strain improvement programs have raised the penicillin production titers by three orders of magnitude [3]. Many of the modifications responsible for the increased productivity in high-producing strains have been characterized, such as the amplification of the region including the penicillin biosynthetic genes [4], the increase in the number of peroxisomes [5,6], the overexpression of those genes encoding enzymes responsible for the biosynthesis of the amino acid precursors as well as those gene encoding peroxisomal proteins [5]. The high producing strains show a complex rebalancing of global metabolism, including redox reactions, production of energy, biosynthesis of amino acid precursors, virulence or secondary metabolism [7].

Regulatory aspects of penicillin biosynthesis have been also subject of interest for researchers, especially since no penicillin pathway-specific regulators have been found in the region that

Abbreviations: ACV, δ(L-α-aminoadipyl)-L-cysteinyl-D-valine; IAT, IPN acyltransferase; IPN, isopenicillin N; PPTase, 4′-phosphopantetheinyl transferase.

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contains the penicillin gene cluster (so-called cluster-situated regulators (CSR)) [8,9]. This cluster comprises three genes (*pcbAB*, *pcbC* and *penDE*) encoding the biosynthetic enzymes, which have been largely described and characterized from the molecular and biochemical point of views (for a recent review see Ref. [10]. Briefly, the *pcbAB* gene encodes the multienzyme δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase, which condensates by a non-ribosomal mechanism L-α-aminoadipic acid, L-cysteine and L-valine, thus giving rise to the tripeptide ACV. In the second step of the biosynthetic pathway, the product of the *pcbC* gene, named Isopenicillin N (IPN) synthase (cyclase), catalyses the cyclization of ACV to form IPN, which is the first bioactive compound of the pathway [11,12]. In the last step of the pathway, the IPN acyltransferase (IAT, encoded by the *penDE* gene) substitutes the L-α-aminoadipyl side chain of IPN by activated aromatic acyl side chains, thus forming hydrophobic penicillins. In addition to the *pcbAB*, *pcbC* and *penDE* genes, three ancillary genes are also required for penicillin biosynthesis. The *ppt* gene encodes the 4′-phosphopantetheinyl transferase (PPTase), which activates the ACV synthetase through the addition of a 4′-phosphopantetheine moiety derived from CoA [13]. Another ancillary gene (more precisely a two-component system comprising *trxA* and *trxB*) encodes the NADPH-dependent thioredoxine disulfide reductase (TrxAB), which catalyses the reduction of the oxidized bis-ACV into monomeric ACV so that it can be cyclized by IPN synthase [14]. Finally, acyl-CoA ligases (the main one encoded by *phlA*, formerly named *phl*), are required for the activation of the precursor acyl molecules as CoA thioesters so that they can be incorporated by the IAT during the biosynthesis of hydrophobic penicillins [15]. It is also well-established that penicillin biosynthesis occurs in the cytosol (the first two steps) and peroxisomes, where the acyl-CoA ligases and the IAT are located (reviewed in [6,16]).

In the absence of pathway-specific regulators, penicillin biosynthesis seems to be controlled by several transcription factors in response to environmental and nutritional conditions. It has been known since long that the biosynthesis of this beta-lactam antibiotic is subjected to carbon catabolite regulation [17]. However, it was not until very recently when CreA (the main wide domain regulator responsible for carbon repression in filamentous fungi) was confirmed to participate in carbon repression of penicillin biosynthesis and expression of the *pcbAB* gene [18]. The external pH controls penicillin biosynthesis by means of the transcription factor PacC [19], whereas nitrogen regulation of the penicillin biosynthetic genes is likely mediated by the NRE transcription factor [20,21].

Penicillin biosynthesis is also controlled by global regulators. LaeA is a nuclear protein with a methyltransferase domain that regulates the expression of the penicillin genes and other secondary metabolites in *P. chrysogenum* [22]. LaeA is one of the major components of the velvet-like complex, which comprises at least ten different proteins [23]. Four members of this complex have been characterized so far in *P. chrysogenum*. Velvet A was reported to play an essential role in the biosynthesis of penicillin and in different developmental processes together with LaeA [24]. More recently, other subunits of the velvet complex were identified, namely PcVelB, PcVelC and PcVosA [25]. PcVelC is a strong activator of penicillin biosynthesis and acts in *P. chrysogenum* together with PcLaeA and PcVelA controlling the production of secondary metabolites. In contrast, PcVelB represses penicillin biosynthesis. In addition, PcVelB and PcVosA promote conidiation, whereas PcVelC has an inhibitory effect [25]. Another global regulator that has been recently characterized in *P. chrysogenum* is PcRFX1 [26]. This transcription factor, which is an ortholog of the regulatory factors CPCR1 (*Acremonium chrysogenum*) and RfxA (*Penicillium marneffei*), controls penicillin biosynthesis through the regulation of *pcbAB*, *pcbC* and *penDE* transcription and seems to be involved in the control of several pathways of primary metabolism [26].

In *A. chrysogenum*, CPCR1 positively regulates cephalosporin C biosynthesis by binding at least two sequences at the *pcbAB-pcbC* intergenic region [27,28] and associates with the C-terminus of AcFKH1, which is a member of the forkhead family of proteins [29]. Within the bidirectional *pcbAB-pcbC* promoter, AcFKH1 recognizes two binding sequences [29], which match the consensus 5′-RYMAAYA-3′ determined from 17 different sequences [30]. This suggests a role for AcFKH1 in the biosynthesis of cephalosporin C. Therefore, it is of great interest to study the ortholog of AcFKH1 in *P. chrysogenum* in order to test whether similar regulatory mechanisms operate in those two beta-lactam producers to gain more information on the regulatory aspects of penicillin biosynthesis.

2. Materials and methods

2.1. Strains, media and culture conditions

P. chrysogenum Wisconsin 54-1255 (reference strain for the genome sequencing project) has been re-identified as *P. rubens* [2], although we will use in this article *P. chrysogenum* for consistency with our previous works. *P. chrysogenum* Wisconsin 54-1255 pyrG- (a uridine auxotroph derived from the Wisconsin 54-1255 strain) was also used in this work. These strains were grown in solid Power sporulation medium [31] for 7 days at 28 °C. Flasks cultures for penicillin production were carried out inoculating fresh spores of *P. chrysogenum* in 100 ml of complex inoculum medium CIM [13] without phenylacetate. After incubation at 25 °C for 20 h in an orbital shaker (250 rpm), aliquots (5%) were inoculated in CP complex penicillin production medium [13] with 0.4% potassium phenylacetate and incubated under the same conditions for up to 72 h. Uridine auxotrophs were grown in the presence of 140 μg/ml uridine.

Escherichia coli DH5α cells were used for plasmid amplification, whereas *E. coli* XL1-Blue cells were used for the expression of recombinant PcFKH1. Bacterial cells were grown in Luria-Bertani medium with ampicillin (100 μg/ml).

2.2. Plasmid constructs

Plasmid pJL43-RNAi-Pc*fkh1*, which was used to generate the Pc*fkh1* knock-down transformants, was constructed as follows: Plasmid pJL43-RNAi [32], which contains the *ble* gene marker (for phleomycin resistance), was digested with *Nco*I. Oligonucleotides FKH2silF and FKH2silR (see Supplementary Table S1) were used to amplify a 449-bp exon fragment from Pc*fkh1*, which was digested with *Nco*I and cloned into pJL43-RNAi, thus yielding pJL43-RNAi-*fkh1*. This plasmid was used to transform the *P. chrysogenum* Wisconsin 54-1255 strain.

Plasmid pIBRC43-Pc*fkh1* was used for the overexpression of the *P. chrysogenum* Pc*fkh1* gene and was constructed as follows: Oligonucleotides FKH2NcoIF and FKH2PvuIIR (see Supplementary Table S1) were used to amplify the 2302-bp Pc*fkh1* gene, which was digested with *Nco*I and *Pvu*II. Then, it was inserted between the strong *Aspergillus awamori gdh* gene promoter and the *Saccharomyces cerevisiae cyc*1 transcriptional terminator into pIBRC43 [33], which was previously digested with *Nco*I-*Stu*I. *P. chrysogenum* Wisconsin 54-1255 PyrG-was cotransformed with this plasmid and with the "helper" plasmid pBG [34], which includes the *P. chrysogenum pyrG* gene.

Plasmid pQE-30-Pc*fkh1* was used for the heterologous expression of PcFKH1 in *E. coli* XL1-Blue and was constructed as

follows: The two introns of Pc*fkh*1 gene were removed by site-directed mutagenesis (Stratagene) following manufacturer's instructions. With this purpose, plasmid pIBRC43-Pc*fkh*1 (template) and primers fkhΔintron1F, fkhΔintron1R, fkhΔintron2F, fkhΔintron2R were used (see Supplementary Table S1), thus generating plasmid pIBRC43-Pc*fkh*1*cDNA*. The Pc*fkh*1 cDNA was amplified by PCR using pIBRC43-Pc*fkh*1*cDNA* as template and primers *BamH*IcDNAfkhF and *Hind*IIIcDNAfkhR (see Supplementary Table S1). The amplified DNA was digested with *BamH*I and *Hind*III and inserted into the *BamH*I and *Hind*III sites of the expression vector pQE-30. The resultant plasmid, pQE-30-Pc*fkh*1 was sequenced to check the correct in-frame insertion.

2.3. Transformation of P. chrysogenum protoplasts, extraction of genomic DNA and Southern blotting

Protoplasts were obtained and transformed following our standard laboratory protocol as previously described [35]. After transformation, protoplasts were grown in Czapek minimal medium [13]. Transformant clones were selected by the complementation of uridine auxotrophy or by resistance to phleomycin (final concentration 30 μg/ml).

DNA isolation and Southern blotting hybridization were carried out as previously described [8,15].

2.4. RNA extraction, RT-PCR and qPCR assays

RNA was extracted from cultures of *P. chrysogenum* at 48 h using "RNeasy Mini Kit" columns (Qiagen), following the manufacturer's instructions. Total RNA was treated with "Ambion® RNA by life technologies", following the manufacturer's instructions and quantified using a NanoDrop ND-1000 spectrophotometer. Prior to reverse transcription, the absence of contaminant DNA in the samples was tested by PCR.

RT-PCR was performed using 200 ng of total RNA and the "SuperScript One-Step RT-PCR with Platinum Taq" system (Invitrogen Corporation), following the manufacturer's instructions. Several primers were designed for this purpose (see Supplementary Table S1). The signals provided by the RT-PCR assays were quantified by densitometry using the "Gel-Pro Analizer" software (Media Cybernetics). The transcript levels were normalized comparing the intensity of each mRNA signal to the γ-actin mRNA signal.

Gene expression was also analysed by qPCR using a StepOnePlus™ Real-Time PCR system (Applied Biosystems) as previously described [26]. Primers are listed in Supplementary Table S2. The relative expression of each gene was calculated following the mathematical algorith based on the $2^{-\Delta\Delta CT}$ Method [36]. Results were expressed as the mean and standard deviation of three biological replicates and three technical replicates each.

2.5. Overexpression of recombinant PcFKH1 in E. coli

E. coli XL1Blue transformed with pQE-30-Pc*fkh*1 was cultured at 37 °C in 20 ml of Luria-Bertani medium supplemented with 100 μg/ml of ampicillin. After overnight cultivation, 2 ml were inoculated into a 500 ml flask containing 100 ml of the same medium and grown at 37 °C and 250 rpm to an OD_{600} of 0.6. Once induced with IPTG (0.5 mM), the culture was incubated for additional 5 h at 37 °C and 250 rpm. Then, cells were harvested and broken by sonication (18–20 pulses of 10 s each). Purification of His-tagged PcFKH1 was performed from soluble extracts using His-Trap HP columns (GE Healthcare) following the manufacturer's instructions. Purified proteins were conserved in 40% glycerol at −80 °C.

2.6. Western blotting

Proteins were extracted from the mycelia of *P. chrysogenum* Wisconsin 54-1255 and Pc*fkh1* knock-down transformant 2 (see Results Section) following the protocol previously described [26]. Immunological detection of IAT was performed as reported elsewhere [37] using strains and antibodies raised against the β-subunit of IAT [38]. Immunological detection of α-tubulin was used as control as it was previously reported [26]. Western bands were quantified by densitometry using the "Gel-Pro Analizer" software (Media Cybernetics). Protein levels were normalized comparing the intensity of each IAT signal to the corresponding α-tubulin signal.

2.7. Electrophoretic mobility shift assay (EMSA)

The DNA fragments used as probes for EMSA were amplified by PCR using the primers listed in Supplementary table S1. The amplicon was labeled at both ends with digoxigenin with the DIG Oligonucleotide 3′-End Labeling Kit, 2nd Generation (Roche Applied Science). The unspecific probe consisted of a 132-bp region within the *pcbAB/pcbC* promoter that does not contain PcFKH1 binding sequences. This unspecific probe was obtained by PCR using plasmid pZ2bAB containing a deletion in the PcRFX1 binding box [26] as template, and oligonucleotides K7 and K9 (see Supplementary Table S1) as primers.

The binding reaction consisted of 0.045 ng labeled DNA, 4 mM EDTA, 0.1–10 μg protein, 1 μl of 1 μg/μl poly dI-dC and 1 μl binding buffer [27] in a volume of 20 μl. After incubation at 25 °C for 30 min, the samples were loaded onto a 5% polyacrylamide (29:1) native gel in 0.5xTBE buffer. After electrophoresis (80 V, 4 °C), DNA was electroblotted onto a nylon membrane (HyBond-N, Amersham Biosciences) in 0.5xTBE buffer (30 min, 200 mA). The DNA was fixed by UV cross-linking, detected with anti-digoxigenin antibodies, and developed by chemiluminescence with the CDP-StarTM reagent (Roche Applied Science).

2.8. Determination of IPN and benzylpenicillin by HPLC

Analysis and quantitation of benzylpenicillin and IPN were carried out by HPLC as previously described [32,34]. Differences were considered as significant according to the standard deviation and when the *p*-value provided by the ANOVA test was $P < 0.05$.

3. Results

3.1. Characterization of the AcFKH1 ortholog in P. chrysogenum (PcFKH1)

In silico analysis of the *P. chrysogenum* genome [5] allowed the identification of the gene encoding the AcFKH1 ortholog (Pc18g00430). This gene, hereafter referred to as Pc*fkh1* (2302 bp, 2 introns), codes a protein (PcFKH1) of 718 amino acids with strong similarity to forkhead transcription factor Fkh1/2 from different filamentous fungi (e.g. 82% similarity and 76% identity to forkhead transcription factor Fkh1/2 from *Aspergillus fumigatus*) and 48% similarity and 34% identity to the *A. chrysogenum* FKH1 protein (see Discussion). Analysis of the DNA-binding domain of the *P. chrysogenum* and *A. chrysogenum* FKH1 orthologues revealed 75% similarity and 68% identity among these two protein domains. In addition, the N-terminal region of the PcFKH1 showed a 63% similarity and 47% identity to the conserved N-terminal forkead-associated domain from AcFKH1, which might be involved in phospho–protein interactions [29].

Expression of Pc*fkh1* in *P. chrysogenum* was tested by RT-PCR using RNA extracted at 24 h, 48 h and 72 h from cultures grown in CP medium, as indicated in Materials and Methods. One band was visible after reverse transcription and amplification of the Pc*fkh1* gene transcripts (Fig. 1A). The higher expression levels were obtained at 48 h (Fig. 1B).

3.2. Identification of the putative DNA binding sites in the promoter region of the penicillin biosynthetic genes

Using the consensus sequence 5′-RYMAAYA-3′ reported by Kaufmann and co-workers [30], the *P. chrysogenum* bidirectional *pcbAB-pcbC* promoter region and the *penDE* gene promoter were analysed in search for putative FKH1 binding sites. Two putative binding sites were identified in each of the *pcbC* and *penDE* gene promoters, whereas no PcFKH1-binding sites were found in the promoter region of the *pcbAB* gene (divergent from *pcbC*).

In order to test whether PcFKH1 binds the promoter region of the penicillin biosynthetic genes, EMSA experiments were conducted using the purified recombinant PcFKH1 fused to a six-His tag, which was obtained and purified from soluble extracts of *E. coli*/pQE-30-Pc*fkh*1.

Two different probes including the 5′-RYMAAYA-3′ consensus sequence from the *pcbC* promoter region (positions −173 and −1004; Fig. 2A) were designed (see Materials and Methods). With both probes the electrophoretic mobility was shifted (Fig. 2B and C). In order to confirm the specificity of the DNA binding activity, competition experiments using specific and unspecific probes (added in 100- and 1000-fold excess to the standard protein-DNA binding reaction) were carried out. The specific probes added in excess (1000-fold) showed very slight competition in both cases and the specificity of the binding of PcFKH1 protein to both DNA fragments seemed to be very low (Fig. 2B and C). These results indicate that PcFKH1 interacts in a non-specific way with probes C-173 and C-1004 (see Discussion).

In order to confirm the binding of the recombinant PcFKH1 to the promoter of the *penDE* gene, one probe including both 5′-RYMAAYA-3′ motifs (positions −113 and −265; Fig. 3A) was designed (see Materials and Methods). After electrophoresis (Fig. 3B), the retarded complexes were clearly observed and two complexes with different mobility were formed. The specificity of retarded complexes was confirmed by competition experiments using specific and unspecific probes. A 100-fold molar excess of the specific probe prevented the formation of the PcFKH1-DNA complexes, whereas the addition of unspecific probe (either in 100- or 1000-fold excess) was unable to modify the intensity of the bands representing such complexes (Fig. 3B). These results clearly demonstrate that PcFKH1 specifically recognizes the 5′-GCCAATA-3′ and 5′-GTCAATA-3′ binding sites present in the *penDE* gene promoter.

Taken together, these results confirmed that PcFKH1 interacts with the promoter region of *penDE* (see Discussion).

3.3. PcFKH1 controls the expression of penDE, but not pcbAB or pcbC

In order to confirm the regulatory role of PcFKH1, gene silencing experiments were conducted using plasmid pJL43-RNAi-Pc*fkh1*, which includes a 433-bp exon fragment from the Pc*fkh1* gene (see Materials and Methods). *P. chrysogenum* protoplasts were transformed with this construct and several phleomycin-resistant transformants were obtained and analysed by PCR to confirm the presence of the silencing cassette (data not shown). Nine transformants that showed a correct amplification pattern were analysed by Southern blotting after the digestion of genomic DNA with *Sph*I and *Hind*III (Fig. 4A). Blots were hybridised to the DIG-labelled exon fragment included in the silencing cassette. All transformants and the parental strain showed the 6-kbp band containing the endogenous Pc*fkh1* gene copy. Unlike transformants 2, 12 and 23, which only contained the 1815-bp band from the silencing cassette, transformants 1, 3, 24, 29, 37 and 43 showed additional hybridization bands, likely because of the ectopic random integration of partial fragments from the silencing cassette within the genome. In order to obtain concluding results, up to seven transformants (1, 2, 12, 23, 24, 29 and 43) were tested in RT(reverse transcription)-qPCR gene expression experiments.

With this purpose, those transformants were grown in CP medium and after 48 h (the time-point with the highest Pc*fkh1* expression level as it was indicated before) RNA was extracted and analysed to confirm the expression of Pc*fkh1*. As it can be observed in Fig. 4B, transformants 2, 23, 24, 29 and 43 showed a significant reduction in the Pc*fkh1* expression (with fold changes ranging from 1.34 in transformant 24 to 2.85 in transformant 43), thus confirming that they were authentic knock-down transformants.

Expression of the penicillin biosynthetic genes was also tested by RT-qPCR (Fig. 4C). Only transformant 2 produced a small (1.5-fold) reduction in the *pcbAB* transcripts, but since it was the only transformant providing a significant effect, it can be concluded that the transcription of the *pcbAB* gene was not significantly affected in the selected transformants. In a similar way, the expression of the *pcbC* gene did not vary in the Pc*fkh1* knock-down transformants. By contrast, when the *penDE* gene expression was analysed, it was

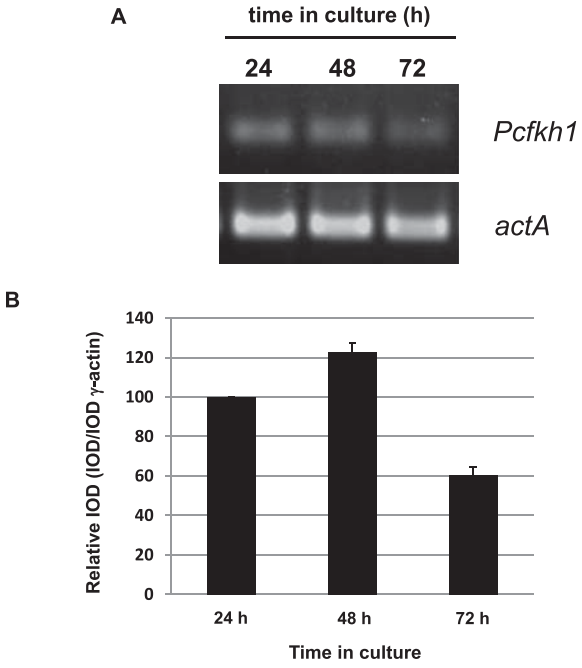

Fig. 1. Expression of the Pc*fkh1* gene along the cultivation time. (**A**) Semiquantitative RT-PCR showing the expression profiles of Pc*fkh1* in *P. chrysogenum* Wisconsin 54-1255 at 24 h, 48 h and 72 h. The signal provided by the γ-actin gene (*actA*) was used as control. (**B**) The intensity of the bands (IOD) obtained in panel A was determined by densitometry. Those values corresponding to the expression of the Pc*fkh1* gene at 24 h were set to 100%. Results correspond to the mean plus standard deviation of three independent measurements.

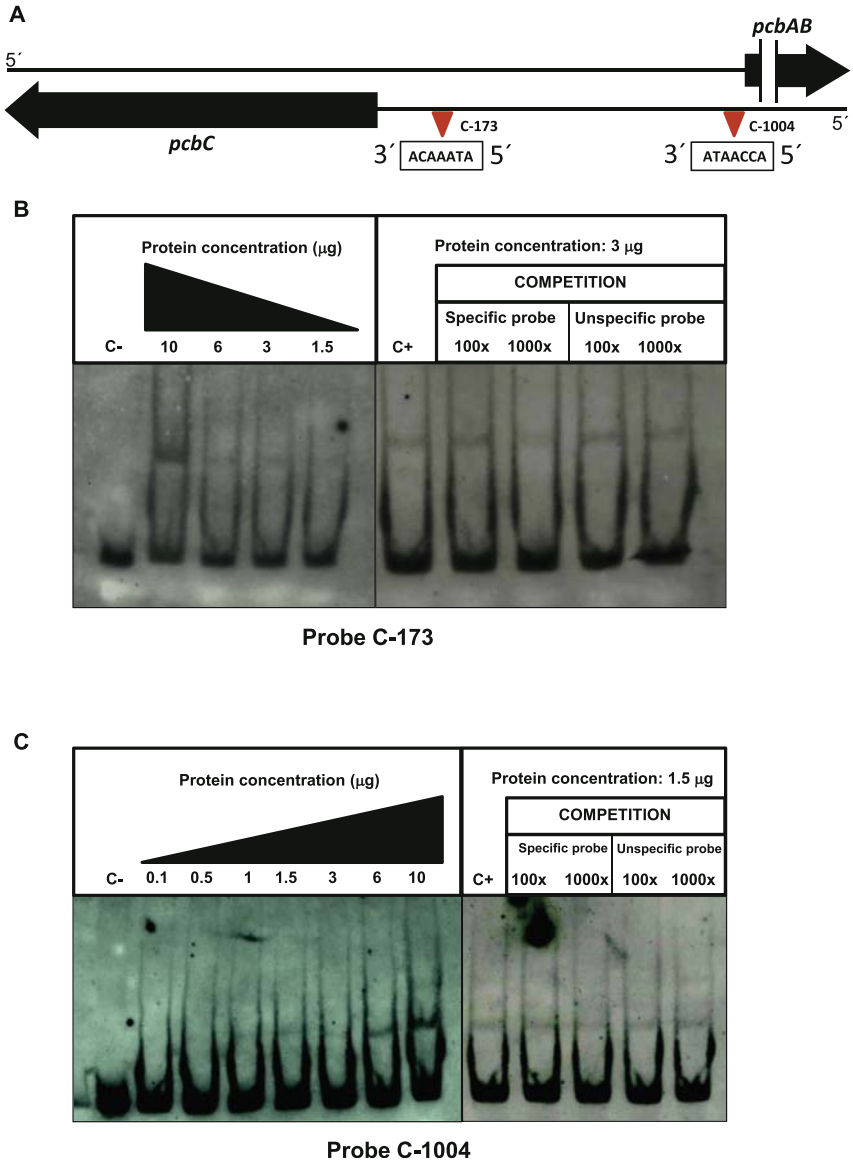

Fig. 2. Binding experiments of the recombinant PcFKH1 to the promoter of the *pcbC* gene in the *pcbAB-pcbC* divergent region. (**A**) Schematic representation of the *pcbC* gene promoter with the two putative PcFKH1 binding sequences (positions −173 and −1004). EMSA experiments performed with the probe containing either the C-173 box (88 bp) (**B**) or the C-1004 box (125 bp) (**C**). Increasing protein concentrations were used in those experiments. The positive control (C+) included the recombinant PcFKH1 purified from *E. coli* protein extracts, whereas the negative control (C-) included protein extracts (subjected to the same purification protocol as the protein extracts used for the positive control) from *E. coli* transformed with an "empty" pQE-30 plasmid. A 132-bp fragment from the *pcbAB/pcbC* promoter region, which lacks binding sites for PcRFX1 or PcFKH1, was used as unspecific probe in the competition experiments (see Materials and methods).

significantly reduced in transformants 2, 23, 24, 29 and 43 (with the only exception of transformants 1 and 12, which were poorly silenced in a non-significant way); this silencing effect was more evident in transformants 2 and 43, which showed a 2.6-fold reduction. This result is in agreement with the fact that those five transformants also showed a reduction in the P*cfkh1* gene expression levels (Fig. 4B), providing evidence of the regulatory role of PcRFKH1 in the expression of the *penDE* gene.

3.4. Gene-silencing of Pcfkh1 leads to a decrease in the benzylpenicillin titers and to an increase in the IPN levels as a consequence of IAT depletion

The involvement of PcFKH1 in the regulation of the penicillin biosynthetic process was confirmed using submerged cultures. Five P*cfkh1* knock-down transformants (2, 23, 24, 29 and 43) were cultured in CP medium for 48 h and 72 h and supernatants from five

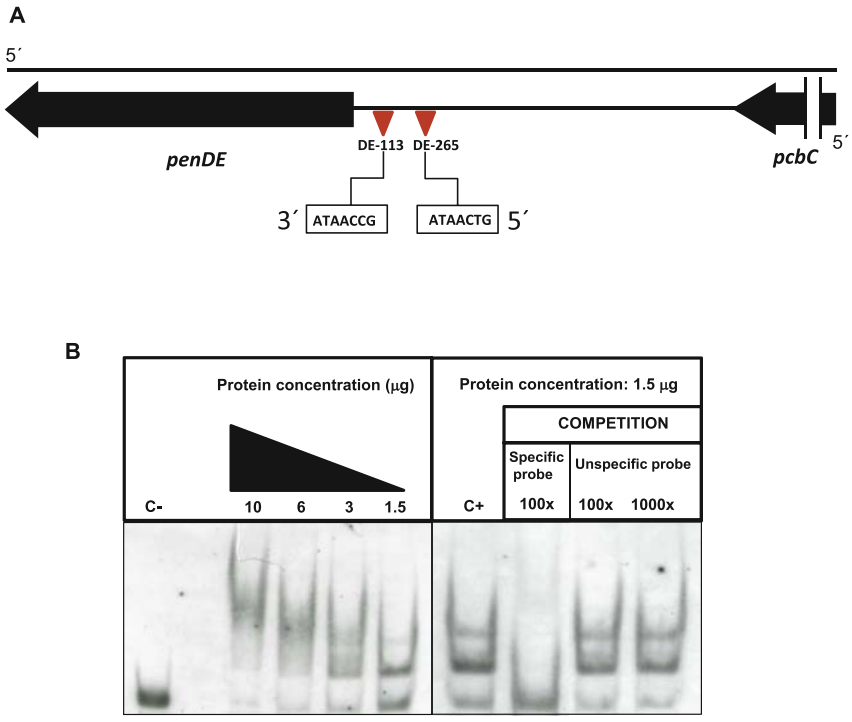

Fig. 3. Binding experiments of the recombinant PcFKH1 to the promoter of the *penDE* gene. (**A**) Schematic representation of the *penDE* gene promoter with the two putative PcFKH1 binding sequences (positions −113 and −265). (**B**) EMSA experiments performed with the probe containing both the DE-113 box and the DE-265 boxes (198 bp). Increasing protein concentrations were used in those experiments. The positive control (C+) included the recombinant PcFKH1 purified from *E. coli* protein extracts, whereas the negative control (C-) included protein extracts (subjected to the same purification protocol as the protein extracts used for the positive control) from *E. coli* transformed with an "empty" pQE-30 plasmid. A 132-bp fragment from the *pcbAB/pcbC* promoter region, which lacks binding sites for PcRFX1 or PcFKH1, was used as unspecific probe in the competition experiments (see Materials and methods).

biological replicates were analysed by HPLC to assess the production of IPN and benzylpenicillin.

When benzylpenicillin production was determined, the production of this antibiotic was significantly reduced in Pc*fkh1* knock-down transformants 2, 29 and 43. Knock-down transformant 2 provided the lowest benzylpenicillin titers, with a 50% decrease in production compared to the Wisconsin 54-1255 strain (Fig. 5A). IPN levels raised in all knock-down transformants at 48 h and 72 h. This increase was significant in transformants 2, 24, 29 and 43 at 48 h and in transformants 2 and 43 at 72 h. This increase was especially relevant in transformant 2, which accumulated two-fold IPN levels, with respect to control culture values (Fig. 5B).

These results may be explained taking into account the expression experiments described before, which suggested that Pc*fkh1* knock-down transformants have reduced steady-state levels of *penDE* transcripts. Since the product of the *penDE* gene (the IAT) is involved in the conversion of IPN into benzylpenicillin, Pc*fkh1* knock-down transformants are expected to have reduced levels of IAT. This would lead to IPN accumulation and to a reduction in the benzylpenicillin titers due to a bottleneck in the IAT-mediated step of the penicillin biosynthetic pathway. To test this, knock-down transformant 2, which provided the highest IPN levels, the lowest benzylpenicillin titers and low *penDE* expression levels was analysed by western blotting. Protein extracts taken from cultures of this transformant grown in CP medium for 48 h were resolved in SDS-PAGE, transferred to PVDF membranes and incubated with antiIAT antibodies. Fig. 5C shows that IAT levels (normalized to those provided by α-tubulin) were approximately two-fold lower in the knock-down transformant than those observed in the control strain, thus confirming this hypothesis.

3.5. Overexpression of Pcfkh1 slightly increases benzylpenicillin production and does not significantly affect the expression of the penicillin biosynthetic genes

Overexpression transformants were also prepared to characterize PcFKH1. With this aim, the *P. chrysogenum* Wis54-1255 PyrG− strain was cotransformed with plasmids pIBRC43-Pc*fkh1* (Fig. 6A) and pBG (containing the *pyrG* gene) (see Materials and methods). Those transformants complementing the uridine auxotrophy were selected and analysed by PCR to confirm the presence of the full 3290-bp overexpression cassette, which was amplified from the DNA of transformants 3, 6 and 19 (Fig. 6A) using primers PgdhF and TcycEndR. These transformants were also tested by Southern blot after the digestion of genomic DNA with *Sph*I and *Hind*III and using part of the Pc*fkh1* gene as probe (Fig. 6A). Results confirmed the presence of a 2021-bp band, corresponding to part of the overexpression cassette, in the genome of those transformants. In addition to the 5444-bp hybridization band corresponding to the

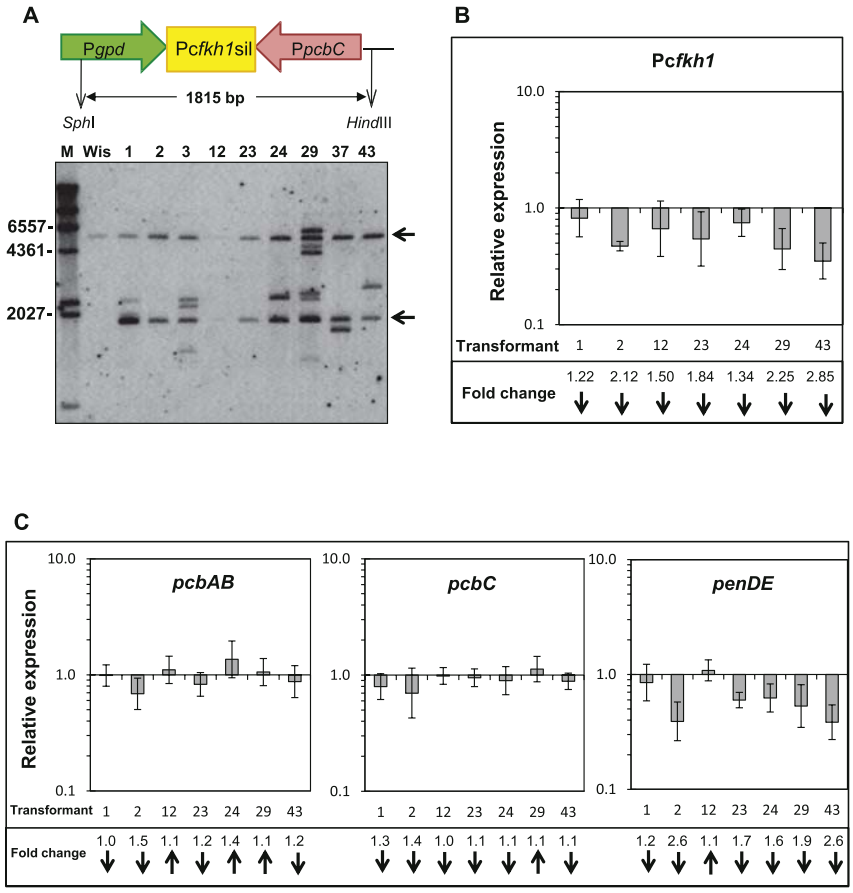

Fig. 4. Gene silencing of P*cfkh1*. (**A**) Southern blot analysis of different transformants and the parental *P. chrysogenum* Wisconsin 54-1255 strain (Wis) showing the integration of the 1815-bp silencing cassette (lower arrow). Note the presence of the 5444-bp genomic band containing the endogenous P*cfkh1* gene (upper arrow). The silencing fragment was used as probe. (**B**) Relative expression (quantified by RT-qPCR) of P*cfkh1* in different transformants compared to the Wisconsin 54-1255 strain (reference value set to 1). Values correspond to the mean plus standard deviation of three independent experiments. Fold change is indicated for each transformant. (**C**) Relative expression (quantified by RT-qPCR) of *pcbAB*, *pcbC* and *penDE* in different transformants compared to the Wisconsin 54-1255 strain (reference value set to 1). Values correspond to the mean plus standard deviation of three independent experiments. Fold change is indicated for each transformant. The increase or decrease in relative expression is represented by vertical arrows.

endogenous P*cfkh1* allele that was detected in all transformants and in the parental strain, they also showed a 9189-bp band that likely corresponds to a partially-digested fragment including the endogenous P*cfkh1* allele as it is indicated in Fig. 6A.

Transformants 3, 6 and 19 were cultured in CP medium and after 48 h, RNA was extracted and P*cfkh1* gene overexpression in the transformants was confirmed by RT-qPCR (Fig. 6B). All transformants exhibited a drastic increase in the P*cfkh1* transcript levels, an effect that was especially relevant in transformant 3, which showed an 11.93-fold increase in the steady-state levels of the P*cfkh1* mRNAs regarding control values. However, when the expression of the benzylpenicillin biosynthetic genes was tested in the P*cfkh1* overexpression transformants, no significant differences were found with respect to the control strain (Fig. 6C) (see Discussion).

The production of benzylpenicillin and IPN was analysed by HPLC in the P*cfkh1* overexpression transformants cultured in CP medium. When the supernatants from five biological replicates were analysed at 48 h and 72 h, a significant increase (20%) in the benzylpenicillin titers was observed in the overexpression transformants 3 and 6 (Fig. 7A), which suggest that an excess of PcFKH1 has slight positive effects on penicillin production (see Discussion). However, no significant differences in IPN were found between those transformants and the parental strain (Fig. 7B), indicating that the increase in the IAT activity in these transformants is modest and does not lead to a reduction in the IPN levels.

3.6. Role of PcFKH1 in the control of conidiation, spore pigmentation, morphology and cell division

With the aim of a further characterization of PcFKH1, the role of this regulator on conidiation and spore pigmentation was assessed using the knock-down transformant 2 and overexpression transformant 3. They were compared to the Wisconsin 54-1255 transformed with the “empty” pJL43-RNAi plasmid and to the PyrG-strain cotransformed with both the “empty” pIBRC43 and pBG plasmids.

With this purpose, 5×10^7 spores from each strain were spread on solid Power medium in triplicate. After a period of 5 days at 28 °C, spores were collected with 10 ml of 0.9% NaCl and counted

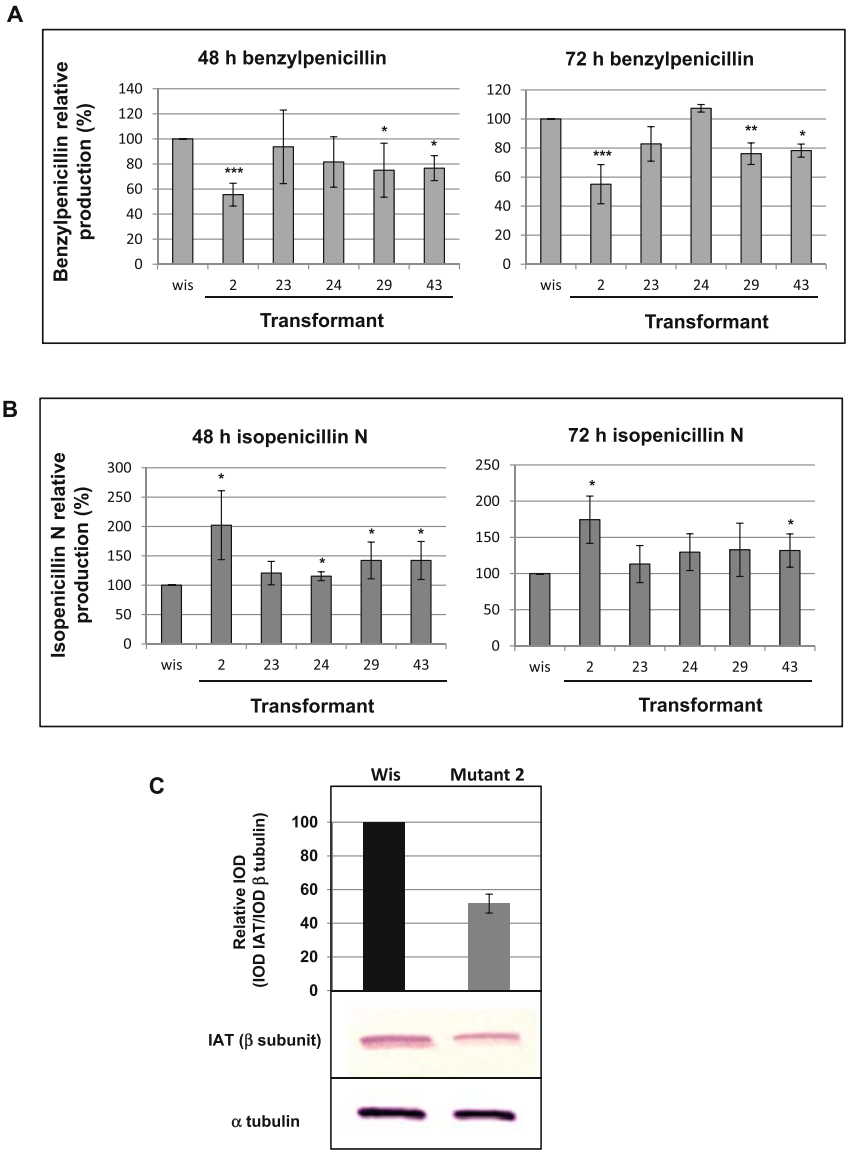

Fig. 5. Antibiotic production in the P*cfkh1* knock-down transformants. Relative production of (**A**) benzylpenicillin and (**B**) IPN in the Wisconsin 54-1255 strain (wis) and in the P*cfkh1* knock-down transformants grown in complex CP medium for 48 h and 72 h. Those values (μg/mg dry weight) provided by the Wisconsin 54-1255 strain (benzylpenicillin 48 h: 7.79 ± 0.21; benzylpenicillin 72 h; 19.52 ± 0.81; IPN 48 h: 5.84 ± 0.95; IPN 72 h: 10.74 ± 1.24) were set to 100%. Results correspond to the mean plus standard deviation of five biological replicates carried out in triplicate. Statistical significance is represented as follows: "*" ($0.01 \leq P < 0.05$); "**" ($0.001 \leq P < 0.01$); "***" ($P < 0.001$). (**C**) Synthesis of IAT in *P. chrysogenum* Wisconsin 54-1255 strain (Wis) and in the P*cfkh1* knock-down transformant 2. The immunoreactive band of α-tubulin was used as control. The intensity of the western blot bands (IOD) was determined by densitometry. IAT values were normalized to those provided by α-tubulin. Values corresponding to the IAT in the Wisconsin 54-1255 strain (Wis) were set to 100%. Values correspond to the mean plus standard deviation of three independent measurements.

using a haemocytometer. As shown in Fig. 8A, the number of spores in the knock-down transformant underwent a significant 2.5-fold reduction in comparison with the Wisconsin 54-1255 strain transformed with the "empty" pJL43-RNAi plasmid (as expected, the spore number of this control strain did not vary regarding the values obtained with the Wisconsin 54-1255 strain). On the other hand, the overexpression transformant produced 60% more spores than the control Wisconsin 54-1255 PyrG-strain cotransformed with the "empty" pIBRC43 and pBG plasmids (Fig. 8A). From these results, it can be concluded that PcFKH1 controls conidiation in *P. chrysogenum*.

In addition, after growing for 5 days at 28 °C in solid Power medium, the knock-down transformant showed a faint spore colour in comparison with the rest of strains, although the colour of the vegetative mycelium did not vary significantly (Fig. 8B). These results suggest that PcFKH1 also controls spore pigmentation.

Regarding morphology, we did not observe modifications in hyphal length, width or branching between transformants and

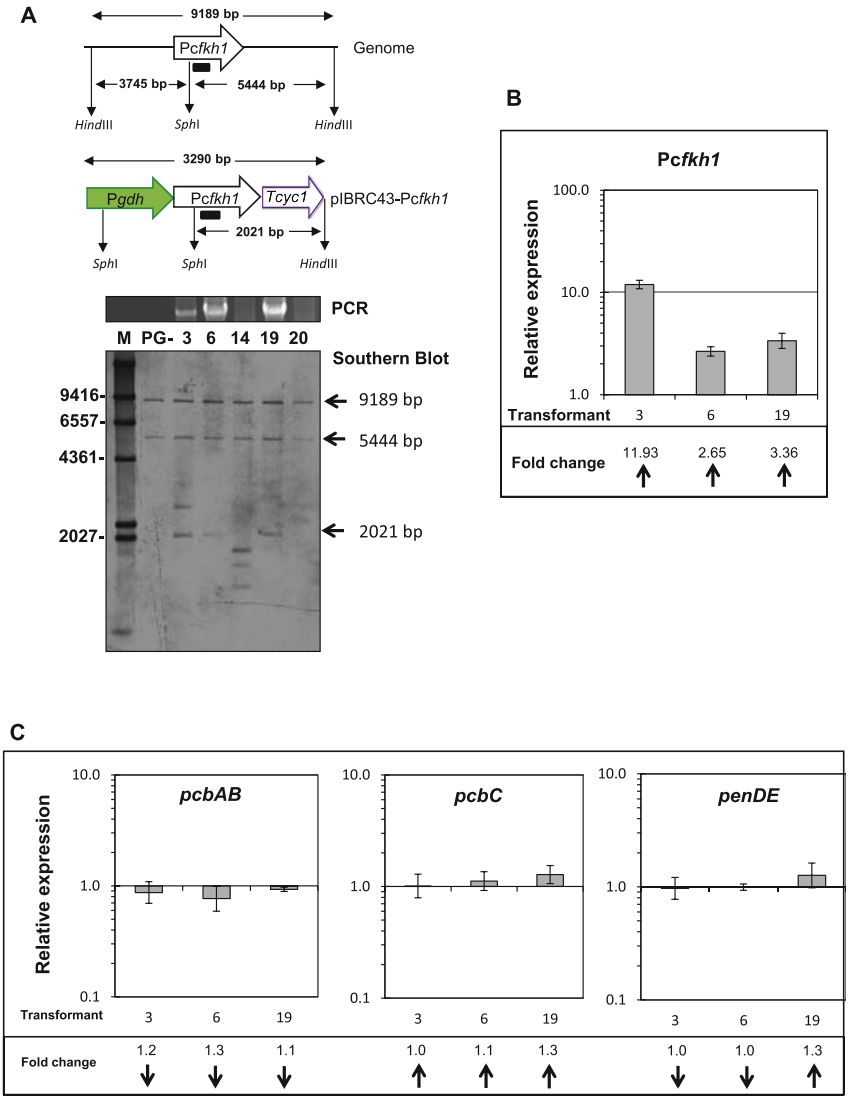

Fig. 6. Overexpression of the P*cfkh1* gene. (**A**) Southern blot and PCR analyses of five transformants and the parental *P. chrysogenum* Wisconsin 54-1255 PyrG-strain (PG-). The PCR was designed to amplify the full 3290-bp overexpression cassette. The Southern blot shows the integration of part of the overexpression cassette (2021 bp), since the restriction enzymes used for this experiment cut in the middle of this cassette. The endogenous gene is included in the 5444-bp and 9189-bp hybridizing bands, as a result of partial digestion (see Results). The fragment used as probe is represented by a black rectangle. (**B**) Relative expression (quantified by RT-qPCR) of P*cfkh1* in different transformants compared to the Wisconsin 54-1255 PyrG-strain (reference value set to 1). Values correspond to the mean plus standard deviation of three independent experiments. Fold change is indicated for each transformant. (**C**) Relative expression (quantified by RT-qPCR) of *pcbAB*, *pcbC* and *penDE* in different transformants compared to the Wisconsin 54-1255 PyrG-strain (reference value set to 1). Values correspond to the mean plus standard deviation of three independent experiments. Fold change is indicated for each transformant. The increase or decrease in relative expression is represented by vertical arrows.

control strains in submerged cultures at 24 h, 48 h or 72 h (data not shown). Radial growth remained similar between transformants and control strains (data not shown).

The role of PcFKH1 in cell division was assessed by means of the expression analysis of genes encoding proteins that have been reported to be involved in cell division in *P. marneffei*; namely Cdc4, Cdc15, KipB, SldA, BimB, Mob1, Mph1, NimA and Src1 [39]. The promoter regions of those genes (up to 900 bp upstream of the start codon and in the sense strand) were analysed in order to find consensus DNA binding sequences for FKH1 [30]. Four out of the nine genes included putative PcFKH1 binding sites (*mph1*, *src1*, *cdc15* and *mob1*; see Table 1) and their expression was analysed in the knock-down and overexpression transformants by RT-PCR using RNA extracted at 48 h. With the exception of *mob1*, which did not underwent amplification under the conditions tested, the other three genes provided clear amplification bands (data not shown). No significant changes were observed in the expression levels of *src1* and *mph1* with respect to control values, unlike *cdc15*, whose expression dramatically increased (400%) in the P*cfkh*1 knock-down transformant (Fig. 8C) (see Discussion).

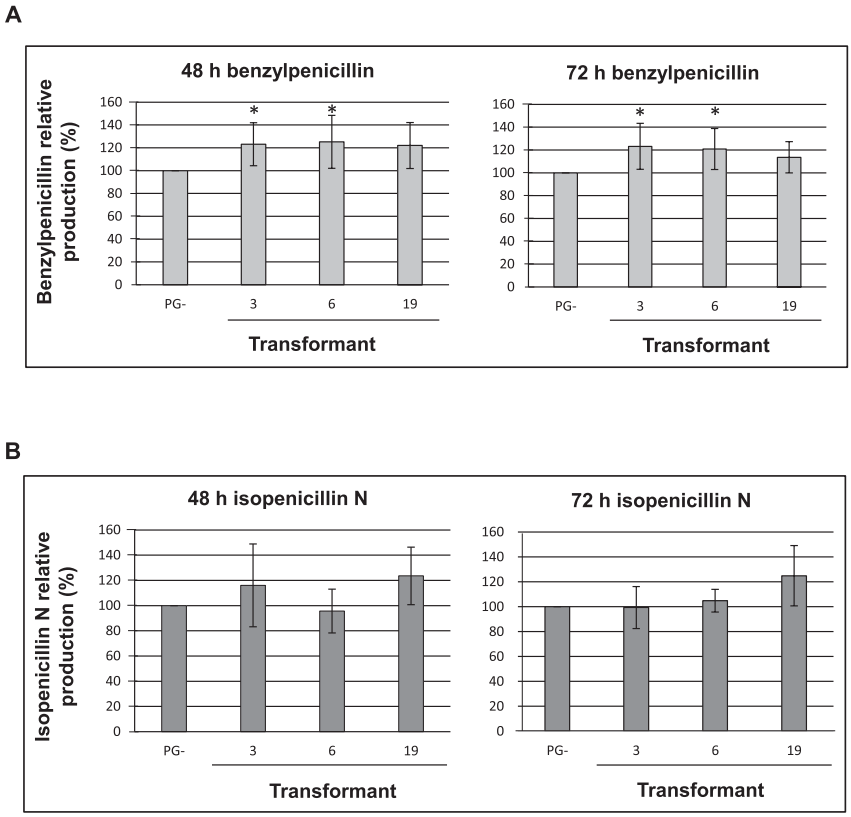

Fig. 7. Antibiotic production in the P*cfkh1* overexpression transformants. Relative production of (**A**) benzylpenicillin and (**B**) IPN in the Wisconsin 54-1255 PyrG-strain (PG-) and in the P*cfkh1* overexpression transformants grown in complex CP medium for 48 h and 72 h. Those values (μg/mg dry weight) provided by the Wisconsin 54-1255 PyrG-strain (benzylpenicillin 48 h: 10.01 ± 1.62; benzylpenicillin 72 h; 17.45 ± 2.40; IPN 48 h: 7.18 ± 1.07; IPN 72 h: 8.26 ± 0.74) were set to 100%. Results correspond to the mean plus standard deviation of five biological replicates carried out in triplicate. Statistical significance is represented as follows: "*" ($0.01 \leq P < 0.05$).

3.7. Other biological processes potentially controlled by PcFKH1

We decided to test whether the penicillin biosynthesis ancillary genes were controlled by PcFKH1 after confirming the presence of PcFKH1 putative binding motifs in their promoter regions (Table 2). The expression of the *ppt* gene encoding PPTase, *trxB* encoding one of the subunits of the NADPH-dependent thioredoxine disulfide reductase TrxAB (the promoter region of *trxA* did not include a PcFKH1 binding region) and *phlA* encoding the main phenylacetyl CoA ligase involved in penicillin biosynthesis, was analysed in the knock-down and overexpression transformants by RT-PCR using RNA extracted at 48 h. No significant differences were observed in the expression levels of those ancillary genes in the knock-down transformant (Fig. 9). However, when *Pcfkh1* was overexpressed a significant increase was observed in the expression of *phlA* and *ppt* genes (40% and 80%, respectively) (Fig. 9), which indicates that upregulation of PcFKH1 may play a positive role in penicillin biosynthesis at several levels of the biosynthetic process (see Discussion).

In an attempt to provide more putative targets for PcFKH1 and to understand the cellular processes potentially controlled by this regulator, we did an *in silico* analysis of the *P. chrysogenum* genes that contain a putative PcFKH1 DNA binding sequence. For this study we used the consensus sequence 5′-RYMAAYA-3′ [30]. Firstly, sites with a FKH1 regulation pattern were found in the whole *P. chrysogenum* genome using the program Patmatch [40]. These sites were filtered in order to obtain only those sites located in intergenic regions within 900 bp upstream of the corresponding ORF and additionally containing at least one putative binding site for the transcription factor PcRFX1 [26] (see Discussion). At the end of this process, 1752 genes putatively regulated by the promoter element were found, of which 842 had Gene Ontology annotation. These genes were analysed in order to find a common functional signal in the group. Biological Process annotations of Gene Ontology were retrieved from UniProt [41] and a p-value for each functional annotation category in the gene group was calculated by means of a hypergeometric distribution test. The results were filtered in order to avoid the functional categories in the group with less than three genes and with p-values over 0.05. In this way, the characteristic functions of the putative regulated genes were statistically measured and identified.

Table 3 shows the Gene Ontology terms of biological processes, which are over-represented in the group of putative regulated genes. The functional analysis revealed that the PcFKH1 transcription factor has a certain tendency to regulate genes involved in processes of primary metabolism (i.e. carbohydrate catabolism, carboxylic acid metabolic process, chitin catabolism, glycine catabolism, histidine biosynthesis, etc). Interestingly, one of these processes is related to the biosynthesis of pantothenate, which is the precursor of 4′-phosphopantetheine, a prostetic group used by

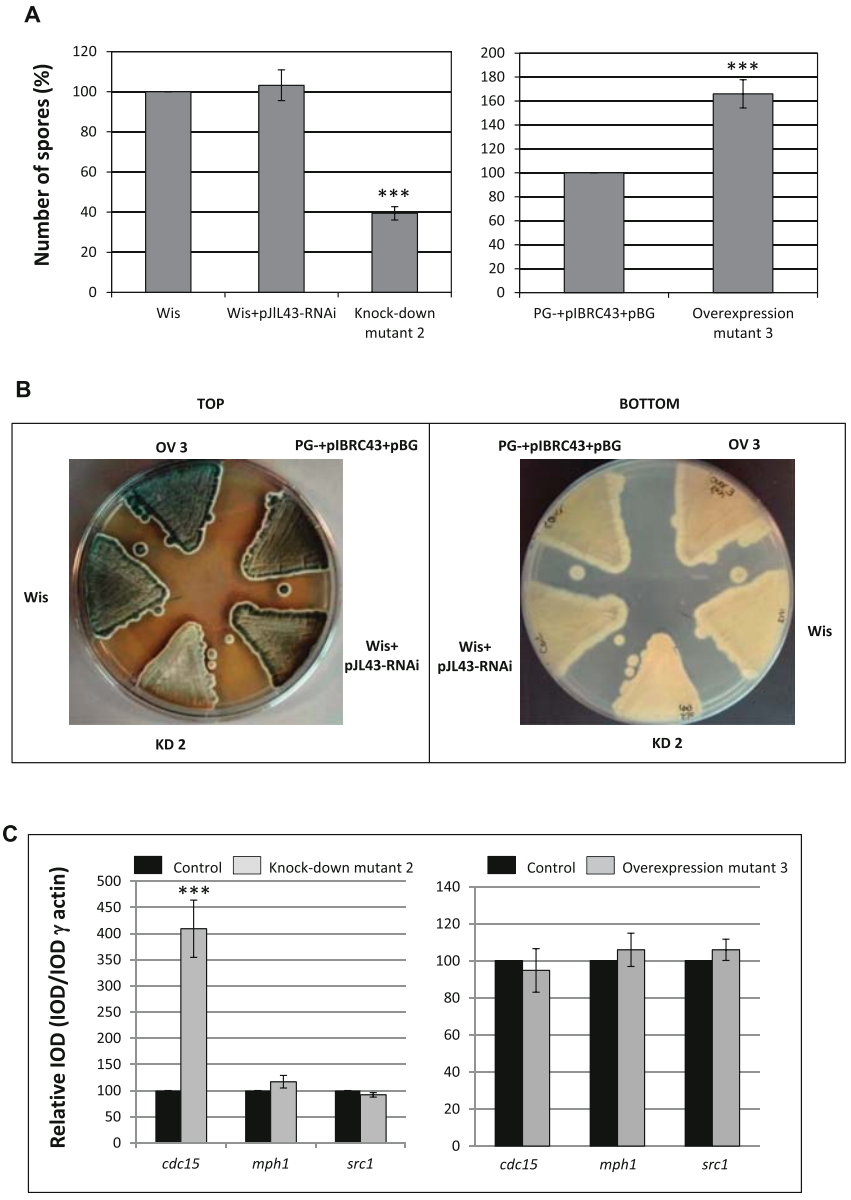

Fig. 8. Role of PcFKH1 in conidiation, pigmentation and cell division. (**A**) The number of spores present in a Petri dish with the knock-down transformant 2 was compared to the number of spores obtained with the Wisconsin 54-1255 (Wis) and with the Wisconsin 54-1255 strain transformed with the "empty" pJL43-RNAi plasmid. In a similar way the number of spores obtained with the overexpression transformant 3 was compared to the number of spores obtained with the PyrG-strain (PG-) cotransformed with both the "empty" pIBRC43 and pBG plasmids. Those values obtained with the Wisconsin 54-1255 and PyrG-strain cotransformed with both the "empty" pIBRC43 and pBG plasmids were set to 100%. Results correspond to the mean plus standard deviation of three independent experiments carried out in triplicate. (**B**) Pigmentation of the knock-down transformant 2 (KD2), overexpression transformant 3 (OV3), Wisconsin 54-1255 (Wis), Wisconsin 54-1255 strain transformed with the "empty" pJL43-RNAi plasmid (Wis + pJL43-RNAi) and Wisconsin 54-1255 PyrG-strain (PG-) cotransformed with both the "empty" pIBRC43 and pBG plasmids (PG- + pIBRC43 + pBG), which were grown for 5 days at 28 °C in solid Power medium. (**C**) Transcriptional analysis of three genes involved in cell division putatively regulated by PcFKH1. Densitometry (IOD) graphs showing the expression profiles (normalized to the γ-actin expression levels) at 48 h of *cdc15*, *mph1* and *src1* after Pc*fkh1* gene silencing (transformant 2) and overexpression (transformant 3). Those values corresponding to the expression of each gene in the control strains *P. chrysogenum* Wisconsin 54-1255 (for knock-down) or Wisconsin 54-1255 strain PyrG- (for overexpression) were set to 100%. Densitometry values correspond to the mean plus standard deviation of three independent measurements. Statistical significance is represented as follows: "***" ($P < 0.001$).

Table 1
Putative PcFKH1 binding sites in the promoter region of several genes of *P. chrysogenum* putatively involved in cell division.

P. chrysogenum ORF	Strand	Protein	PcFKH1 putative binding sites	
Pc20g06510	–	Mph1	−69	
Pc20g14730	–	Mob1	−371	−478
Pc12g11470	–	Src1	−469	−475
Pc13g10210	–	Cdc15	−173	

Table 2
Putative PcFKH1 binding sites in the promoter region of several ancillary genes of the penicillin biosynthetic process.

P. chrysogenum ORF	Strand	Protein	PcFKH1 putative binding sites	
Pc13g04050	–	PPTase	−292	−418
Pc22g02940	+	TrxB	−80	−612
Pc22g14900	–	PhlA	−392	

the ACV synthetase, fatty acid and polyketide synthases among other enzymes (see Discussion).

4. Discussion

P. chrysogenum is one of the best examples of the taming of a microorganism with industrial purposes. In fact, product titers and productivities have increased by three orders of magnitude in the last decades [3]. In addition to this unprecedented success in classical strain improvement, the recent omics techniques have contributed to the understanding of some of the mechanisms responsible for the increased penicillin productivity [5,7,42]. Nevertheless, one of the most interesting and challenging topics that remain to be elucidated is related to the regulatory mechanisms controlling the penicillin biosynthesis and production. In general, fungal secondary metabolism regulation is composed of overlapping and interconnected pathways [43]. In the absence of penicillin pathway-specific CSRs (regulatory genes situated in the *pen* gene cluster), global regulators have been pointed out as the main candidates to control the penicillin biosynthetic process. In fact, some global regulators have been reported to participate in the regulation of penicillin biosynthesis, such as PacC [44], CreA [18], LaeA and the Velvet complex [22,24,25]. Recently, the winged-helix proteins (another family of global regulators) have been described in the regulation of beta-lactam antibiotics. Hitherto, these proteins are represented by CPCR1 [27,28] and its interacting protein AcFKH1 [29] in *A. chrysogenum* and by the CPCR1 ortholog PcRFX1 [26] and the forkhead transcription factor PcFKH1 (characterized in this work) in *P. chrysogenum*.

AcFKH1 contains two conserved domains; i) the forkhead associated domain (FHA), which is involved in phosphoprotein interactions [45] and ii) the DNA binding C-terminal domain (FKH). The comparative analysis of the FKH1 amino acid sequence with orthologs in different fungi revealed a moderate similarity to the *A. chrysogenum* FKH1 (another beta-lactam producing fungus), whereas the similarity was much higher with the orthologs of other *Penicillium* or *Aspergillus* species. This result indicates that *fkh1* has evolved in parallel with other phylogenetic parameters (i.e. rRNA), but is not phylogenetically linked to the beta-lactam genes.

We have demonstrated that the recombinant PcFKH1 interacts with the consensus sequences 5′-RYMAAYA-3′[30] present within the *pcbC* and *penDE* gene promoters. Two distinct shifted bands were observed with the *penDE* promoter probe DE-113 DE-265 (Fig. 3), which indicates that two DNA-protein complexes were formed. Since two binding sites for PcFKH1 are included in the same probe (due to the proximity of such sites), once one binding site is occupied (lower band), further protein can bind the other site, thus accounting for the upper DNA-protein band. Interestingly, binding of recombinant PcFKH1 to the *pcbC* and *penDE* promoters showed different affinities (Figs. 2 and 3). Unlike the *penDE* gene promoter probe, both *pcbC* gene promoter probes did not provide a specific interaction according to the competition experiments. This suggests a differential regulatory role of PcFKH1 in the expression of these penicillin biosynthetic genes. In fact, only the *penDE* transcripts were downregulated in the Pc*fkh1* knock-down transformants (Fig. 4), which confirms that PcFKH1 exerts a stronger control on the *penDE* gene. This is consistent with the phenotype observed in the knock-down transformants, which increased the IPN levels and reduced the production of benzylpenicillin as a consequence of IAT depletion (Fig. 5). Interestingly, overexpression of the Pc*fkh1* gene did not upregulate the expression of the

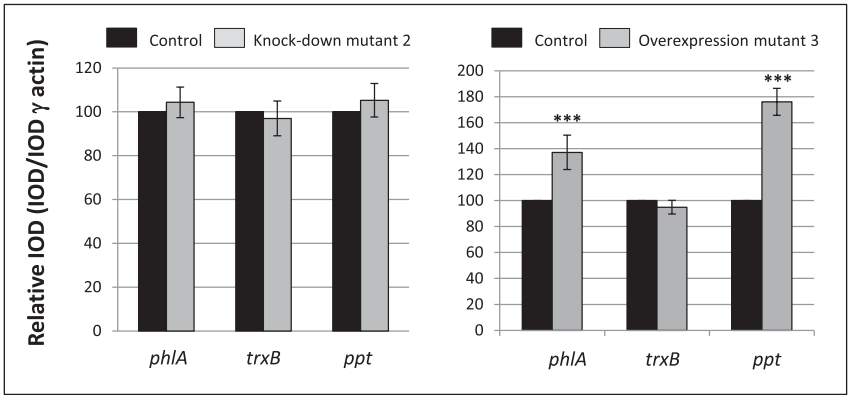

Fig. 9. Transcriptional analysis of three penicillin biosynthesis ancillary genes putatively regulated by PcFKH1. Densitometry (IOD) graphs showing the expression profiles (normalized to the γ-actin expression levels) at 48 h of *phlA*, *trxB* and *ppt* after Pc*fkh1* gene silencing (transformant 2) and overexpression (transformant 3). Those values corresponding to the expression of each gene in the control strains *P. chrysogenum* Wisconsin 54-1255 (for knock-down) or Wisconsin 54-1255 strain PyrG- (for overexpression) were set to 100%. Densitometry values correspond to the mean plus standard deviation of three independent measurements. Statistical significance is represented as follows: "***" ($P < 0.001$).

Table 3
Cellular processes potentially controlled by PcFKH1 and PcRFX1.

GO ID	GO Description	N of putative regulated genes with this GO	N of putative regulated genes with GO annotation	N of genes with this GO	N of genes with GO annotation	*p*-Value
6529	Asparagine biosynthetic process	2	842	3	6546	0.0021
9082	Branched chain family amino acid biosynthetic process	3	842	8	6546	0.0124
5975	Carbohydrate metabolic process	30	842	176	6546	0.0406
19752	Carboxylic acid metabolic process	2	842	5	6546	0.0173
6032	Chitin catabolic process	3	842	6	6546	0.0033
6546	Glycine catabolic process	2	842	3	6546	0.0021
105	Histidine biosynthetic process	3	842	6	6546	0.0033
6886	Intracellular protein transport	10	842	46	6546	0.0284
6564	L-serine biosynthetic process	2	842	3	6546	0.0021
7067	Mitosis	3	842	11	6546	0.0426
6334	Nucleosome assembly	3	842	11	6546	0.0426
15940	Pantothenate biosynthetic process	2	842	5	6546	0.0173
51056	Regulation of small GTPase mediated signal transduction	2	842	6	6546	0.0314
6278	RNA-dependent DNA replication	4	842	14	6546	0.0257

penicillin biosynthetic genes (Fig. 6). One explanation to this phenomenon might be the tight regulation of such genes, where the physiological levels of PcFKH1 are sufficient to control the expression of the biosynthetic genes and therefore, an excess of this transcription factor (due to overexpression) is unable to exert a bigger regulatory effect. However, we have also observed that overexpression of Pc*fkh1* slightly increased penicillin production (Fig. 7A). These results are explained by the positive effect observed on the expression of ancillary genes, such as *phlA* (encoding phenylacetyl CoA ligase) and *ppt* (encoding PPTase) (Fig. 9). The positive effect of the overexpression of these two genes on benzylpenicillin production has been previously reported [13,15].

Forkhead transcription factors are a subclass of winged-helix transcription regulators [46] that in humans and yeasts are involved in different processes such as cell cycle regulation, cell death control, pre-mRNA processing or morphogenesis [47,48], and [49]. In fact, AcFKH1 is not directly involved in the fragmentation of hyphae, but its presence seems to be necessary for CPCR1 function in *A. chrysogenum* morphogenesis [50]. It has been suggested that CPCR1 and AcFKH1 behave as a molecular link between secondary metabolism (antibiotic production) and morphogenesis and therefore, they represent two very important factors in the control of fungal growth during metabolite production processes. However, neither PcRFX1 [26] nor PcFKH1 (this work) seem to be involved in morphological differentiation in *P. chrysogenum* under the conditions tested. It is worth noting that the strain used for these experiments (Wisconsin 54-1255) is not a wild-type strain, but a laboratory reference strain that has undergone several rounds of classical mutagenesis during early strain improvement programs, where morphological differentiation may have been slightly modified, although it shows normal morphology. Therefore, a possible role of PcFKH1 in morphological development cannot be completely ruled out.

Forkhead transcription factors also regulate sexual development and cell cycle in fungi [51] and [52]. One of the genes involved in cell cycle is *cdc15*. This gene has been reported to be important for growth, septation, asexual development and pathogenicity in *Magnaporthe oryzae* [53] and plays a key role in the regulation of the assembly and contraction of the actomyosin ring during cytokinesis in *Schizosaccharomyces pombe* [54] and in *Saccharomyces cerevisiae* [55]. Deletion of *fkh1* upregulated the expression of *cdc15* in *M. oryzae* [52] and in *S. pombe* [56], a phenomenon that has been also observed in *P. chrysogenum* (Fig. 8C). However, the increased *cdc15* mRNA levels in the Pc*fkh1* knock-down transformant were not accompanied by phenotypical effects. This can be explained by the fact that we are working with a *Pcfkh1* knock-down transformant with still basal Pc*fkh1* mRNA levels. In addition, as it was indicated before, the transformant has been obtained in a laboratory reference strain, which can have undergone previous modifications in cell cycle and development during early improvement programs.

We have observed that PcFKH1 positively regulates conidiation and spore pigmentation in *P. chrysogenum* (Fig. 8A and B). The role of this transcription factor on the production of conidia has been reported in other filamentous fungi, such as *M. oryzae,* where the relative abundance of transcripts from the MoFKH1 encoding gene increased during conidiation and/or in conidia, supporting its role in controlling the production of these structures [52,57].

The *in silico* analysis of metabolic processes putatively controlled by PcFKH1 was performed considering those promoter regions that contain at least one putative PcFKH1 and one PcRFX1 DNA binding sequences. This was due to the fact that FKH1 and CPCR1 (the PcRFX1 ortholog) interact in *A. chrysogenum* [29] and therefore, assuming that both transcription factors may work together in *P. chrysogenum* as well, we limited the search to those genes that have binding sites for both proteins in their promoter region to define precisely the candidate genes and processes. One of these processes is related to pantothenate biosynthesis (Table 3) and was represented by PanB (ketopantoate hydroxymethyltransferase), which is the first enzyme in the pantothenate biosynthesis pathway, and PanE (ketopantoate reductase), the latter being the second enzyme in the pathway. Pantothenate is the precursor of 4′-phosphopantetheine, an essential prosthetic group that is transferred to the ACV synthetase by means of the PPTase [13]. Taking into account that the expression of the *ppt* gene encoding PPTase is controlled by PcFKH1 (Fig. 9), the putative positive regulation of *panB* and *panE* may represent an additional beneficial effect of PcFKH1 on penicillin biosynthesis.

This global analysis will help us to investigate the link between primary metabolism, cell cycle and the biosynthesis of penicillin. This information can be of great interest to shed light on the mechanisms underlying beneath the complex regulatory network of this important beta-lactam antibiotic.

5. Conclusions

Using overexpression and gene silencing techniques (the latter being an alternative for the study of either essential genes whose knock-out may give rise to nonviable strains, or regulatory genes putatively involved in the control of growth and morphology

whose deletion may cause crippling effects in the strain), we have reported here that the *P. chrysogenum* AcFKH1 ortholog (PcFKH1) controls the expression of the *penDE* gene, thus playing a role in penicillin biosynthesis. It also plays a role in conidiation and spore pigmentation, but it does not seem to be involved in hyphal morphology. A global map of processes putatively coregulated by PcFKH1 and PcRFX1 in *P. chrysogenum* has been provided, which will be helpful for the understanding of the complex regulatory network underlying the process of penicillin biosynthesis.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2015.05.015.

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SUPPLEMENTARY MATERIAL

Journal: BIOCHIMIE

Article Title: "PcFKH1, a novel regulatory factor from the forkhead family, controls the biosynthesis of penicillin in *Penicillium chrysogenum*"

Supplementary Table S1. List of primers used in this work.

Primer name	Sequence (5´- 3´)	Use
FKH2NcoIF	GGATATTCTACGCCATGGATGCCCCTCACAAAC[1]	Amplification of the 2302-bp Pc*fkh1* gene. Sequencing of the full gene and overexpression.
FKH2PvuIIR	GCCGCTACTTGACAGCTGCTACGATGGTGC[2]	
FKH2silF	GGTTGCCATGGGAGATCTTTTATGATG[1]	Amplification of a 449-bp Pcfkh*1* exon fragment. Gene silencing. RT-PCR experiments.
FKH2silR	GCCTCCCATGGAAAGCGGGGACTGTTTAG[1]	
PgdhF	GGACTCCCTAATGGATTCCGAG	Amplification of a 3290-bp overexpression cassette.
TcycEndR	CAAATTAAAGCCTTCGAGCG	
RTactAF	CTGGCCGTGATCTGACCGACTAC	Amplification of 457 bp from the *actA* -encoding β-actin gene. RT-PCR experiments.
RTactAR	GGGGGAGCGATGATCTTGACCT	
KA	CATCTGGGCTGCAAGCGT	Amplification of 88 bp from the *pcbAB* /*pcbC* promoter. EMSA experiments. Specific probe C-173.
KB	ACCTGAGCTCTCTTCCTG	
K10	AGACCAATGCAGCAGGCC	Amplification of 125 bp from the *pcbAB* /*pcbC* promoter. EMSA experiments. Specific probe C-1004.
KPREX	GTCATGTCTGTCAATGAC	
PpenDEFKHbox1F	CTCTTCATGCAAGAGGTCA	Amplification of 198 bp from the *penDE* gene promoter. EMSA experiments. Specific probe DE-113/DE-265.
PpenDEFKHbox2R	GAACTGCATGCTGAGATTTA	
K7	TGCACGTTGCCTACTCTC	Amplification of 132 bp from the pcbAB/pcbC promoter without binding sites for PcRFX1 and PcFKH1. EMSA experiments. Unspecific probe.
K9	GGGCAGAGCAACACTCCC	
RPc12g11470	GGGAGTCGATACGCGAGGAGAGC	Amplification of 414 bp from Pc12g11470 (*src* 1). RT-PCR experiments.
FPc12g11470	CACTTGCCCTCGGTGGCCTAGTG	
RPc20g06510	GAGCTGCCGCCTCTACCAATG	Amplification of 399 bp from Pc20g06510 (*mph* 1). RT-PCR experiments.
FPc20g06510	CTCGAAGGAACAACCACAACCC	
FPc20g14730	GCCTTCATCTCGGGACATGTCTG	Amplification of 464 bp from Pc20g14730 (m*ob* 1). RT-PCR experiments.
RPc20g14730	ATGTCTGTCCCCGCTACCCCTC	
FPc13g10210	GCCTTTCTTTGTCATGGCGACTC	Amplification of 418 bp from Pc13g10210 (*cdc15*). RT-PCR experiments.
RPc13g10210	CAGCAGCCTCCTATGCAACAGC	
FPc22g14900	GACCCATACACCTGTGGTATTAC	Amplification of 357 bp from Pc22g14900 (*phlA*). RT-PCR experiments.
RPc22g14900	GTAGATTCTGTTCTTCGGGAGAC	
FPc22g02940	GCTCCAGCCAGTCCTTTACG	Amplification of 490 bp from Pc22g02940 (*trxB*). RT-PCR experiments.
RPc22g02940	CTTGCGAACGAGAACAGTCAC	
FPc13g04050	CGAGATCACAATGCAATGGTAG	Amplification of 351 bp from Pc13g04050 (*ppt*). RT-PCR experiments.
RPc13g04050	GAGACCAGCCTGATGGCTGAC	
fkhΔintron1F	CCGCAAAGAAGCGCAAGGTTGACCGC	Deletion of the first intron of Pc*fkh* 1. Site-directed mutagenesis.
fkhΔintron1R	GTGTGGCGGTCAACCTTGCGCTTCTTT	
fkhΔintron2F	GTTTGATCTTGCTAGGGGCTTCGAACC	Deletion of the second intron of Pc*fkh* 1. Site-directed mutagenesis.
fkhΔintron2R	CAATAGGTTCGAAGCCCCTAGCAAGA	
BamHIcDNAfkhF	CTTCAGAGGATCCATGCCCCTCACAAA[3]	Amplification of the Pc*fkh* 1 cDNA with *BamH* I and *Hind* III.
HindIIIcDNAfkhR	TCTCCGAAGCTTTCAAGTAGCGGCCG[4]	

[1] **The sequence corresponding to the *Nco* I restriction site is underlined**
[2] **The sequence corresponding to the *Pvu* II restriction site is underlined**
[3] **The sequence corresponding to the *BamH* I restriction site is underlined**
[4] **The sequence corresponding to the *Hind* III restriction site is underlined**

Supplementary Table S2. Primers used for qPCR experiments.

Pc21g21390 (*pcbAB*)

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	3323	20	59.93	50.00	5´-atggggacaatctcacttcg-3´
RIGHT PRIMER	3457	20	59.99	50.00	5´-cgttcatatcacaccgttcg-3´

Pc21g21380 (*pcbC*)

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	545	20	60.11	50.00	5´-cctcggttgttctgattcgt-3´
RIGHT PRIMER	668	20	60.14	55.00	5´-aggacggtaatgagcgacac-3´

Pc21g21370 (*penDE*)

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	118	20	59.99	50.00	5´-acgaagaagacggacgaaga-3´
RIGHT PRIMER	253	20	59.79	50.00	5´-tgacaatctcggagacatcg-3´

Pc20g11630 (*actA*)

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	9	20	58.91	50.00	5´-agaagttgctgctctcgtca-3´
RIGHT PRIMER	106	20	60.86	45.00	5´-cgacaatggaagggaaaaca-3´

Pc18g00430 (fkh1)

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	1561	20	60.12	55.00	5´-aaccaccactccagcatctc-3´
RIGHT PRIMER	1696	20	60.19	50.00	5´-cgaggagggttcttcattg-3´

Artículo 3

Direct involvement of the CreA transcription factor in penicillin biosynthesis and expression of the *pcbAB* gene in *Penicillium chrysogenum*

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Direct involvement of the CreA transcription factor in penicillin biosynthesis and expression of the *pcbAB* gene in *Penicillium chrysogenum*

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Abstract The transcription factor CreA is the main regulator responsible for carbon repression in filamentous fungi. CreA is a wide domain regulator that binds to regulatory elements in the promoters of target genes to repress their transcription. Penicillin biosynthesis and the expression of penicillin biosynthetic genes are subject to carbon repression. However, evidence of the participation of CreA in this regulation is still lacking, and previous studies on the promoter of the *pcbC* gene of *Aspergillus nidulans* indicated the lack of involvement of CreA in its regulation. Here we present clear evidence of the participation of CreA in carbon repression of penicillin biosynthesis and expression of the *pcbAB* gene, encoding the first enzyme of the pathway, in *Penicillium chrysogenum*. Mutations in *cis* of some of the putative CreA binding sites present in the *pcbAB* gene promoter fused to a reporter gene caused an important increase in the measured enzyme activity in glucose-containing medium, whereas activity in the medium with lactose was not affected. An RNAi strategy was used to attenuate the expression of the *creA* gene. Transformants expressing a small interfering RNA for *creA* showed higher penicillin production, and this increase was more evident when glucose was used as carbon source. These results confirm that CreA plays an important role in the regulation of penicillin biosynthesis in *P. chrysogenum* and opens the possibility of its utilization to improve the industrial production of this antibiotic.

Keywords Carbon repression · CreA · Filamentous fungi · *Penicillium chrysogenum* · Penicillin · Transcriptional regulation

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Introduction

Carbon source regulation is a general mechanism that operates in bacteria and fungi preventing the synthesis and/or activity of enzymes required for assimilation of a wide variety of carbon sources when a more readily utilized carbon source is available (Ronne 1995). Biosynthesis of many secondary metabolites and antibiotics is also subject to carbon source regulation (Sánchez et al. 2010). Glucose and other repressing carbon sources exert their control at various levels, from transcriptional repression to enzyme inhibition (Klein et al. 1998). The term carbon repression refers to the repressing effect that glucose and other readily utilized carbon sources exert on the expression of different genes. In *Saccharomyces*

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cerevisiae, the final effector responsible for repression of glucose-regulated genes is the transcription factor Mig1 (Nehlin and Ronne 1990). Genes orthologous to *MIG1* have been found in filamentous fungi and have been named *creA*/*cre1* (for Carbon REpression); these include *Aspergillus nidulans creA* (Dowzer and Kelly 1991), *Trichoderma reesei cre1* (Strauss et al. 1995), *Neurospora crassa cre-1* (de la Serna et al. 1999), *Gibberella fujikuroi creA* (Tudzynski et al. 2000) and *Fusarium oxysporum cre1* (Jonkers and Rep 2009). A consensus binding sequence, SYGGRG, for *A. nidulans* CreA was defined by DNase I footprinting (Cubero and Scazzochio 1994; Kulmburg et al. 1993), which mostly coincided with that for Mig1: GCGGRG. In the proline utilization gene cluster of *A. nidulans*, mutations or deletion of the CreA binding sites in the intergenic region of the divergently transcribed *prnB* and *prnD* genes results in specific derepression of the *prn* cluster (Cubero and Scazzochio 1994; Sophianopoulou et al. 1993).

Penicillin biosynthesis is subject to carbon source regulation in *Penicillium chrysogenum* and *A. nidulans* (Espeso and Peñalva 1992; Martin 2000; Revilla et al. 1984). In *P. chrysogenum*, glucose represses in vivo formation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) and isopenicillin N synthase activity (Revilla et al. 1986). Gutiérrez et al. (1999) showed that RNA transcript levels of genes *pcbAB* (encoding ACV synthetase), *pcbC* (encoding isopenicillin N synthase) and *penDE* (encoding acyl-CoA: isopenicillin N acyltransferase) were drastically reduced by glucose. Genes *pcbAB* and *pcbC* are transcribed divergently from a common intergenic region both in *P. chrysogenum* (Díez et al. 1990) and in *A. nidulans* (MacCabe et al. 1990). Using reporter genes in *A. nidulans*, Brakhage et al. (1992) showed that glucose repressed transcription from the *ipnA* (*pcbC*) gene promoter but had a much weaker effect on the *acvA* (*pcbAB*) promoter. Similar results regarding repression of the *ipnA* gene by glucose were obtained by Espeso and Peñalva (1992). Pérez-Esteban et al. (1993) carried out a deletion analysis of the *ipnA* gene promoter in search for regulatory sequences and found a region involved in sucrose repression. Two near consensus CreA binding sites are present in this region, within a 29-bp sequence that was protected in DNase I footprint analysis using a GST::CreA fusion protein (Espeso et al. 1993). However, deletion of this 29-bp sequence did not result in derepression of *ipnA* expression in medium with sucrose (Espeso et al. 1993). In addition, extreme *creA* loss-of-function mutations had very little effect on carbon repression of *ipnA* gene expression (Espeso and Peñalva 1992). These results suggest that carbon source regulation of penicillin biosynthesis in *A. nidulans* is not directly mediated by CreA.

Therefore, it was of great interest to study the possible involvement of CreA in the biosynthesis of penicillin in *P. chrysogenum*, the fungus used for industrial production of this antibiotic, since carbon regulation is a possible target for improvement of the production process. We used the reporter gene *lacZ* fused to the *pcbAB* gene promoter, integrated in single copy at the *pyrG* site, to test functionality of CreA binding sites present in the intergenic *pcbAB-pcbC* region. We also used an RNAi strategy to attenuate expression of CreA and analyse its effect on penicillin production. Ullán et al. (2008) showed that expression of small interfering RNA (siRNA) targeted against specific genes is a suitable method for effective gene silencing in *P. chrysogenum*. In addition, Janus et al. (2009) found evidence for a Dicer-dependent RNA interference mechanism in this fungus. Our results show that CreA is the main regulator responsible for carbon repression of penicillin biosynthesis and transcription of the *pcbAB* gene.

Materials and methods

Fungal and bacterial strains

P. chrysogenum Wisconsin 54-1255 (ATCC 28089) was used as recipient strain for transformation with the CreA-silencing plasmid pCreA-RNAi. *P. chrysogenum npe*10 *pyrG*$^{-}$ was used as host strain to obtain single copy transformants of the promoters fused to the *lacZ* reporter gene. This strain is a uridine auxotroph obtained by mutation of the *npe*10 strain (Díez et al. 1987), which is a deletion mutant derived from *P. chrysogenum* Wisconsin 54-1255 lacking the entire penicillin gene cluster (Cantoral et al. 1993; Fierro et al. 1996a). *Escherichia coli* DH5α was used as host strain for plasmid manipulations.

Culture conditions

P. chrysogenum was grown on power solid medium (García-Rico et al. 2008) for 5 to 7 days to obtain conidia for inoculation of liquid medium cultures. Flask cultivations for the determination of β-galactosidase activity in the single copy P*pcbAB*::*lacZ* transformants were performed as follows: conidia at a concentration of 1×10^7 mL^{-1} were inoculated in flasks containing 100 mL of complex seed (CS) medium (g L^{-1}: corn steep solids, 20; sucrose, 20; yeast extract, 10; $CaCO_3$ 5; pH 5.7), which were incubated for 24 h at 25 °C and 250 rpm in a rotary shaker. Eight millilitres from the seed cultures were then inoculated in 500 mL flasks containing 100 mL of complex production (CP) medium (g L^{-1}: phenylacetic acid, 4; $(NH_4)_2SO_4$, 4; $CaCO_3$, 5; Pharmamedia®, 20; pH 6.1) with either lactose or glucose (30 g L^{-1}) as the only added carbon source, and they were incubated for 48 h at 25 °C and 250 rpm. Pharmamedia® is a flour made from the embryo of cottonseed (Archer Daniels Midland Company, Decatur, IL).

Flask cultivations for the determination of penicillin production in *creA* knock-down mutants (harbouring plasmid pCreA-RNAi) were performed as follows: conidia at a concentration of 1×10^7 mL^{-1} were inoculated in flasks containing

50 mL of defined inoculation (DI) medium (Casqueiro et al. 1999), which were incubated for 36 h at 25 °C and 220 rpm in a rotary shaker. Eight millilitres from the seed cultures were transferred to 500 mL flasks containing 100 mL of CP medium with either lactose or glucose (30 g L^{-1}) as the only added carbon source. Flasks, in duplicate, were incubated for 120 h at 25 °C and 220 rpm. Every 24 h, 5 mL samples were taken to determine penicillin G by bioassay, pH and dry weight as previously described (García-Rico et al. 2007).

Cultures at controlled pH were carried out in BIOSTAT B 5-L bioreactors (Braun). Conidia at a concentration of 1×10^7 mL^{-1} were inoculated in flasks containing 100 mL of DI medium with 3 % lactose and 1 % glucose (*w*/*v*), which were incubated for 24 h at 25 °C and 250 rpm in a rotary shaker. A volume of 3,333 mL of CP medium, with either lactose or glucose (30 g L^{-1}) as carbon source, was inoculated with 267 mL (8 %*v*/*v*) of the seed culture, for a total volume of 3.6 L in the bioreactor. The culture was incubated for 72 h at 25 °C with an initial agitation speed of 300 rpm, which was gradually increased during the cultivation period to maintain a constant oxygen supply, and a constant pH value of 6.0, maintained throughout the culture time by the addition of HCl 5 N or KOH 5 N. Samples of 80 mL were taken at 24, 36, 48 and 72 h for β-galactosidase assays.

Cell-free extracts and β-galactosidase assays

β-Galactosidase activity was determined in extracts from mycelia grown in CP medium as described above. Mycelia were harvested by filtration through nylon filters, washed with 0.9 % NaCl, dried between filter papers and frozen in liquid nitrogen. The mycelial cake was ground to a fine powder in liquid nitrogen on a mortar, resuspended in 1.5 mL of extraction buffer (50 mM sodium phosphate buffer pH 7.0; 1 mM EDTA; 20 μM phenylmethylsulfonyl fluoride) and kept on ice. The extracts were centrifuged at 4,000 rpm for 10 min at 4 °C, and the supernatants centrifuged again at 14,000 rpm for 10 min at 4 °C. The final supernatants were stored on ice until used for the assays.

β-Galactosidase assays were carried out with 5 to 200 μL of clear extracts and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate, as described by Miller (1972). Protein concentration in the extracts was determined with the Bradford dye reagent (BioRad) according to the manufacturer's specifications.

Plasmid constructions

Plasmid pCreA-RNAi is a derivative of pJL43-RNAi (Ullán et al. 2008) and confers resistance to phleomycin. Oligonucleotides creAi-F: 5′-TACGCCATGGTTCTCCAACTACGCCAACCACA-3′ and creAi-R: 5′-TACGCCATGGTGACCCTGATTCGGGCTGTAAT-3′ (tails containing *Nco*I restriction sites underlined) were used to amplify a 533-bp exon fragment from the *P. chrysogenum creA* gene (gene Pc20g13880 from the *P. chrysogenum* genome database at the NCBI). The PCR product was digested with *Nco*I and cloned into *Nco*I-digested pJL43-RNAi to yield pCreA-RNAi. This allows the generation of a siRNA by transcription from two opposite promoters flanking the 533-bp *creA* gene fragment. Plasmid pCreA-RNAi was used to transform the *P. chrysogenum* Wisconsin 54-1255 strain.

Plasmids containing the P*pcbAB*::*lacZ* gene fusions carrying different mutated CreA binding sites were constructed using the vector pZ2b (Gutiérrez et al. 1999), which contains the *P. chrysogenum pyrG* gene mutated at the *Bam*HI site enabling selection of single copy transformants targeted to the mutated *pyrG* gene locus of strain *P. chrysogenum npe*10 *pyrG*, as described by Gutiérrez et al. (1999). It also contains the *lacZ* gene of *E. coli*, with a *Bam*HI restriction site next to the ATG start codon, and the *trpC* terminator downstream of *lacZ*. Plasmid constructions were made as follows: a 1,161-bp *Nco*I fragment containing the intergenic *pcbAB-pcbC* region was inserted, after *Nco*I filling in with Klenow, into *Bam*HI-digested+Klenow-filled in pBluescript KS+ (Stratagene-Agilent) to give rise to plasmid pBKS+AB. Different mutations of CreA binding sites in the *pcbAB-pcbC* intergenic region were performed with the QuickChange® Site-Directed Mutagenesis Kit (Stratagene-Agilent) using the oligonucleotides described in Table 1 and plasmid pBKS+AB as template. The mutated promoters were then excised by *Bam*HI digestion and ligated to *Bam*HI-digested pZ2b, giving rise to P*pcbAB*::*lacZ* gene fusions carrying different mutated CreA binding sites. An in-frame translational fusion is obtained, and the 5′ end of the *pcbAB* gene is retained, so that a hybrid ACV synthetase::β-galactosidase protein is formed, containing the first 49 amino acids of the ACV synthetase. Plasmids containing the gene fusions with the mutated CreA sites are described in Table 2.

Transformation of *P. chrysogenum* protoplasts and isolation of single copy transformants

Protoplast isolation and transformation of *P. chrysogenum* Wisconsin 54-1255 with plasmid pCreA-RNAi were performed as previously described (Cantoral et al. 1987). Clones from transformed protoplasts were selected by growing on Czapek minimal medium containing 30 μg/mL of phleomycin.

Isolation of single copy transformants with the P*pcbAB*::*lacZ* gene fusions carrying mutated CreA binding sites was achieved as described by Gutiérrez et al. (1999), by targeted integration at the *pyrG* locus of the uridine auxotrophic strain *P. chrysogenum npe*10 *pyrG* and the pZ2b-derived plasmids described in Table 2. Single copy transformants were confirmed by Southern blot analysis (Online Resource 1); they are described in Table 2.

Table 1 Oligonucleotides used for mutation of different CreA binding sites in the *pcbAB* promoter

Target CreA box	Type of mutation	Oligonucleotide name	Oligonucleotide sequence
CreA-1	Deletion	ΔCreA-1F	TTGCCCACTGCCAAGTCCCGCTTGGCTGTCCCTG
		ΔCreA-1R	CAGGGACAGCCAAGCGGGACTTGGCAGTGGGCAA
CreA-1	Substitution GGGG→ACTA	CreA-1MUT-F	GCCCACTGCCAAGTCGCACTACCGCTTGGCTGTCCC
		CreA-1MUT-R	GGGACAGCCAAGCGGTAGTGCGACTTGGCAGTGGGC
CreA-5	Substitution GGGG→ACTA	CreA-5MUT-F	GCCTGAGCGGGGGCACTATGTTTATGCTGAGAC
		CreA-5MUT-R	GTCTCAGCATAAACATAGTGCCCCCGCTCAGGC
CreA-6	Substitution GGGG→ACTA	CreA-6MUT-F	CCTGTGTGCCTGAGCACTAGCGGGGTGTTTATG
		CreA-6MUT-R	CATAAACACCCCGCTAGTGCTCAGGCACACAGG

Genomic DNA extraction and Southern blotting

Isolation of total DNA from *P. chrysogenum* was carried out essentially as previously described (Fierro et al. 1996b). Southern blotting was performed by standard procedures (Sambrook et al. 1989), using either [α^{32}P]dCTP and the Nick Translation System (Life Technologies) or digoxigenin and the DIG-High Prime Kit (Roche) to label the DNA probes, following manufacturer's instructions. In the latter case, detection was carried out by chemiluminescence using the CDP-Star kit (Roche).

RNA extraction

Total RNA was extracted from cultures of the *P. chrysogenum* transformants harbouring plasmid pCreA-RNAi at 48 h of cultivation in CP medium with lactose as carbon source. "RNeasy Mini Kit" columns (Qiagen, Hilden, Germany) were used according to the manufacturer's instructions. Total RNA was treated with "RQ1 RNase-Free DNase" (Promega Corporation, Madison, WI, USA), following the manufacturer's indications, and quantified using a NanoDrop ND-1000 spectrophotometer. Prior to RT-PCR, the absence of contaminant DNA in the samples was tested by conventional PCR.

RT-PCR assays

RT-PCR was performed using 200 ng of total RNA and the "SuperScript One-Step RT-PCR with Platinum Taq" system (Invitrogen Corporation, Carlsbad, CA, USA), following the manufacturer's instructions. Primers creAi-F and creAi-R (see above) were used for this purpose. As an internal control for messenger RNA (mRNA) amount in the different samples, a 457-bp fragment of the *P. chrysogenum* γ-actin gene was amplified using primers RTactAF: 5′-CTGGCCGTGATCTG ACCGACTAC-3′, and RTactAR: 5′-GGGGGAGCGATGAT CTTGACCT-3′. The signals provided by the RT-PCR assays were quantified by densitometry using the "Gel-Pro Analyzer" software (Media Cybernetics). The transcript levels were normalized comparing the intensity of each *creA* mRNA signal to the γ-actin (*actA*) mRNA signal.

Results

Mutation of the CreA-1 site causes derepression of transcription from promoter *pcbAB* with glucose as carbon source

Six putative CreA binding sites (sequence SYGGRG) are present in the intergenic *pcbAB-pcbC* region of *P. chrysogenum*. We

Table 2 Plasmids carrying the *pcbAB* promoter fused to the *lacZ* gene with different mutations in CreA binding sites and single copy transformants obtained with these plasmids. See "Materials and methods" for details

Promoter reporter gene	CreA site mutation	Plasmid	Transformant	Reference
lacZ gene without promoter	N/A	pZ2b	0::Z	Gutiérrez et al. (1999)
P*pcbAB*::*lacZ*	No mutations	pZ2bAB	AB::Z	Gutiérrez et al. (1999)
P*pcbAB*::*lacZ*	ΔCreA-1	pZAB-ΔCreA1	AB::ZΔCreA-1	This work
P*pcbAB*::*lacZ*	CreA-1 GCGGGG→GCACTA	pZAB-CreA1	AB::ZCreA-1	This work
P*pcbAB*::*lacZ*	CreA-5 GCGGGG→GCACTA	pZAB-CreA5	AB::ZCreA-5	This work
P*pcbAB*::*lacZ*	CreA-6 GCGGGG→GCACTA	pZAB-CreA6	AB::ZCreA-6	This work

have named them CreA-1 to CreA-6 starting from the site closer to the *pcbAB* gene; thus, the CreA-1 and CreA-6 sites are located 318 and 856 bp upstream from the *pcbAB* start codon, respectively. All but the CreA-2 sequence are located on the *pcbAB* sense strand. Sequences CreA-5 and CreA-6 are located together in a tandem array (GCGGGGGCGGGG), 155 bp upstream from the start codon of the *pcbC* gene.

We first deleted by in vitro mutagenesis the six nucleotides of the CreA-1 site and obtained single copy transformants of the P*pcbAB::lacZ* fusion integrated at the *pyrG* locus of strain *P. chrysogenum npe*10 *pyrG*, which lacks the endogenous *pen* cluster (see "Materials and methods") (Online Resource 1). We selected one of these transformants and named it AB::ZΔCreA-1. As control, we used transformant AB::Z, previously obtained by Gutiérrez et al. (1999), which contains the wild-type *pcbAB* promoter. A second control for background β-galactosidase activity consisted of a single copy transformant with the pZ2b plasmid (transformant 0::Z), which contains the *lacZ* reporter gene but no promoter sequences.

β-Galactosidase activity in transformant 0::Z was 23.4± 3.2 U/mg of protein in mycelium grown with the non-repressing carbon source lactose and 0.61±0.21 U/mg in mycelium grown with glucose, which is in the range of previously measured endogenous β-galactosidase in strain *P. chrysogenum npe*10 (34.0±12.4 and 0.8±0.3 U/mg of protein, respectively). This result indicates that there is virtually no expression of the exogenous *lacZ* gene in the transformants if *pcbAB* promoter sequences are absent upstream of the reporter gene.

As shown in Fig. 1, deletion of site CreA-1 causes partial derepression of transcription from the *pcbAB* promoter in glucose-containing medium. Once endogenous β-galactosidase was subtracted from total β-galactosidase activity, the resulting activities indicate that with glucose as carbon source the *pcbAB* promoter with the CreA-1 site deleted recovered about 57 % of the transcriptional activity present in non-repressing conditions, i.e. with lactose, and 50 % if we take as reference the transcription of the wild-type promoter. In the wild-type *pcbAB* promoter, transcriptional activity with glucose is only 15 % of that with lactose.

Next, in order to avoid possible structural effects in the promoter caused by the deletion of the CreA-1 binding site, we mutated the CreA-1 sequence replacing GCGGGG with GCACTA (without deleting any nucleotide) and obtained a single copy transformant with the CreA-1-mutated *pcbAB* promoter fused to *lacZ* (Online Resource 1), named AB::ZCreA-1. This mutation had a similar effect to the ΔCreA-1 mutation, with some quantitative differences (Fig. 1). First, the promoter activity with lactose was 25 % higher when the CreA-1 site was mutated instead of deleted, which may be due to structural changes in the ΔCreA-1 promoter affecting other transcriptional factors. In addition, the promoter with the mutated CreA-1 site recovered in glucose-containing medium 32 % of its activity in lactose medium (35 % taking the wild-type promoter as reference), in contrast to the 57 % recovered by the ΔCreA-1 *pcbAB* promoter.

Therefore, the CreA-1 site plays an important role in glucose repression of the *pcbAB* gene transcription. However, full promoter activity, i.e. equal to that with the non-repressing carbon source lactose, is not recovered in promoters with the CreA-1 site mutated or deleted. This may be due to one of the

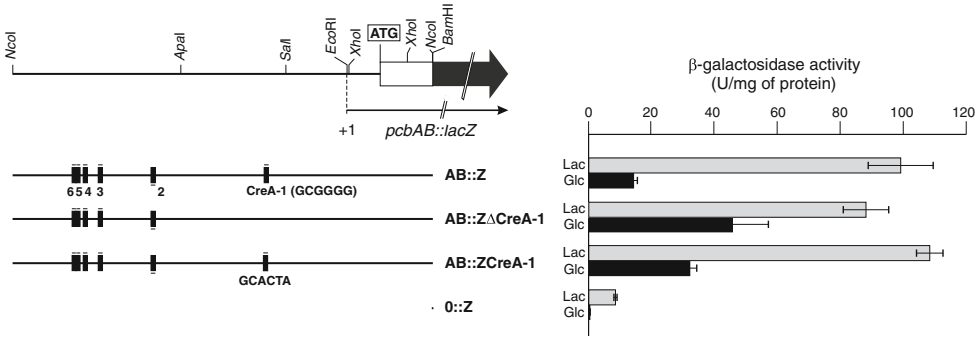

Fig. 1 Transcriptional activity of the wild-type *pcbAB* promoter (transformant AB::Z) and the promoter with the CreA-1 site deleted (transformant AB::ZΔCreA-1) or mutated (transformant AB::ZCreA-1), in mycelium grown for 48 h in flask cultures with CP medium and glucose or lactose as carbon source. Transformant 0::Z lacks promoter sequences and was used as control for endogenous/background β-galactosidase activity. At the *top left of the figure*, the scheme of the *pcbAB* promoter and the *lacZ* reporter gene fusion is shown; the *black line* represents sequences of the promoter, the *white box* sequences from the beginning of the *pcbAB* gene encoding 49 amino acids and the *black arrow* sequences from the *lacZ* gene. The transcription start site of the *pcbAB* gene is indicated with +1. The *pcbAB* promoter with the CreA binding sites in the different transformants is shown below. Consensus CreA binding sites are indicated by a *black rectangle*, numbered from 1 to 6. A *line above* or *below the rectangle* indicates the orientation of the CreA binding site, on the upper (*pcbAB* coding sequence) or lower strand, respectively. At the *right*, the β-galactosidase activities of the different transformants in glucose- or lactose-containing medium are shown. *Error bars* represent standard deviations from three independent cultures and two different assays of each of them

following effects, or a combination of both: (1) other CreA binding sites may also have an effect and contribute to the repression and (2) an effect of the pH. The *pcbAB* promoter is subject to pH regulation (Chu et al. 1997), and pH regulation mediated by the transcription factor PacC has been shown to occur in the *pcbC* gene (Suárez and Peñalva 1996). After 48 h of cultivation in flasks, the pH of the medium with lactose was about 1.5 units higher than that in glucose medium (7.5 and 6.0, respectively) in all transformants. Therefore, the pH increase in lactose-containing medium could be responsible for the still higher activity of the ΔCreA-1 promoter observed with lactose as carbon source with regard to the activity with glucose.

To avoid the pH effect, new batch cultures were carried out in bioreactors maintaining a constant pH (Fig. 2). Transformants AB::Z, AB::ZΔCreA-1 and AB::ZCreA-1 were grown in CP medium with glucose or lactose and at a constant pH of 6.0, so that the possible inducing pH effect mediated by PacC does not take place. In these conditions,

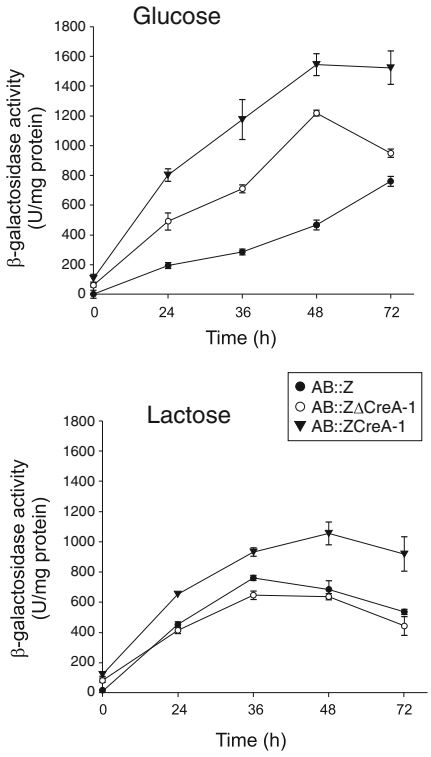

Fig. 2 Transcriptional activity of the wild-type *pcbAB* promoter (transformant AB::Z) and the promoter with the CreA-1 site deleted (transformant AB::ZΔCreA-1) or mutated (transformant AB::ZCreA-1), in mycelium grown in bioreactor cultures at a constant pH of 6.0 and with glucose or lactose as carbon source. *Error bars* represent standard deviations from two independent experiments and two different assays of each sample

glucose regulation of the wild-type *pcbAB* promoter is clear but not as strong as when pH was not controlled; transcriptional activity in glucose-containing medium was 43 % of that in lactose-containing medium at 24 h of cultivation, 38 % at 36 h and 68 % at 48 h, as compared to the 15 % at 48 h when pH was not controlled in flask cultivations. This result confirms the existence of a carbon source regulation of this promoter independent from the pH regulation. When the CreA-1 site is mutated or deleted, there is again a derepressing effect in glucose-containing medium, but much stronger than in flask cultures with free pH; in both AB::ZΔCreA-1 and AB::ZCreA-1 transformants, the transcriptional activity of the *pcbAB* promoter is higher in glucose- than in lactose-containing medium throughout the cultivation time, especially at 48 and 72 h. This result confirms the importance of the CreA-1 site for carbon source regulation of the *pcbAB* gene expression. When the two transformants are compared in both glucose- and lactose-containing media, transcription is higher in transformant AB::ZCreA-1 than in AB::ZΔCreA-1, which, as mentioned before, may be the result of other transcription factors being affected by structural changes in the ΔCreA-1 promoter.

Role of the CreA-5 and CreA-6 sites in transcription from the *pcbAB* promoter

We then mutated the CreA-5 and CreA-6 sites, whose sequence is identical to that of the CreA-1 site and are also situated on the sense strand of the *pcbAB* gene. In both cases, the sequence GCGGGG was changed to GCAC TA, maintaining the number of nucleotides, and single copy transformants were obtained with the mutated promoters fused to the *lacZ* reporter gene (Table 2) (Online Resource 1). The different transformants were cultured in flasks with CP medium to analyse expression of the *pcbAB* promoter measured as β-galactosidase activity present in the mycelium.

Transcriptional activity of the *pcbAB* promoter in glucose-containing medium was derepressed by mutations in both the CreA-5 and CreA-6 sites, but to a lesser extent than in transformants with the mutated CreA-1 site. In transformant AB::ZCreA-5, expression of the *pcbAB* promoter in mycelium grown with glucose was 25 % of its expression in lactose-containing medium (Fig. 3a), and in transformant AB::ZCreA-6, it was 14 % (Fig. 3b); as shown before (Fig. 1), the derepressing effect in transformants AB::ZCreA-1 and AB::ZΔCreA-1 was clearly higher, since transcriptional activity with glucose was 32 and 57 %, respectively, of that with lactose as carbon source. The increase of transcriptional activity in glucose-containing medium of transformant AB::ZCreA-5 with respect to the transformant with the wild-type *pcbAB* promoter (AB::Z) was 114 %, and the increase in transformant AB::ZCreA-6 was 28 %; in contrast, the increase of

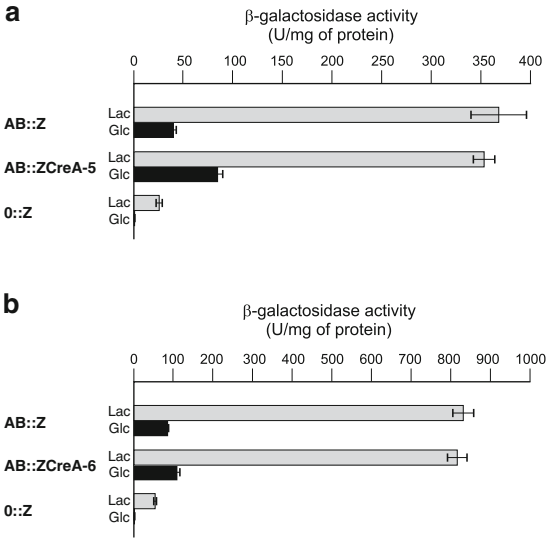

Fig. 3 Transcriptional activity of the *pcbAB* promoter with mutations in the CreA-5 site (**a**) or the CreA-6 site (**b**). Mycelium from transformants was grown for 48 h in flask cultures with CP medium and glucose or lactose as carbon source. *Error bars* represent standard deviations from three independent cultures and two different assays of each of them. Experiments with transformant AB::ZCreA-5 and with transformant AB::ZCreA-6 were carried out separately, hence the differences in the absolute β-galactosidase activities (note the different scale in **a** and **b**), due to sample manipulation for obtaining the cell-free extracts and different batches of the substrate and compounds used in the assays

transcriptional activity in glucose-containing medium of transformant AB::ZCreA-1 with respect to transformant AB::Z was 127 %, and in transformant AB::ZΔCreA-1, 225 % (Fig. 1). These results indicate that the CreA-5 site, and to a lesser extent the CreA-6 site, have a role in carbon source regulation of transcription from the *pcbAB* promoter, but this role is not as important as that of site CreA-1, which seems to be the main *cis*-acting element regulating carbon repression of the *pcbAB* gene.

Isolation of *P. chrysogenum* transformants expressing a *creA* siRNA

In a search of the *P. chrysogenum* genome database at NCBI (van den Berg et al. 2008), we found the gene Pc20g13880 as the main candidate to be orthologous to other filamentous fungi *creA*/*cre1* genes, such as *A. nidulans creA*. The protein encoded by the *P. chrysogenum* Pc20g13880 gene shares an overall identity of 79 % in its amino acid sequence with the *A. nidulans* CreA. We cloned a fragment of 14.6 kb from a genomic library in a lambda phage vector, which contained this gene, and constructed a plasmid vector for the replacement of the endogenous *creA* gene by a phleomycin resistance gene. After numerous attempts, following strategies that had been successfully applied to delete other *P. chrysogenum* genes in our lab (García-Rico et al. 2008; Naranjo et al. 2004), no *creA*-deleted strain could be isolated. Some candidate transformants with extreme slow growing phenotypes were obtained, but they were not stable and were lost after transfer to fresh plates. Therefore, we concluded that the *creA* gene is essential in *P. chrysogenum* (see "Discussion").

A strategy based on *creA* silencing was then used. Based on the DNA sequence of the gene, we designed primers to amplify a 533-bp exon fragment that was used to construct plasmid pCreA-RNAi. This plasmid was transformed into strain *P. chrysogenum* Wisconsin 54-1255 to generate a siRNA aiming to silence expression of the *creA* gene. Nine transformants, named Wis/CreAi-1 and subsequent numbers, were isolated. With the exception of transformant Wis/CreAi-6, all transformants showed a clear positive band pattern in a Southern blot analysis (Fig. 4a) and were next submitted to RT-PCR analysis (Fig. 4b). Seven of the eight transformants showed a reduction of *creA* gene expression, between 25 and 40 % with respect to the parental strain, whereas transformant Wis/CreAi-1 showed a similar expression level. The eight transformants were used for cultures in flasks to study the effect of *creA* attenuation in penicillin production. As control, we used the parental Wisconsin 54-1255 strain untransformed and transformed with the empty vector pJL43-RNAi (strain Wis/pJL43-RNAi).

Attenuation of *creA* causes derepression of penicillin biosynthesis with glucose as carbon source

The transformants with attenuated expression of the *creA* gene and the control strains Wisconsin 54-1255 and Wis/pJL43-RNAi were cultured for 120 h in flasks with CP medium, with either lactose or glucose as carbon source. All transformants showed similar penicillin-specific production rates (no significant differences among them at each time of each condition) except transformant Wis/CreAi-1, which behaved similarly to the control strains Wis 54-1255 and Wis/pJL43-RNAi (Online Resource 2). As indicated above, transformant Wis/CreAi-1 had not shown a significant attenuation of *creA* gene expression (Fig. 4b).

Control strains showed carbon source regulation of penicillin biosynthesis, producing at 96 h of cultivation about 23.5 μg/mg (penicillin/dry weight) in lactose-containing medium and about 15 μg/mg in glucose-containing medium (Fig. 5). In the *creA*-attenuated transformants, penicillin titers were in the range of 26.3–28.8 μg/mg dry weight in lactose medium at 96 h and in the range of 23.5–25.3 μg/mg dry weight in glucose medium (Fig. 5), which is 57 to 92 % higher than in the control strains in glucose medium. Hence, there is a clear derepressing effect on penicillin production caused by the reduced activity of CreA when glucose is used as carbon source. Penicillin titers in these conditions reach and even

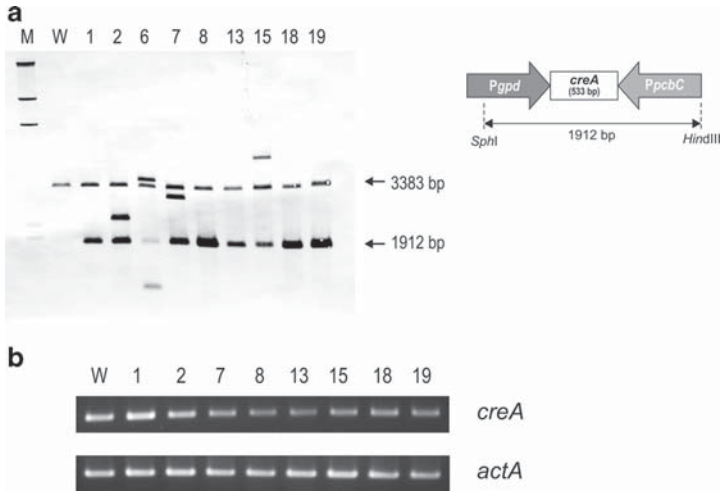

Fig. 4 **a** Isolation of *P. chrysogenum* Wisconsin 54-1255 transformants with the pCreA-RNAi plasmid. The *scheme at the right* shows the gene silencing cassette, with two opposite promoters from which two complementary reverse RNA strands from the *creA* gene fragment are synthesized, generating a siRNA. Southern blotting was carried out with DNA from the parental Wisconsin 54-1255 strain (*lane W*) and nine different transformants, named Wis/*creA*-RNAi-1 to 19. The DNA was digested with the restriction enzymes *Sph*I and *Hin*dIII, which release the gene silencing cassette (1,912 bp). The *creA* 533-bp gene fragment from an exon sequence was used as probe. The endogenous *creA* gene produces a hybridization signal of 3,383 bp, whereas transformants show an additional band of 1,912 bp, of different intensity according to the number of integrated copies. Eventually other bands are visible, which probably correspond to integrations by crossing-over occurring at some point within the 1,912-bp region. The size marker is *Hin*dIII-digested lambda phage DNA (*lane M*). **b** Analysis of the attenuation of *creA* gene expression by RT-PCR. RNA from the parental strain and the transformants was obtained as described in "Materials and methods", after 48 h of cultivation in CP medium with lactose as carbon source, and the RT-PCR was performed using primers RTactAF and RTactAR

surpass those of the control strains grown in non-repressing conditions, with lactose as carbon source. An increase in the production is also observed in the *creA*-silenced transformants under non-repressing conditions, but of only 12–22 % with respect to the control strains. ANOVA statistical analysis indicates that the derepressing effect in the *creA*-attenuated transformants is significant ($p<0.01$) from 48 h of cultivation onwards in glucose medium, whereas in lactose medium, the increase of production observed in the knock-down transformants is significant from 72 h.

Discussion

For many years it has remained unclear whether the transcriptional regulator CreA controls the expression of penicillin biosynthesis genes. In this article, we have shown that the wide domain regulator CreA is responsible for the carbon source regulation of penicillin biosynthesis in *P. chrysogenum*. This conclusion is based on experimental evidence obtained from two sources: the in vivo analysis of the functionality of CreA binding sites present in the intergenic *pcbAB-pcbC* region and the study of the differential effect that *creA* attenuation by siRNA has on penicillin production in glucose- or lactose-containing medium.

Former studies in *A. nidulans* had led to the conclusion that in this fungus an as yet uncharacterized CreA-independent mechanism was the main responsible for carbon source regulation of penicillin biosynthesis (Espeso and Peñalva 1992; Espeso et al. 1993). A DNA region found to be involved in sucrose repression of the *ipnA* gene contains two nearly consensus CreA binding sites, situated within a 29-bp sequence protected by DNase I footprint (Espeso et al. 1993). However, when the 29-bp sequence was deleted, no derepressing effect of *ipnA* expression was observed (Espeso et al. 1993). In addition, different *creA* loss-of-function mutations had only a minor effect on *ipnA* gene expression (Espeso and Peñalva 1992), and in a microarray analysis of a *creA*-deleted mutant vs. a reference strain, no penicillin biosynthetic gene showed a significant change in its expression level (Mogensen et al. 2006). Nevertheless, a functional in vivo analysis of the three consensus CreA binding sites situated in the intergenic *acvA-ipnA* region has not been performed, and Espeso and Peñalva (1994) observed in vitro binding of a GST::CreA fusion protein to these sites. Therefore, it cannot be totally ruled out that CreA has some degree of involvement in carbon source regulation of penicillin biosynthesis in *A. nidulans*; actually some *creA* mutants did show a slight derepression of the *ipnA* gene transcription (Espeso and Peñalva 1992).

In *P. chrysogenum*, there are six consensus CreA binding sites in the *pcbAB-pcbC* intergenic region. This number is higher than that expected to occur at random: one site every 512 bp. In this work, we have found that the site CreA-1, the most proximal to the *pcbAB* gene, has a very important function in glucose repression of this gene. When the site

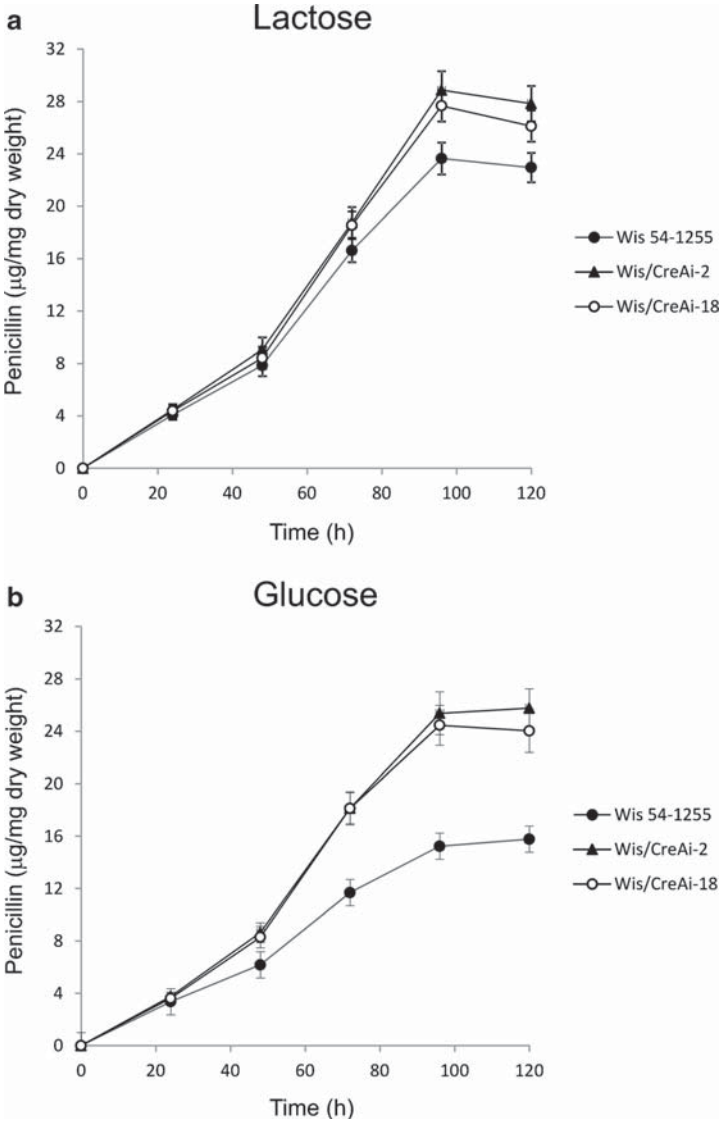

Fig. 5 Specific penicillin production of two of the *creA*-attenuated transformants and the parental strain Wisconsin 54-1255 during cultivation in flasks with CP medium and glucose or lactose as carbon source. The two transformants are representative of the behaviour of all transformants except Wis/CreAi-1 (see text). For complete numerical data on penicillin production of all transformants, see Online Resource 2. *Error bars* represent standard deviations from three independent experiments, with two flasks per condition and two bioassays for each sample

CreA-1 is deleted or mutated, the expression of the *pcbAB* gene in glucose-containing medium at an uncontrolled pH is derepressed up to 50 % of the expression in non-repressing conditions, whereas at a controlled constant pH of 6.0, full derepression is achieved. Site CreA-5 has also a role in the regulation, but quantitatively lower than that of CreA-1, whereas site CreA-6 has only a minor role. The different derepression level observed in the CreA-1-mutated/deleted promoters when the pH is left free or at a constant pH of 6.0 is probably the result of the pH regulation of the *pcbAB* promoter, which is independent from the CreA-mediated control. Transcription of the *pcbAB* gene is regulated by pH, being increased at alkaline pH (Chu et al. 1997). The growth of mycelium in glucose-containing medium leads to a decrease of the pH, whereas in lactose-containing medium, pH is increased. This implies that when the pH is not controlled, the repressor effect of glucose and the lack of activation by alkaline pH are concurrent and cause a very low transcriptional activity of the *pcbAB* promoter. However, in lactose-containing medium, the lack of carbon repression is added to the activating effect of the higher pH, resulting in high transcription levels. When the pH is kept constant at a value of 6.0, there is no activating pH effect, and thus, differences in transcription of the wild-type promoter between glucose and

lactose medium are less marked (Figs. 1 and 2), and when the CreA-1 site is mutated or deleted, the relative derepressing effect is consequently more evident.

The importance of CreA binding sites in the regulation of penicillin biosynthetic gene expression is supported by the findings described by van den Berg (2011); simultaneous mutation of sites CreA-4, CreA-5 and CreA-6 caused an important derepressing effect on the *pcbC* gene transcription in glucose-containing medium as measured by RT-PCR. On the other hand, the third gene of the penicillin biosynthetic pathway, *penDE*, is also subject to carbon source regulation, and its transcription is repressed by glucose, although to a lower extent than genes *pcbAB* and *pcbC* (Gutiérrez et al. 1999; Martin 2000). There are a total of 10 consensus CreA binding sites in the promoter region of the *penDE* gene; therefore, very likely, this gene is also regulated by CreA.

In the *creA*-attenuated transformants analysed in this work, full derepression of penicillin biosynthesis was observed when they were grown in glucose-containing medium, that is, specific penicillin production in the *creA*-attenuated transformants growing with glucose were similar to those of the parental strain growing with lactose as carbon source (Fig. 5). This result, together with the results on the regulation of penicillin genes by *cis*-acting CreA binding sites, leads us to conclude that CreA is either the only or the main factor responsible for carbon repression of penicillin biosynthesis in *P. chrysogenum*. CreA might also have additional functions affecting directly or indirectly penicillin production; in lactose-containing medium, *creA*-attenuated mutants showed a slightly but significantly higher penicillin production than the parental strain (Fig. 5), and transcriptional activity of the *pcbAB* promoter with a mutated CreA-1 site is likewise higher than that of the wild-type promoter (Fig. 2).

In *Acremonium chrysogenum*, another β-lactam antibiotic producing fungus, the transcription factor Cre1, homologous to CreA, regulates the transcription of cephalosporin genes in response to carbon sources, such as glucose. In a cephalosporin-improved production strain, expression of the *pcbC* gene has lost the glucose repression present in the wild type, but repression was restored after transformation with multiple copies of the *cre1* gene, which also increased repression of the *cefEF* gene expression (Jekosch and Kück 2000).

As described in the "Results" section, no *creA*-deleted mutant could be isolated after many attempts, which indicates that *creA* is probably essential in *P. chrysogenum*. CreA is a wide domain regulator that controls the expression of a high number of genes (Mogensen et al. 2006; Portnoy et al. 2011). CreA null mutants have been obtained in some fungi, either by mutation or gene deletion. These null mutants show important growth and development defects such as very small colony diameter and reduced sporulation, as described in *A. nidulans* (Shroff et al. 1997). A similar phenotype was observed in a Δ*cre1* strain of *T. reesei* (Nakari-Setälä et al. 2009). In *N. crassa*, a Δ*cre-1* strain presented reduced growth and changes in the morphology of hyphae and colony, which became compact (Ziv et al. 2008). In *F. oxysporum*, Jonkers and Rep (2009) could not obtain a *CRE1*-deleted mutant after the analysis of up to 1,000 transformants, which was interpreted as full-length deletion of *CRE1* being lethal to the fungus. Therefore, there seems to be differences in the precise function of CreA/Cre1 among fungal species.

The existence of a CreA-mediated carbon source regulation of penicillin biosynthesis makes the *creA* gene a possible target for strain improvement of *P. chrysogenum*. On the other hand, it will be of great interest to study the possible interactions between CreA-mediated carbon source regulation and other regulatory circuits controlling the expression of the penicillin biosynthetic genes and to gain insight in the signalling pathway of carbon repression and how CreA is activated/deactivated in *P. chrysogenum*.

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Conflict of interest The authors declare that they have no conflict of interest.

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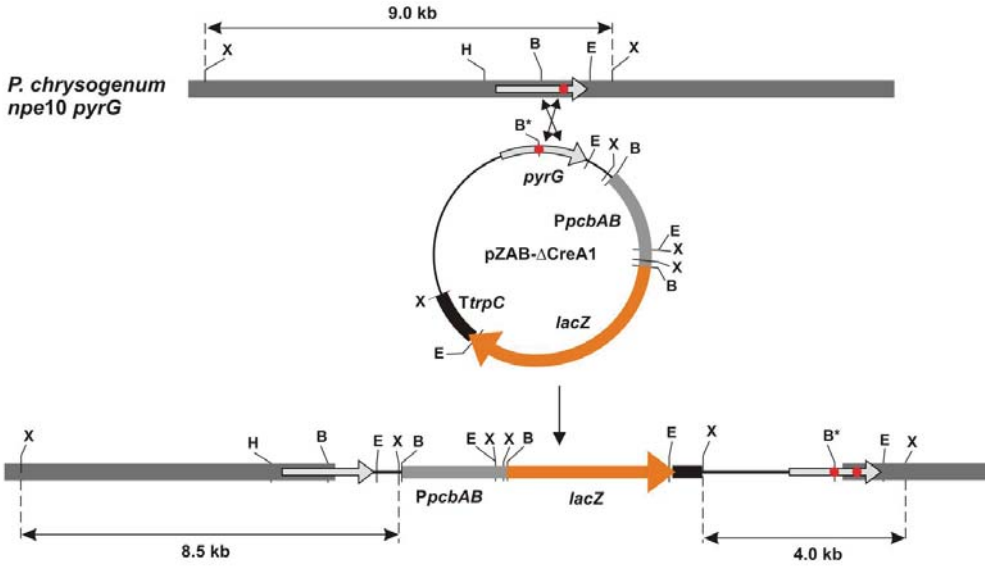
SUPPLEMENTARY MATERIAL

Journal: APPLIED MICROBIOLOGY AND BIOTECHNOLOGY

Article Title: "Direct involvement of the CreA transcription factor in penicillin biosynthesis and expression of the *pcbAB* gene in *Penicillium chrysogenum*"

a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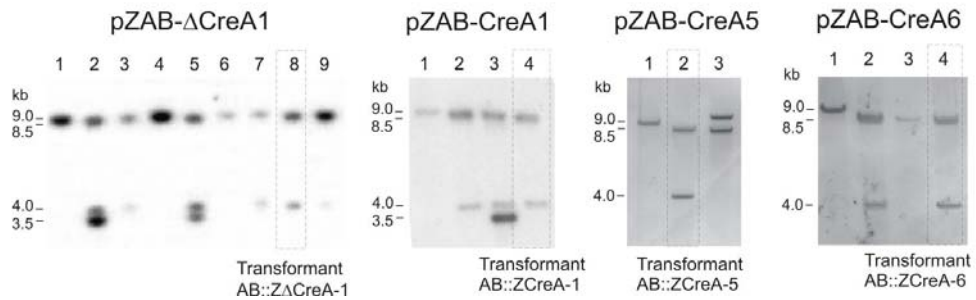
b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Online Resource 1 Isolation of single copy transformants with the pZ2b-derived plasmids containing the *pcbAB* promoter fused to the *lacZ* gene and mutations of CreA binding sites, following the strategy described by Gutiérrez et al. (1999). **a.** Scheme of the integration event of the pZAB-ΔCreA1 plasmid (as model for all other plasmids in Table 2) at the *pyrG* locus of strain *P. chrysogenum npe*10 *pyrG*. Loss-of-function mutations in the *pyrG* gene present in the genome of the recipient strain and in the pZ2b-derived plasmids are indicated by a red dot. A single crossing-over in the DNA region between both mutations (indicated with a crossed double arrow) enables targeted integration at the genomic *pyrG* locus with reconstitution of a functional *pyrG* gene, which allows selection of transformants in minimal medium. Restriction enzymes are: *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H) and *Xho*I (X). The *Bam*HI site in the *pyrG* gene of plasmid pZ2b was mutated to get a non-functional *pyrG* gene, and is indicated with an asterisk (B*). **b.** Selection by Southern blot of single copy transformants with the P*pcbAB*::*lacZ* fusion integrated at the *pyrG* locus. Total DNA from transformants was digested with *Xho*I. A 360 bp *Sal*I-*Eco*RI fragment from the 3'-end of the *pyrG* gene was used as probe in all hybridization experiments. When a single integration event at the *pyrG* locus occurred two bands appear in the blots, with sizes of 8.5 and 4.0 kb, as happens in lane 8 of the left panel for instance. Additional bands of 3.5 kb or other sizes reveal the presence of additional copies integrated elsewhere in the genome. The plasmid used for transformation is indicated at the top of each panel, and the selected transformant is highlighted with a rectangle. Lane 1 in all panels contains DNA from the recipient strain *npe*10 *pyrG*, which produces a hybridization signal of 9.0 kb.

Lactose

Strain	Penicillin (μg/mg dry weight) ± standard deviation				
	24 h	48 h	72 h	96 h	120 h
Wis 54-1255	4.067 ± 0.365	7.860 ± 0.821	16.613 ± 0.886	23.638 ± 1.213	22.944 ± 1.115
Wis/pJL43-RNAi	3.945 ± 0.383	7.932 ± 0.819	17.168 ± 1.016	23.555 ± 1.253	22.542 ± 1.188
Wis/CreAi-1	4.890 ± 0.339	7.786 ± 0.712	16.944 ± 0.976	23.863 ± 1.168	23.268 ± 1.226
Wis/CreAi-2	4.476 ± 0.424	9.056 ± 0.931	18.756 ± 1.167	28.864 ± 1.418	27.833 ± 1.345
Wis/CreAi-7	4.578 ± 0.395	8.896 ± 0.896	19.111 ± 0.968	28.222 ± 1.395	27.562 ± 1.291
Wis/CreAi-8	4.321 ± 0.410	8.240 ± 0.753	18.444 ± 0.922	27.764 ± 1.267	26.733 ± 1.323
Wis/CreAi-13	4.279 ± 0.353	8.249 ± 0.777	18.185 ± 1.064	27.543 ± 1.312	26.023 ± 1.377
Wis/CreAi-15	4.179 ± 0.383	8.052 ± 0.902	18.267 ± 1.207	27.117 ± 1.279	26.555 ± 1.224
Wis/CreAi-18	4.378 ± 0.404	8.417 ± 0.832	18.543 ± 1.045	27.668 ± 1.207	26.115 ± 1.188
Wis/CreAi-19	3.257 ± 0.377	7.644 ± 0.818	18.111 ± 0.952	26.378 ± 1.188	25.927 ± 1.253

Glucose

Strain	Penicillin (μg/mg dry weight) ± standard deviation				
	24 h	48 h	72 h	96 h	120 h
Wis 54-1255	3.356 ± 0.417	6.172 ± 0.644	11.682 ± 0.968	15.224 ± 1.075	15.765 ± 1.182
Wis/pJL43-RNAi	3.215 ± 0.425	6.231 ± 0.634	11.553 ± 0.867	14.952 ± 1.263	15.276 ± 1.281
Wis/CreAi-1	3.178 ± 0.397	6.675 ± 0.588	10.973 ± 0.886	15.065 ± 0.988	14.867 ± 1.203
Wis/CreAi-2	3.749 ± 0.347	8.632 ± 0.762	18.126 ± 1.183	25.367 ±1.643	25.765 ± 1.474
Wis/CreAi-7	3.852 ± 0.389	8.938 ± 0.855	18.231 ± 1.329	24.962 ± 1.557	25.043 ± 1.615
Wis/CreAi-8	3.772 ± 0.410	8.032 ± 0.715	17.753 ± 0.933	24.156 ± 1.372	24.115 ± 1.532
Wis/CreAi-13	3.592 ± 0.340	8.228 ± 0.644	17.270 ± 1.217	23.774 ± 1.515	23.259 ± 1.479
Wis/CreAi-15	3.826 ± 0.374	8.428 ± 0.613	16.855 ± 0.988	23.955 ± 1.345	23.568 ± 1.774
Wis/CreAi-18	3.613 ± 0.312	8.283 ± 0.817	18.116 ± 1.263	24.451 ± 1.523	24.026 ± 1.645
Wis/CreAi-19	3.449 ± 0.357	7.886 ± 0.738	17.439 ± 1.182	23.584 ± 1.430	22.788 ± 1.365

Lactose

Strain	pH				
	24 h	48 h	72 h	96 h	120 h
Wis 54-1255	6.35	6.50	6.55	7.10	7.53
Wis/pJL43-RNAi	6.29	6.48	6.53	7.03	7.46
Wis/CreAi-1	6.32	6.56	6.62	7.12	7.42
Wis/CreAi-2	6.47	6.54	6.63	7.31	7.66
Wis/CreAi-7	6.50	6.43	6.60	7.25	7.61
Wis/CreAi-8	6.38	6.52	6.59	7.10	7.50
Wis/CreAi-13	6.35	6.54	6.63	7.09	7.55
Wis/CreAi-15	6.41	6.48	6.56	7.16	7.47
Wis/CreAi-18	6.28	6.42	6.52	7.11	7.39
Wis/CreAi-19	6.44	6.57	6.59	6.91	7.41

Glucose

Strain	pH				
	24 h	48 h	72 h	96 h	120 h
Wis 54-1255	6.18	5.50	5.38	5.29	5.17
Wis/pJL43-RNAi	6.15	5.47	5.36	5.32	5.15
Wis/CreAi-1	6.12	5.53	5.31	5.25	5.20
Wis/CreAi-2	6.20	5.55	5.42	5.38	5.25
Wis/CreAi-7	6.18	5.60	5.48	5.40	5.29
Wis/CreAi-8	6.10	5.57	5.45	5.36	5.23
Wis/CreAi-13	6.07	5.41	5.56	5.20	5.14
Wis/CreAi-15	6.14	5.52	5.35	5.29	5.22
Wis/CreAi-18	6.08	5.49	5.38	5.30	5.18
Wis/CreAi-19	6.11	5.58	5.46	5.39	5.27

Online Resource 2 Specific penicillin production of *creA*-attenuated transformants and the control strains Wisconsin 54-1255 and Wis/pJL43-RNAi, at five different time points during the culture. Cultures were performed in flasks as described in Materials and Methods. Data represent the mean and standard deviation of three independent experiments, with two flasks per condition, and two bioassays for each sample. The two lowermost panels show the pH of the medium; the initial pH was 6.1 for both the glucose- and the lactose-containing medium.

3. Resultados y Discusión

Capítulo 1

Resultados y Discusión Capítulo 1

La penicilina es uno de los metabolitos secundarios más importantes producidos por microorganismos. Durante las últimas décadas, el esfuerzo conjunto llevado a cabo entre la industria y los organismos de investigación ha contribuido no sólo a la mejora de cepas y a la optimización de la producción industrial de estos antibióticos β-lactámicos, sino también a entender algunos de los mecanismos responsables de este incremento en la productividad (Barreiro y col., 2012; van den Berg, 2011; van den Berg y col., 2008). Sin embargo, a pesar de este esfuerzo, aún permanecen mecanismos reguladores globales sin esclarecer.

La caracterización de reguladores de la ruta de biosíntesis de penicilina es especialmente importante para la optimización de los procesos de producción. Es conocido que el crecimiento y la producción de penicilina en *P. chrysogenum* están controlados por procesos reguladores complejos (Martín y col., 1999). Hasta la fecha, no se ha encontrado ningún regulador específico de la ruta de penicilina, indicando que este "*cluster*" puede estar controlado solamente por reguladores globales del metabolismo secundario, como LaeA (Kosalková y col., 2009) o el complejo Velvet (Hoff y col., 2010). CPCR1 es otro regulador global identificado en *A. chrysogenum* (Schmitt y Kück, 2000) y el hecho de que este factor transcripcional se uniese al menos a dos secuencias presentes en la región intergénica de los genes *pcbAB*/*pcbC* regulando positivamente la biosíntesis de cefalosporina C (Schmitt y Kück, 2000; Schmitt y col., 2004b), sugirió que este regulador también podría estar presente en *P. chrysogenum* controlando la biosíntesis de penicilina. De hecho, previamente Schmitt y Kück (2000) habían identificado una secuencia parcial incompleta del gen que codifica el homólogo de CPCR1 en *P. chrysogenum*. Sin embargo, esta secuencia tenía varias modificaciones de nucleótidos respecto a la mostrada en el genoma de *P. chrysogenum* Wisconsin 54-1255 para el gen Pc20g01690 (van den Berg y col., 2008). En nuestro trabajo, hemos mostrado que la expresión del gen Pc*rfx1* de *P. chrysogenum* es similar en la cepa silvestre (*P. chrysogenum* NRRL 1951) y la cepa de referencia (*P. chrysogenum* Wisconsin 54-1255), indicando que la secuencia y la

expresión del gen Pc*rfx1* permaneció sin modificarse durante la mejora de cepas productoras de penicilina.

La combinación de las secuencias de unión al ADN caracterizadas previamente para ortólogos de RFX1 (Bugeja y col., 2010; Emery y col., 1996; Schmitt y col., 2004a), nos permitió la identificación de supuestos sitios de unión al ADN para PcRFX1 en la región promotora bidireccional de los genes *pcbAB* y *pcbC*, aunque no se identificó el supuesto sitio de unión al ADN para PcRFX1 en el promotor del gen *penDE* con aquellas secuencias consenso. Sin embargo, los resultados proporcionados por los análisis de expresión de los transformantes de silenciamiento génico de PcRFX1, indicaron que los niveles de transcriptos de los tres genes del "*cluster*" de biosíntesis de penicilina eran menores cuando la expresión del gen Pc*rfx1* estaba silenciada, sugiriendo que el gen *penDE* también estaba controlado por Pc*rfx1*. Esta hipótesis, también estuvo apoyada por el hecho de que el supuesto sitio de unión para PcRFX1 en la región promotora del gen *penDE* era capaz de unir proteínas de extractos proteicos de *P. chrysogenum*, como se puede observar en los estudios de EMSA. Ante estos resultados, se determinó una nueva secuencia consenso, basándonos en las secuencias anteriormente mencionadas y teniendo en cuenta las secuencias de los promotores de los genes biosintéticos de penicilina. Con esta secuencia consenso (NNRCCNNRSHWAY), se encontró un supuesto sitio de unión para PcRFX1 en la región promotora del gen *pcbC*, otro en la región promotora del gen *penDE* y dos supuestas secuencias de unión para PcRFX1, las cuales se solapan en cuatro nucleótidos, en la región promotora del gen *pcbAB*. Cabe destacar que existe una caja GATA en la convergencia de las supuestas cajas de unión para PcRFX1 en el promotor del gen *pcbAB* y que los últimos cinco nucleótidos para el supuesto sitio de unión para PcRFX1 en el promotor del gen *pcbC* también incluyen la caja CCAAT.

Los ensayos de gen reportero confirmaron la funcionalidad de las secuencias de unión al ADN para PcRFX1. Sin embargo, se produjo la eliminación de la caja GATA presente en la convergencia de los dos supuestos sitios de unión al ADN para PcRFX1 en el promotor del gen *pcbAB* tras la sustitución o deleción de una de las supuestas secuencias de unión para PcRFX1. La caja GATA representa la secuencia

consenso para el factor transcripcional NRE en *P. chrysogenum* (Haas y Marzluf, 1995). Se han encontrado seis secuencias GATA en la región intergénica bidireccional *pcbAB*/*pcbC* en *P. chrysogenum* (cuatro en la hebra con sentido para *pcbAB* y dos en la hebra con sentido para *pcbC*). En dos de estos motivos GATA (uno en cada hebra de ADN, separados por 27 pb y organizados con la orientación cabeza-cabeza), se ha demostrado la interacción con el factor transcripcional NRE (Haas y Marzluf, 1995). Los sitios de unión para ambos factores (NRE y PcRFX1) se solapan y pueden estar compitiendo en las funciones de regulación. El hecho de que el motivo GATA eliminado en nuestros experimentos corresponda a la segunda de estas cajas GATA (la cual está en sentido al gen *pcbAB*) puede ser relevante para la interpretación de los resultados de la actividad β-galactosidasa obtenidos para el promotor bidireccional en sentido al gen *pcbAB*. La misma situación tiene lugar con la sustitución o deleción de la supuesta secuencia de unión al ADN para PcRFX1 en el promotor en dirección al gen *pcbC*, la cual lleva a la modificación o deleción del motivo CCAAT. Este motivo es la secuencia consenso para la unión del factor transcripcional AnCF (anteriormente denominado PENR1). Cabe indicar que existen seis motivos CCAAT en la región intergénica *pcbAB*/*pcbC* en *P. chrysogenum* (cuatro de ellos en la hebra con sentido para el gen *pcbAB* y dos dispuestos en la hebra con sentido para el gen *pcbC*). Aunque existen evidencias *in vitro* de la unión de AnCF a esta región (Bergh y col., 1996), la funcionalidad de la caja CCAAT que ha sido modificada o delecionada en los experimentos de gen reportero no ha sido confirmada hasta ahora. Por tanto, basándonos únicamente en las evidencias, no podemos descartar que el descenso de la actividad β-galactosidasa observado en los ensayos de gen reportero sea una consecuencia directa de la sustitución o deleción de la supuesta secuencia de unión al ADN para PcRFX1 presente en el promotor del gen *pcbC*.

Los experimentos de silenciamiento y sobreexpresión génica de Pc*rfx1* proporcionaron más evidencias, confirmando el papel de Pc*rfx1* en el control de la biosíntesis de penicilina. En los transformantes de silenciamiento y sobreexpresión de dicho factor de transcripción, la producción de IPN y penicilina G se encuentra modificada. En *A. chrysogenum*, la interrupción del gen *cpcR1*, el cual codifica CPCR1 (ortólogo de PcRFX1), reduce los niveles de penicilina N y la actividad de la IPN

sintasa (probablemente también disminuye los niveles de IPN). En cambio, los niveles de cefalosporina permanecen constantes (Schmitt y col., 2004a). Esto indica que CPCR1 no está involucrado en la regulación de los genes tardíos de la ruta de biosíntesis de cefalosporina en *A. chrysogenum*. Por el contrario, el proceso completo de biosíntesis de penicilina sí parece estar controlado por PcRFX1 en *P. chrysogenum*.

Existe otra diferencia entre *A. chrysogenum* y *P. chrysogenum* en relación al desarrollo morfológico. En el hongo productor de cefalosporina *A. chrysogenum*, CPCR1 también está implicado en el control del desarrollo morfológico, es necesario para la fragmentación de las hifas y por tanto, para la formación de artrosporas (Hoff y col., 2005). Por consiguiente, el factor transcripcional CPCR1 parece ser el enlace molecular que controla la biosíntesis de cefalosporina y la morfogénesis. Este papel de conexión también se ha sugerido en el hongo patógeno oportunista *P. marneffei*. En este hongo, donde la transición desde una forma de crecimiento en forma de hifa a una forma de espora unicelular es análoga a la artroconidiación de *A. chrysogenu*m, RfxA (homologo a CPCR1 y PcRFX1) regula la proliferación y la diferenciación celular. Por tanto se ha sugerido que RfxA actúa como enlace entre la división celular y la morfogénesis, sobre todo durante la conidiación y el crecimiento como levadura (Bugeja y col., 2010), ya que en el transformante de silenciamiento génico de *rfxA* se muestra una incapacidad de transición desde la forma micelial a la forma de levadura infecciosa. A diferencia de lo que sucede en *P. marneffei*, el cual sufre una clara diferenciación, *P. chrysogenum* no experimenta un crecimiento como levadura y de hecho, los transformantes de silenciamiento y sobreexpresión génica de Pc*rfx1* mostraron una morfología y una formación de conidióforos similares a las cepas control respectivas. Únicamente la esporulación parece estar ligeramente afectada después del silenciamiento génico de Pc*rfx1*. Además, el nivel de expresión de los ocho genes supuestamente implicados en división celular, donde solamente tres de ellos contienen la secuencia de unión al ADN para PcRFX1 propuesta en este trabajo, no estaba modificado después del silenciamiento o sobreexpresión génica de Pc*rfx1*. Estos resultados deben tomarse con precaución, ya que aunque sugieren que PcRFX1 no está implicado en la diferenciación y desarrollo de *P. chrysogenum*, hemos de tener en cuenta que la cepa *P. chrysogenum* Wisconsin 54-1255 ha sido sometida a diferentes rondas de mutagénesis clásica durante los programas industriales de

mejora de cepas, las cuales pueden haber afectado a diferentes genes. Por tanto, no podemos descartar que el desarrollo morfológico en las cepas silvestres de *P. chrysogenum* pueda estar controlado por PcRFX1.

Para concluir, la publicación del genoma de la cepa *P. chrysogenum* Wisconsin 54-1255 (van den Berg y col., 2008) nos posibilitó realizar un estudio global de los supuestos genes regulados por PcRFX1. Este estudio indicó que, además de la función de PcRFX1 en la regulación del "*cluster*" génico de biosíntesis de penicilina, este factor tiende a regular genes implicados en el metabolismo primario (i.e. catabolismo de carbohidratos, catabolismo de la quitina, catabolismo de polisacáridos, biosíntesis de histidina, metabolismo lipídico, metabolismo de compuestos de un-carbono, biosíntesis de fosfolípidos). Este análisis funcional prepara el camino para estudiar los procesos metabólicos potencialmente controlados por este factor de transcripción y para dilucidar su relación con la producción de penicilina.

Capítulo 2

Resultados y Discusión Capítulo 2

P. chrysogenum es uno de los mejores ejemplos de la mejora de un microorganismo con fines industriales. Los niveles de productividad han aumentado en tres órdenes de magnitud en las últimas décadas (Hersbach y col., 1984). Además de este éxito en la mejora clásica de cepas, las recientes técnicas ómicas han contribuido a la comprensión de algunos de los mecanismos responsables del aumento de producción de penicilina (van den Berg y col., 2008; van den Berg, 2011; Jami y col., 2010). Aun así, uno de los asuntos más interesantes y desafiantes que permanece sin esclarecerse, está relacionado con los mecanismos reguladores que controlan la biosíntesis y producción de penicilina. En general, la regulación del metabolismo secundario en hongos está compuesta por rutas interconectadas y solapadas (Lim y Keller, 2014). Ante la ausencia de un regulador específico de la ruta de biosíntesis de penicilina en *P. chrysogenum*, se ha señalado a reguladores globales como los principales candidatos para el control de la producción de penicilina en este hongo filamentoso. De hecho, se ha demostrado que algunos reguladores globales, tales como PacC (Suárez y Peñalva, 1996), CreA (Cepeda-García y col., 2014), LaeA y el complejo Velvet (Kosalková y col., 2009; Hoff y col., 2010; Kopke y col., 2013), participan en el control de la biosíntesis de penicilina. Recientemente, se ha descrito otra familia de reguladores globales (proteínas "*winged helix*") que está implicada en la regulación de la biosíntesis de antibióticos β-lactámicos. Hasta la fecha, dicha familia está representada por las proteínas CPCR1 (Schmitt y Kück, 2000; Schmitt y col., 2004a) y la proteína AcFKH1 que interacciona con la anterior (Schmitt y col., 2004b) en *A. chrysogenum* y por PcRFX1, ortólogo de CPCR1 (Domínguez-Santos y col., 2012), y el factor transcripcional PcFKH1 (caracterizado en este trabajo) en *P. chrysogenum*.

AcFKH1 contiene dos dominios conservados: i) el dominio de asociación a "*forkhead*" (FHA), el cual está implicado en la interacción con fosfoproteínas (Li y col., 2000), y ii) el dominio C-terminal de unión al ADN (FKH). El análisis comparativo de la secuencia de aminoácidos de la proteína FKH1 en diferentes

hongos mostró una moderada similitud a la proteína FKH1 de *A. chrysogenum* (otro hongo productor de antibióticos β-lactámicos), mientras que la similitud fue mucho mayor con los ortólogos de otras especies de *Penicillium* o *Aspergillus*. Este resultado indicó que *fkh1* ha evolucionado en paralelo con otros parámetros filogenéticos (i. e. ARNr), pero no filogenéticamente ligados a los genes β-lactámicos.

En este trabajo hemos demostrado que la proteína recombinante PcFKH1 interacciona con las secuencias consenso 5`-RYMAAYA-3` (Kaufmann y col., 1995) presentes en los promotores de los genes biosintéticos *pcbC* y *penDE*. En estos estudios de interacción ADN-proteína se observaron dos bandas distintas de retraso de la movilidad electroforética con la sonda que incluye los sitios de unión al ADN en las posiciones DE-113 y DE-265 del promotor del gen *penDE*, lo cual indica que se estaban formando dos complejos proteína-ADN. Dado que los dos sitios de unión al ADN para PcFKH1 estaban incluidos en la misma sonda (debido a la proximidad de ambos sitios de unión), una vez que un sitio de unión era ocupado (banda inferior), más proteína podía unirse al otro sitio, explicando el retraso observado en la banda superior. Es interesante indicar que la unión de la proteína recombinante PcFKH1 a los promotores de los genes *pcbC* y *penDE* presentó afinidades diferentes. A diferencia de la sonda localizada en la región promotora del gen *penDE*, las dos sondas ensayadas ubicadas en la zona promotoras del gen *pcbC* no proporcionaron una interacción específica conforme a los experimentos de competición. Esto sugiere un papel regulador diferente de PcFKH1 en la expresión de estos genes biosintéticos. De hecho, sólo se observó una disminución de los transcriptos del gen *penDE* (y no del gen *pcbC*) en los transformantes de silenciamiento génico de Pc*fkh1*. Esto concuerda con el fenotipo observado en los transformantes de silenciamiento génico de Pc*fkh1*, el cual se caracteriza por un aumento del nivel de IPN y una reducción de la producción de penicilina G como consecuencia de una reducción en los niveles de la proteína IAT. Curiosamente, la sobreexpresión del gen Pc*fkh1* no produjo un aumento de la expresión de los genes biosintéticos de penicilina. Una explicación para este fenómeno podría ser la ajustada regulación a la que están sometidos estos genes, donde el nivel fisiológico de PcFKH1 es suficiente para controlar la expresión de los genes biosintéticos y por tanto, un exceso de este factor transcripcional (debido a la sobreexpresión) no es capaz de ejercer un efecto regulador mayor. Sin

embargo, también se observó que la sobreexpresión de P*cfkh1* produjo un ligero aumento en la producción de penicilina. Estos resultados se pueden explicar por el efecto positivo que ejerce la sobreexpresión de P*cfkh1* sobre la expresión de los genes auxiliares de la biosíntesis de penicilina, tales como *phlA* (que codifica la fenilacetil CoA ligasa) y *ppt* (que codifica la 4`-fosfopanteteinil transferasa). El efecto positivo de la sobreexpresión de estos dos genes sobre la producción de penicilina ya se había descrito con anterioridad por nuestro grupo de investigación (Lamas-Maceiras y col., 2006; García-Estrada y col., 2008a).

Los factores transcripcionales de tipo "*forkhead*" son una subclase de la familia de reguladores transcripcionales "*winged helix*" (Gajiwala y Burley, 2000) que en humanos y levaduras están implicados en diferentes procesos, tales como la regulación del ciclo celular, el control de la muerte celular, el procesamiento de pre-ARNm o la morfogénesis (Burgering y Kops, 2002; Carlsson y Mahlapuu, 2002; Morillon y col., 2003). De hecho, AcFKH1 no está implicado directamente en la fragmentación de las hifas, pero su presencia parece ser necesaria para que CPCR1 realice su función reguladora en la morfogénesis de *A. chrysogenum* (Hoff y col., 2005). Se ha sugerido que CPCR1 y AcFKH1 funcionan como un enlace molecular entre el metabolismo secundario (producción de antibióticos) y la morfogénesis y por tanto, representan dos factores muy importantes en el control del crecimiento del hongo durante los procesos de producción de metabolitos. Sin embargo, ni PcRFX1 (Domínguez-Santos y col., 2012) ni PcFKH1 parecen estar implicados en la diferenciación morfológica en *P. chrysogenum* bajo las condiciones estudiadas. Es conveniente destacar que la cepa utilizada para estos experimentos (*P. chrysogenum* Wisconsin 54-1255) no es la cepa silvestre, sino una cepa de referencia que ha sufrido varias rondas de mutagénesis clásica durante el programa de mejora de cepas, donde la capacidad de diferenciación morfológica puede haber sido ligeramente modificada. Por tanto, no puede ser descartado completamente el posible papel de PcFKH1 en el desarrollo morfológico de *P. chrysogenum*.

Los factores transcripcionales "*forkhead*" también regulan el desarrollo sexual y el ciclo celular en hongos (Lee y col., 2005; Park y col., 2014). Uno de estos genes implicados en el ciclo celular es *cdc15*. Este gen es importante para el crecimiento,

septación, desarrollo asexual y patogenicidad en *Magnaporthe oryzae* (Goh y col., 2011) y juega un papel clave en la regulación del ensamblaje y contracción del anillo de actomiosina durante la citoquinesis en *Schizosaccharomyces pombe* (Fankhauser y col., 1995) y en *S. cerevisiae* (Lippincott y Li, 1998). La deleción de *fkh1* aumenta la expresión del gen *cdc15* en *M. oryzae* (Park y col., 2014) y en *S. pompe* (Bulmer y col., 2004), un fenómeno que también se observó en *P. chrysogenum*. Sin embargo, el incremento de los niveles de ARNm de *cdc15* en los transformantes silenciados en el gen Pc*fkh1* no estuvo acompañado por efectos fenotípicos. Esto puede ser explicado por el hecho de que estamos trabajando con transformantes silenciados en el gen Pc*fkh1*, los cuales presentan unos niveles basales de expresión del gen Pc*fkh1*. Además, como hemos indicado antes, los transformantes se obtuvieron a partir de la cepa de referencia Wisconsin 54-1255, la cual puede haber sufrido modificaciones previas en el desarrollo y en el ciclo celular durante los programas de mejora de cepas.

También se observó que PcFKH1 regula positivamente la conidiación y la pigmentación de las esporas en *P. chrysogenum*. En otros hongos filamentosos se ha confirmado la implicación de este factor de transcripción en la producción de conidios, como es el caso de *M. oryzae*, donde la abundancia relativa de los transcriptos del gen que codifica a MoFKH1 aumenta durante la conidiación y/o en conidios, respaldando su papel en el control de la producción de estas estructuras (Park y col., 2014, 2013).

El análisis *in silico* de los supuestos procesos metabólicos controlados por PcFKH1 fue llevado a cabo considerando aquellas regiones promotoras que contenían al menos una supuesta secuencia de unión para PcFKH1 y una para PcRFX1. Esto fue debido al hecho de que AcFKH1 y CPCR1 (ortólogo de PcRFX1) interaccionan entre sí en *A. chrysogenum* (Schmitt y col., 2004b) y por tanto, asumiendo que ambos factores de transcripción pueden trabajar también juntos en *P. chrysogenum*, limitamos la búsqueda a aquellos genes que tienen sitios de unión al ADN para ambas proteínas en sus regiones promotoras. El análisis reveló que PcFKH1 tiene tendencia a regular aquellos genes implicados en el metabolismo primario (p. ej. catabolismo de carbohidratos, proceso metabólico de ácidos di y tri-

carboxílico, catabolismo de quitina, catabolismo de glicina, la biosíntesis de histidina, etc.). Curiosamente, uno de los procesos bioquímicos supuestamente controlados por PcRFX1 y PcFKH1 está relacionado con la biosíntesis de pantotenato, regulando los genes que codifican PanB (ketopantoato hidroximetiltransferasa), la cual es la primera enzima de la ruta de biosíntesis de pantotenato, y PanE (ketopantoato reductasa), siendo esta última la segunda enzima de la ruta. El pantotenato es el precursor de 4`-fosfopanteteina, grupo prostético esencial que es transferido a la ACV sintetasa por medio de la PPTasa (García-Estrada y col., 2008a). Teniendo en cuenta que tal y como se indicó anteriormente la expresión del gen *ppt* que codifica la PPTasa está controlada por PcFKH1, la supuesta regulación positiva de los genes *panB* y *panE* puede representar un efecto beneficioso adicional de PcFKH1 sobre la biosíntesis de penicilina.

Los resultados proporcionados tras este análisis global ayudarán a dilucidar la relación existente entre el metabolismo primario, ciclo celular y la biosíntesis de penicilina. Esta información puede ser de gran interés para arrojar luz sobre los mecanismos que subyacen bajo la compleja red reguladora de este importante antibiótico β-lactámico.

Capítulo 3

Resultados y Discusión Capítulo 3

Durante muchos años ha permanecido sin aclaración el posible papel del regulador transcripcional CreA en el control de la expresión de los genes biosintéticos de penicilina. En este trabajo, hemos mostrado que el regulador global CreA es el responsable de la regulación por fuente de carbono de la biosíntesis de penicilina en *P. chrysogenum*. Esta conclusión está basada en las evidencias experimentales obtenidas a partir de dos aspectos: i) el análisis *in vivo* de la funcionalidad de los sitios de unión para CreA presentes en la región intergénica *pcbAB-pcbC*, y ii) el estudio del efecto diferencial que el silenciamiento del gen *creA* mediado por ARN de interferencia tiene sobre la producción de penicilina en medio con glucosa o lactosa como fuentes de carbono.

Los estudios previos realizados en *A. nidulans* concluyeron que existe un mecanismo aún no caracterizado e independiente de CreA, el cual es el responsable principal de la regulación por fuente de carbono de la biosíntesis de penicilina (Espeso y Peñalva, 1992; Espeso y col., 1993). En estos trabajos se encontró una región de ADN que contiene dos sitios de unión de CreA al ADN, los cuales se encontraban situados en una secuencia de 29 pb protegida de la digestión por la ADNasa I (Espeso y col., 1993) involucrada en la represión por sacarosa del gen *ipnA*. Sin embargo, tras la deleción de la secuencia de 29 pb, no se observó ningún efecto desrepresivo de la expresión del gen *ipnA* (Espeso y col., 1993). Además, diferentes mutaciones de pérdida de función del gen *creA* sólo tuvieron un efecto menor sobre la expresión del gen *ipnA* (Espeso y Peñalva, 1992). En el caso del análisis comparativo de microarrays entre un mutante delecionado en el gen *creA* y la cepa de referencia, los genes biosintéticos no mostraron cambios significativos en sus niveles de expresión (Mogensen y col., 2006). Aun así, no se ha realizado un análisis funcional *in vivo* de los tres sitios consenso de unión de CreA al ADN situados en la región intergénica *acvA-ipnA.* Espeso y Peñalva (1994) observaron *in vitro* la unión de la proteína de fusión GST-CreA a estos sitios. Por tanto, no se puede descartar totalmente que en cierto grado, CreA esté involucrado en la regulación por fuente

de carbono de la biosíntesis de penicilina en *A. nidulans*, ya que realmente algunos mutantes *creA* mostraron una ligera desrepresión de la transcripción del gen *ipnA* (Espeso y Peñalva, 1992).

En *P. chrysogenum*, hay seis sitios consenso dentro de la región intergénica *pcbAB-pcbC* para la unión del factor transcripcional CreA. Este número de sitios de unión al ADN es mucho más alto de lo esperado para una probabilidad aleatoria de un sitio por cada 512 pb. En nuestro trabajo, hemos encontrado que el sitio CreA-1, el más próximo al gen *pcbAB*, tiene una función muy importante en la represión por glucosa de este gen. Cuando se delecionó o sustituyó el sitio de unión al ADN CreA-1, la expresión del gen *pcbAB* en medio con glucosa (como fuente de carbono) y con el pH libre estuvo desreprimida hasta un 50% de la expresión en condiciones no represoras (con lactosa como fuente de carbono), mientras que a un pH controlado de 6, se observó una desrepresión total. El sitio CreA-5 también tuvo un papel en dicha regulación, pero cuantitativamente menor que el del sitio CreA-1. Por otro lado, el sitio CreA-6 jugó sólo un pequeño papel en la expresión del gen *pcbAB*. El diferente grado de desrepresión observado en los promotores con la caja CreA-1 delecionada o sustituida, cuando el pH se encontraba libre o fijado a un valor de 6, es probablemente debido al resultado de la regulación por pH sobre el promotor del gen *pcbAB*, la cual es independiente del control ejercido por CreA. La transcripción del gen *pcbAB* está regulada por pH, siendo incrementada dicha expresión a pH alcalino (Chu y col., 1997). El crecimiento del micelio en medio con glucosa produce un descenso del pH, mientras que en medio con lactosa, el pH se incrementa. Esto implica que cuando el pH no es controlado, el efecto represor de la glucosa y la falta de activación por el pH alcalino son simultáneos y causan una muy baja actividad transcripcional del promotor del gen *pcbAB*. Sin embargo, en medio con lactosa, la falta de represión catabólica por carbono se añade al efecto activador del pH alcalino, resultando en una mayor tasa de transcripción. Cuando el pH se mantuvo constante en un valor de 6, no hubo efecto activador por el pH y por tanto, las diferencias en la transcripción del promotor intacto entre los medios que contenían glucosa y lactosa fueron menos patentes. Cuando el sitio de unión al ADN CreA-1 se delecionó o sustituyó, el efecto relativo de la desrepresión fue por consiguiente, más evidente.

La importancia de los sitios de unión para el factor transcripcional CreA en la regulación de la biosíntesis de penicilina está apoyada por lo descrito por van der Berg (2011). En este artículo, las mutaciones simultáneas de los sitios de unión CreA-2, CreA-5 y CreA-6 causaron una desrepresión importante de la transcripción del gen *pcbC* en condiciones represoras. Tras el resultado de la deleción de los elementos de unión en el promotor del gen *pcbC*, se identificó a la proteína CreA como el mayor efector negativo de la transcripción del gen de biosíntesis de penicilina *pcbC* (van den Berg, 2011). Además, el tercer gen de la ruta de biosíntesis de penicilina, *penDE*, también se encuentra sujeto a una regulación por fuente de carbono, y su expresión está reprimida por glucosa, aunque en menor grado que la ejercida sobre los genes *pcbAB* y *pcbC* (Gutiérrez y col., 1999b; Martín, 2000b). Hay un total de 7 supuestos sitios de unión para CreA en la región promotora del gen *penDE*, con lo que muy probablemente, este gen sea también regulado por CreA, ya que este número de sitios de unión es mucho más alto de lo esperado para una probabilidad aleatoria de un sitio cada 512 pb, como ocurre en el promotor bidireccional *pcbAB-pcbC*.

La secuenciación del genoma de *P. chrysogenum* (van den Berg y col., 2008) reveló la presencia de dos ORF adyacentes, Pc20g13880 y Pc20g13890, que supuestamente codificaban el factor transcripcional CreA. Aunque el orden es similar a la situación que se encontró en el genoma de *A. niger* (An02g03830 y An02g03840), la última de estas ORF parecía tratarse de un pseudogén, con un nivel de transcripto constantemente bajo (van den Berg y col., 2008) y con una secuencia demasiado corta en comparación con otros genes *creA* conocidos.

Como se describe en los resultados, no se pudieron aislar mutantes delecionados en el gen *creA* a partir de la cepa *P. chrysogenum* Wisconsin 54-1255. Después de realizar numerosas pruebas, al igual que lo manifestado por otros grupos de investigación, los intentos por eliminar el gen Pc20g13880 llevaron a aislados inestables (Touw y colaboradores, citado en van den Berg, 2011), lo que indica que probablemente el gen *creA* desempeña un papel esencial en el hongo además de regular la biosíntesis de penicilina. En *Fusarium oxysporum*, tampoco se pudo obtener un mutante delecionado en el gen *cre1* después de analizar más de 1000 transformantes (Jonkers y Rep, 2009). En cambio, sí se han obtenido mutantes

nulos en el gen *creA* en algunos hongos, ya sea por mutación o por deleción génica, como es el caso del gen *creA* en *A. nidulans*. Estos mutantes nulos muestran importantes defectos de crecimiento y de desarrollo, tales como colonias de pequeño diámetro y esporulación reducida (Shroff y col., 1997). Un fenotipo similar se observó en la cepa Δ*cre1* de *Trichoderma reesei* descrito por Nakari-Setälä y colaboradores (2009). Lo mismo sucede en la cepa Δ*cre1* de *N. crassa*, la cual presenta un crecimiento reducido y cambios en la morfología de las hifas y las colonias, las cuales se volvían compactas (Ziv y col., 2008). Por tanto, tras estas observaciones parece haber diferencias en la función precisa del gen *creA*/*cre1* entre diferentes especies de hongos, que puede ser esencial o no.

Tras las observaciones del fenotipo de crecimiento extremadamente lento y la incapacidad de mantener los diferentes clones de la deleción del gen *creA* en *P. chrysogenum*, se decidió recurrir a la estrategia de silenciamiento génico mediada por ARN de interferencia. En los transformantes de silenciamiento del gen *creA* se observó una completa desrepresión de la biosíntesis de penicilina, cuando crecían en medio complejo de fermentación con glucosa como fuente de carbono. La producción específica de penicilina en los transformantes realmente silenciados en el gen *creA* cultivados en condiciones represoras fueron similares a los obtenidos por la cepa parental *P. chrysogenum* Wisconsin 54-1255 en condiciones no represoras por fuente de carbono. Estos resultados, junto con los resultados obtenidos sobre la regulación de los genes de biosíntesis de penicilina mediada por los sitios de unión en cis para CreA, nos llevaron a concluir que CreA es el único o el principal factor responsable de la represión catabólica por carbono de la biosíntesis de penicilina en *P. chrysogenum*.

CreA podría también tener funciones adicionales que afectan directa o indirectamente a la producción de penicilina, ya que en medio con lactosa (en condiciones no represoras), los transformantes realmente silenciados en el gen *creA* mostraron un ligero, aunque significativo, incremento en la producción de penicilina con respecto a la cepa parental *P. chrysogenum* Wisconsin 54-1255. Además, la actividad transcripcional del promotor del gen *pcbAB* con el sitio de unión al ADN

CreA-1 mutado fue también mayor en comparación con el promotor intacto en las mismas condiciones no represoras por fuente de carbono.

En otro hongo productor de antibióticos β-lactámicos como es *A. chrysogenum*, el factor transcripcional Cre1, homólogo de CreA, también regula negativamente la transcripción de los genes del "*cluster*" de biosíntesis de cefalosporina en respuesta a fuentes de carbono tales como la glucosa. En la cepa mejorada de producción de cefalosporina, la expresión del gen *pcbC* ha perdido la represión por glucosa existente en la cepa silvestre, pero dicha represión se recuperó cuando se introdujeron múltiples copias del gen *cre1*, lo cual también incrementó la represión de la transcripción del gen *cefEF* (Jekosch y Kück, 2000b).

Tras todo lo indicado en este trabajo, la existencia de una regulación de la biosíntesis de penicilina por la fuente de carbono mediada por el factor transcripcional CreA hace al gen *creA* una posible diana para la mejora de cepas de *P. chrysogenum*. Por otro lado, sería de gran interés el estudio de las posibles interacciones entre la regulación de la biosíntesis de penicilina por la fuente de carbono mediada por CreA y otros circuitos reguladores que controlan la expresión de los genes de biosíntesis de penicilina. Sería además importante esclarecer la ruta de señalización de la represión por carbono y cómo CreA es activado o desactivado en *P. chrysogenum*.

4. Conclusiones

Conclusiones

1. En el desarrollo de este trabajo se han caracterizado tres reguladores globales (PcRFX1, PcFKH1 y CreA) implicados en el control del proceso biosintético de penicilina en *P. chrysogenum*.

2. PcRFX1, factor transcripcional del tipo "*winged helix"*, está implicado en el control positivo de la agrupación completa de biosíntesis de penicilina a través de la interacción con las secuencias consenso (NNRCCNNRSHWAY) de los tres promotores de los genes biosintéticos y el control de la expresión de los genes *pcbAB*, *pcbC* y *penDE* en *P. chrysogenum*.

3. PcRFX1 controla ligeramente la esporulación de la cepa *P. chrysogenum* Wisconsin 54-1255, aunque no parece participar en el control de la morfología y el desarrollo bajo las condiciones estudiadas.

4. PcFKH1, miembro de la familia de factores transcripcionales de tipo "*forkhead*", los cuales también pertenecen a la subclase de factores de transcripción "*winged helix"*, regula positivamente la producción de penicilina a través de la interacción específica con la región promotora del gen *penDE* en *P. chrysogenum*.

5. PcFKH1 también controla la expresión de dos genes auxiliares implicados en el proceso de biosíntesis de penicilina, en concreto los genes *phlA* (que codifica la fenilacetil CoA ligasa) y *ppt* (que codifica la 4`-fosfopanteteinil transferasa).

6. PcFKH1 juega un papel en la conidiación y pigmentación de las esporas, pero no parece estar implicado en el control de la morfología y división celular en la cepa *P. chrysogenum* Wisconsin 54-1255.

7. El factor transcripcional CreA que posee dos dedos de zinc del tipo Cys2His2 es el principal regulador responsable de la represión catabólica por fuente de carbono sobre la biosíntesis de penicilina en *P. chrysogenum*.

8. En el promotor del gen *pcbAB* en *P. chrysogenum* existe una regulación por fuente de carbono dependiente de CreA, en la que participa principalmente la caja CreA-1, y en menor grado los sitios de unión CreA-5 y CreA-6.

9. Los factores transcripcionales globales PcRFX1, PcFKH1 y CreA representan posibles dianas para la optimización de los procesos de producción de penicilina y la mejora de cepas de uso industrial de *P. chrysogenum* a través de ingeniería genética.

5. Anexo

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Capítulo 8

Regulatory Processes Controlling the Biosynthesis of Beta-lactam Antibiotics in *Penicillium chrysogenum* and *Acremonium chrysogenum*

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Regulatory Processes Controlling the Biosynthesis of Beta-lactam Antibiotics in *Penicillium Chrysogenum* and *Acremonium Chrysogenum*

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ABSTRACT

Antibiotics from the beta-lactam group, which includes penicillins, cephalosporins, monolactams, clavulanic acid and carbapenems, are among the most commonly prescribed medications in modern medicine. Penicillium chrysogenum and Acremonium chrysogenum are the filamentous fungi used for the industrial production of penicillins and cephalosporins. During the last decades the joint effort between industry and academia has contributed to gather information on the biosynthetic genes and enzymes as well as on the regulation of the biosynthetic process. The present review discusses modern aspects of the regulatory mechanisms controlling the biosynthesis of penicillins and cephalosporins in P. chrysogenum and A. chrysogenum.

***Key words*:** Penicillins, Cephalosporins, Beta-lactam biosynthesis, Regulation, Transcription factors.

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1. INTRODUCTION

Some filamentous fungi and bacteria produce organic compounds of low molecular weight that selectively inhibit the growth of microorganisms at low concentrations. These secondary metabolites, termed antibiotics, are classified into different families, including beta-lactam antibiotics, macrolides, quinolones, sulfonamides, tetracyclines, aminoglycosides, etc. Beta-lactam antibiotics (mainly penicillins and cephalosporins) stand out from the rest of families due to their high activity and low toxicity and are among the most commonly prescribed drugs.

The discovery of penicillin by Sir Alexander Fleming eighty five years ago is one of the most important milestones in the history of medicine. Penicillin was the first beta-lactam antibiotic discovered and the improvement of strains of *Penicillium chrysogenum* (producing 100,000 times more penicillin than the Fleming´s original isolate) represents one of the best examples of biotechnological taming of one microorganism. Cephalosporins were discovered in 1945, when the fungus *Cephalosporium acremonium* (later renamed *Acremonium chrysogenum*) was isolated by Giuseppe Brotzu from the bay water at Cagliari, Italy. The original natural product, cephalosporin C, has been successfully modified for the production of semisynthetic cephalosporins.

During the past decades, the unprecedented joint effort between industry and academia has contributed to gather information on these antibiotics, including the regulation of the biosynthetic process.

2. CHEMICAL STRUCTURE OF PENICILLINS AND CEPHALOSPORINS

Beta-lactam antibiotics are non-ribosomal peptide-derived antibiotics, which like many other secondary metabolites, have unusual chemical structures. The common structure for this class of antibiotics consists of a four-membered beta-lactam ring closed by an amide bond. With the exception of monolactams, which have only the beta-lactam ring, the rest of beta-lactam antibiotics possess a bicyclic system. The structure of the second ring allows the classification of these antibiotics into penicillins, cephalosporins, clavulanic acid and carbapenems, in addition to the above-mentioned monolactams (O´Sullivan and Sykes, 1986; Aharonowitz *et al*., 1992) (Fig. 1).

Penicillins with a hydrophobic side chain (*e.g.* benzylpenicillin or penicillin G and phenoxymethylpenicillin or penicillin V) are exclusively synthesized by filamentous fungi from the genera *Penicillium* (*e.g. P. chrysogenum*) and *Aspergillus* (*A. nidulans*). On the contrary,

hydrophilic penicillins are synthesized by filamentous fungi (*A. chrysogenum*), actinomycetes (*Streptomyces sp*.) and some Gram-negative bacteria. Cephalosporins are produced by fungi (*A. chrysogenum*, *Paecilomyces persicinus*, *Kallichroma tethys* and some other deuteromycetes), Gram-positive actinomycetes (*Streptomyces clavuligerus* or *Nocardia lactamdurans*) and Gram-negative bacteria (*Lysobacter lactamgenus*) (Liras, 1999; Brakhage *et al*., 2009; Martín *et al*., 2010).

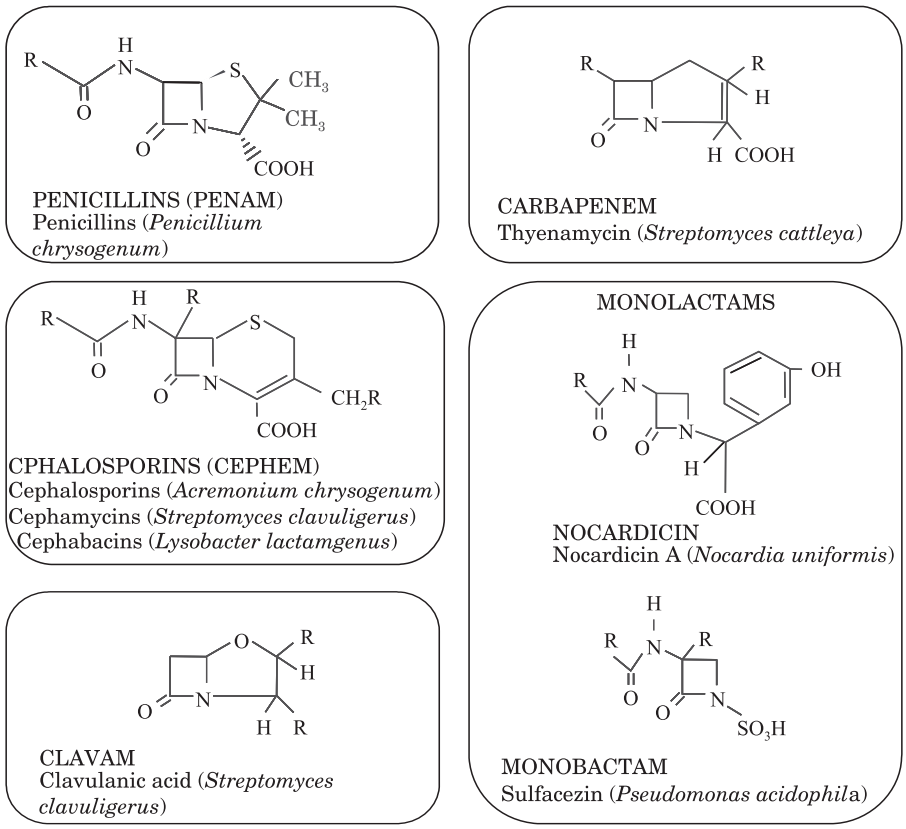

Fig. 1: Chemical structure and classification of beta-lactam antibiotics. Producer microorganisms are indicated.

2.1. Natural, Synthetic and Semisynthetic Penicillins

Penicillins contain a bicyclic "penam" nucleus (Fig. 1), which consists of a beta-lactam ring fused to a sulfur-containing thiazolidine ring and an acyl side chain bound to the amino group at C-6. The side chain depends on the precursors present in the culture medium. In the absence of a

specific side chain precursor, different natural penicillins will be produced, such as penicillin F (D3-hexenoic acid as side chain) and K (octanoic acid as side chain). However, feeding penicillin fermentations with specific side chain precursors gives rise to the synthesis of specific penicillins. This strategy has been followed for decades to obtain the most important synthetic hydrophobic penicillins; penicillin G (phenylacetate as side chain) and penicillin V (phenoxyacetate as side chain).

Penicillin G and penicillin V are the precursors of semisynthetic penicillins and cephalosporins, since chemical or enzymatic release of the side chain gives rise to the 6-aminopenicillanic acid (6-APA) structural core. The development of semisynthetic penicillins began after the isolation of 6-APA from fermented broths in the late 1950s (Kato, 1953; Batchelor *et al*., 1959). Industrial production of 6-APA is currently based on the enzymatic deacylation of either or both biosynthetic penicillins (although penicillin G is the molecule of choice). The addition of different side chains to the 6-APA core leads to the production of semisynthetic penicillins, which can be grouped into five categories: antistaphylococcal penicillins, aminopenicillins, carboxypenicillins, ureidopenicillins and beta-lactamase-resistant penicillins (Oshiro, 1999).

2.2. Cephalosporins: Penicillin-Derived Cephalosporins, Cephalosporin C-Derived Cephalosporins and Semisynthetic Cephalosporins

Cephalosporins contain a six-membered dihydrothiazine ring fused to the beta-lactam ring, which constitute the "cephem" nucleus (Fig. 1). Cephalosporin C has a D-α-aminoadipyl side chain bound to the C-7 amino group, which is identical to that of hydrophilic penicillin N (PenN) but differs from that of hydrophobic penicillins.

Penicillin-derived cephalosporins are mainly based on the chemical expansion of the benzylpenicillin ring followed by an enzymatic deacetylation that removes the aromatic side chain, thus yielding 7-aminodeacetoxycephalosporanic acid (7-ADCA) (Barber *et al*., 2004). Alternative strategies to produce 7-ADCA, adipyl-7-ADCA, adipyl 7-aminocephalosporanic acid (adipyl-7-ACA) or adipyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (ad7-ACCCA), which is an interesting semisynthetic cephalosporin precursor from the stability point of view, have been carried out through the genetic modification of *P. chrysogenum* (Crawford *et al*., 1995; Harris *et al*., 2009).

Cephalosporin C-derived cephalosporins are based on the conversion of cephalosporin C into 7-ACA by either a chemical or an enzymatic process that removes the 7-aminoadipyl side chain. This industrial conversion is currently achieved through a two-step process catalyzed by a D-amino acid oxidase and a glutaryl acylase (Velasco *et al.*, 2000).

Commercial cephalosporins are all semisynthetic and are derived from 7-aminodecetylcephalosporanic acid (7-DAC), 7-ACA, 7-ADCA or the corresponding nuclei of the cephamycins (Demain and Elander, 1999). The addition of a new side chain at position C-7 or alteration of the 3′ side chain will lead to advanced intermediates with modified antibacterial spectrum, beta-lactamase stability and pharmacokinetic properties. The medically useful cephalosporins are categorized as first, second, third, fourth and fifth generation products depending on their spectrum and resistance to enzymatic degradation.

3. MECHANISM OF ACTION OF PENICILLINS AND CEPHALOSPORINS

The mechanism of action of beta-lactam antibiotics is highly related to their chemical structure. These antibiotics are bactericidal agents that inhibit the crosslinking of peptidoglycan chains during the bacterial cell wall biosynthesis. They bind in a covalent manner the active site of PBPs (Penicillin Binding Proteins), mainly DD-transpeptidase (PBP3) and DD-carboxypeptidases (PBP4, PBP5 and PBP6), which are responsible for the last step in the bacterial cell wall biosynthesis. This mechanism of action is due to the structure of beta-lactam antibiotics, which is similar to the last two amino acids acyl-D-alanine-D-alanine (the natural substrate of PBPs) of the pentapeptide that links the peptidoglycan molecule. As a consequence of this binding, PBPs are irreversibly inhibited, with the subsequent instability of the bacterial cell wall and cell autolysis (Giesbrecht *et al.*, 1991; Frère *et al.*, 1993). Beta-lactam antibiotics also have the ability to trigger the activation of cell wall hydrolases and autolysins, which ultimately contribute to destroy the bacteria (Kong *et al.*, 2010).

According to the mode of action of beta-lactams, the activity of these antibiotics is higher against gram-positive bacteria, since the peptidoglycane is the major constituent of their cell wall and PBPs are located on the cytoplasmic membrane exposed to the environment. On the contrary, in gram-negative bacteria PBPs are present in the periplasmic space protected by the external outer membrane, which acts as a barrier for beta-lactam antibiotics. This drawback has been

overcome with the incorporation of new side chains to the penam and cephem nuclei in semisynthetic penicillins and cephalosporins.

4. PENICILLIN AND CEPHALOSPORIN BIOSYNTHETIC PATHWAYS

Beta-lactam antibiotics are peptidic molecules with a common backbone chemical structure constituted by the non-ribosomally synthesized tripeptide δ-L (α-aminoadipyl)-L-cysteinyl-D-valine (ACV). After ACV cyclization, the penam nucleus is formed (early steps), further expanded to the cephem nucleus (intermediate steps) and finally, modified during the late decorating steps (Fig. 2). The early steps are common for penicillin, cephalosporin and cephamycin producers, since isopenicillin N (IPN) is the branch point for the biosynthesis of those compounds, whereas the intermediate and late steps are exclusive of cephalosporin and cephamycin producers.

4.1. Early Enzymatic Steps

Beta-lactam producers have the two early enzymatic steps in common, which lead to the biosynthesis of IPN, the first active compound in the pathway (Fig. 2). Depending on the presence of specific enzymes that carry out the intermediate and late decorating steps, producer microorganisms synthesize different beta-lactam antibiotics.

Penicillin and cephalosporin biosynthesis starts in the cytosol with the non-ribosomal condensation of three amino acids: L-α-aminoadipic acid (L-α-AAA; non-proteinogenic amino acid formed by a specific pathway related to lysine biosynthesis), L-cysteine and L-valine, which constitute the tripeptide ACV. This reaction is catalyzed by the 426-kDa ACV synthetase (ACVS), which contains one module for each amino acid (each one including an adenylate-forming or activation domain, a thiolation or amioacyl carrier domain and a condensation domain) and two additional domains; an epimerase domain (catalysing the conversion of L-valine into D-valine) and a thioesterase domain (involved in the release of the tripeptide from the enzyme) (Baldwin *et al.*, 1991; De Crécy-Lagard *et al.*, 1995; Stachelhaus and Marahiel, 1995; Martín, 2000a; Wu *et al.*, 2012). This enzyme is encoded by the 11-kbp intron-free *pcbAB* (*acvA*) gene (Díez *et al.*, 1990; Smith *et al.*, 1990), which is present in fungal and bacterial penicillin and cephalosporin (and cephamycin) gene clusters.

Since ACVS is synthesized as an inactive apoprotein, activation by an ancillary enzyme is required. This is achieved by the cytosolic

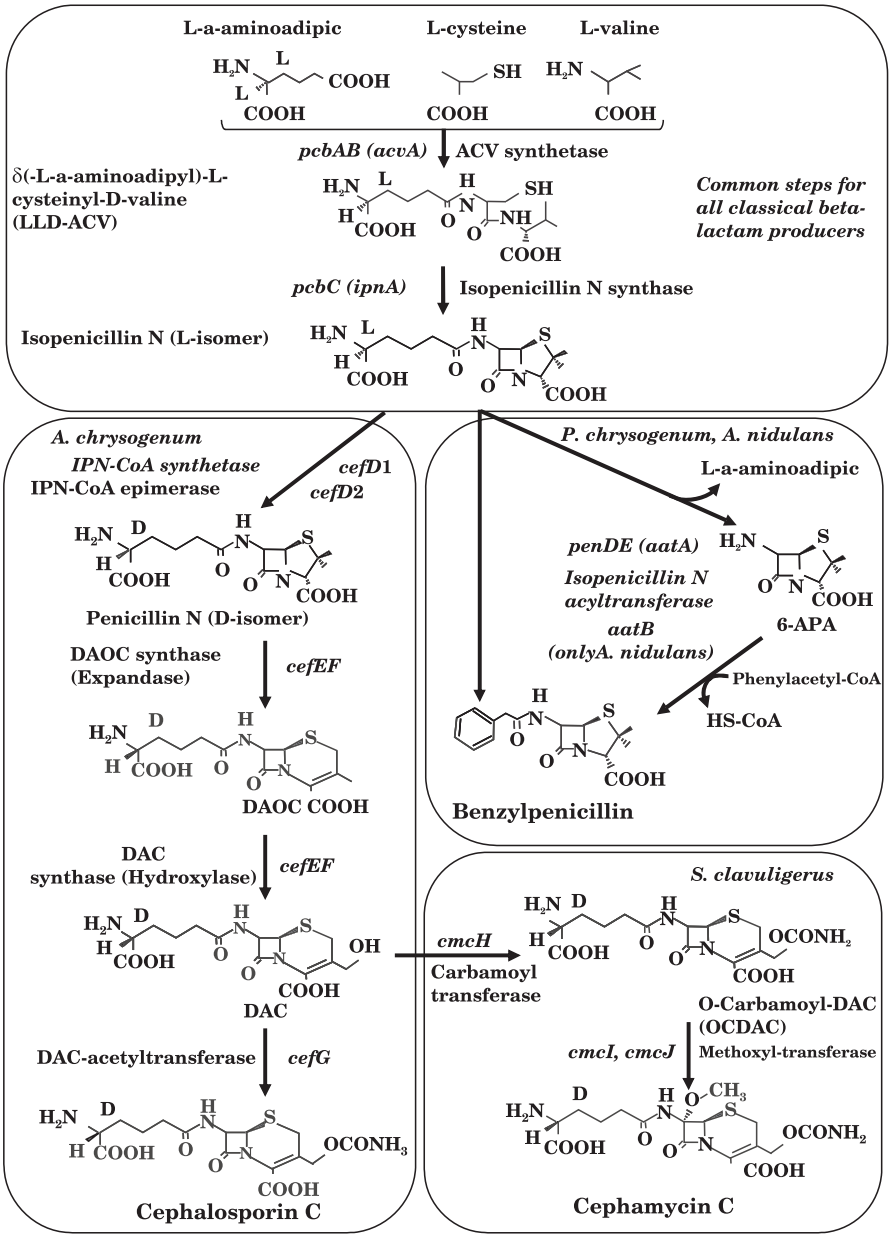

Fig. 2: Fungal benzylpenicillin and cephalosporin C and bacterial cephamycin C biosynthetic pathways.

4′-phosphopantetheinyl transferase (PPTase), which adds a 4′-phosphopantetheine arm derived from CoA to the thiolation domain of ACVS. Therefore, although PPTase does not directly participates in

the biosynthesis of ACV, is necessary for penicillin biosynthesis both in *A. nidulans* (Lambalot *et al*., 1996; Keszenman-Pereyra *et al*., 2003; Márquez-Fernández *et al*., 2007) and in *P. chrysogenum* (García-Estrada *et al*., 2008a).

During the second step of the early biosynthetic stage, four hydrogen atoms from ACV are removed, leading to the oxidative ring closure of the tripeptide and the formation of the bicyclic structure (penam nucleus) of IPN, which constitutes the branch point of penicillin and cephalosporin biosynthesis. This reaction is catalyzed in the cytosol by the 38-kDa IPN synthase or cyclase (encoded by the intronless *pcbC/ ipnA* gene), which is an intermolecular dioxygenase that requires Fe^{2+}, molecular oxygen and ascorbate (Perry *et al*., 1988; Bainbridge *et al*., 1992; Cooper, 1993).

Since IPN synthase only accepts monomeric reduced ACV as substrate (once the ACV tripeptide is synthesized, the aeration conditions that are present inside the culture medium rapidly oxidize the monomer to the disulfide form, thus forming the bis-ACV dimer) the oxidized bis-ACV has to be previously reduced before it can be cyclized. The reduction of bis-ACV is achieved by another ancillary system; the cytosolic NADPH-dependent thioredoxine disulfide reductase (TrxAB), which has been fully characterized in *P. chrysogenum* (Cohen *et al*., 1994).

In those filamentous fungi that produce hydrophobic penicillins (*e.g*. *P. chrysogenum* or *A. nidulans*), IPN enters the peroxisome, which implies the presence of specific transporters in the peroxisomal membrane. In the peroxisomal matrix, the L-α-AAA side-chain of this intermediate is replaced by a hydrophobic acyl molecule (previously activated with CoA). Side chain replacement is catalyzed by the peroxisomal acyl-CoA: IPN acyltransferase (IAT), which is encoded by the *penDE* (*aatA*) gene. This gene, which unlike the *pcbAB* and *pcbC* genes contains three introns, is translated as a preprotein of 40 kDa termed proacyltransferase or proIAT. The proacyltransferase is autocatalytically self-processed to constitute an active heterodimer with 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; Whiteman *et al*., 1990; Tobin *et al*., 1990; 1993; García-Estrada *et al*., 2008b). A two-step enzymatic process has been proposed for side chain replacement reaction (Queener and Neuss, 1982). During the first step, the amidohydrolase activity removes the L-α-aminoadipate side chain of IPN, thus forming 6-APA. Next, the acyl-CoA: 6-APA acyltransferase activity introduces the new activated acyl side chain (Álvarez *et al*., 1993).

Activation of the precursor acyl molecules as CoA thioesters is a prerequisite for the incorporation of acyl side chains during the biosynthesis of hydrophobic penicillins and therefore, another ancillary activity is necessary. Acyl-CoA ligases, which are also present in the peroxisomal matrix, are in charge of this reaction. In fact, a peroxisomal phenylacetyl-CoA ligase (PCL) activity was identified in peroxisomes (Gledhill *et al*., 1997) and some years later, the PCL-encoding *phl* gene was cloned in *P. chrysogenum* (Lamas-Maceiras *et al*., 2006). A second gene (*phlB*) was also cloned and proposed to encode a peroxisomal PCL enzyme involved in the activation of phenylacetic acid (Wang *et al*., 2007). However, recent studies from Koetsier *et al*. (2009; 2010) revealed that the *phlB* gene (also named *aclA*) is not involved in the activation of phenylacetic acid, since it encodes a broad spectrum acyl-CoA ligase protein that activates adipic acid. A more recent study has identified a third gene (*phlC*) in *P. chrysogenum* that encodes a peroxisomal protein with PCL activity (Yu *et al*., 2011).

For many years, genes and enzymes from the penicillin biosynthetic pathway seemed to be fully characterized. However, the genome sequence of *A. nidulans* revealed that this filamentous fungus in addition to the *aatA* gene, contains another gene (named *aatB*), which is not clustered with the rest of the penicillin genes and encodes a cytosolic protein that plays a role in penicillin biosynthesis (Spröte *et al*., 2008). Interestingly, the *P. chrysogenum aatB* homolog (named *ial*) was not expressed and encoded a protein not related to penicillin biosynthesis (García-Estrada *et al*., 2009). Therefore, *aatB* and *ial* genes differ in function and appear to have had a different evolution from a common ancestor.

4.2. Intermediate Enzymatic Steps

The central step of the cephalosporin C biosynthetic pathway (Fig. 2) consists of the conversion of IPN to its D-isomer PenN, which is the precursor of antibiotics containing the cephem nucleus (*e.g.* cephalosporins and cephamycins). In bacteria, this reaction is carried out in a single step by a pyridoxal phosphate-dependent epimerase encoded by the *cefD* gene, which is located in the cephamycin gene cluster (Liras, 1999). In *A. chrysogenum*, the conversion of IPN to PenN is catalyzed by a two-component protein system encoded by the linked genes *cefD1* (four introns) and *cefD2* (one intron) (Ullán *et al*., 2002a). It has been proposed that CefD1 (71 kDa) converts IPN into isopenicillinyl N-CoA, which is isomerized to penicillinyl N-CoA by CefD2. A thioesterase is also required for the release of penicillinyl N-CoA from the enzyme. The CefD1-CefD2 system seems to be located in

peroxisomes, since both proteins contain canonical peroxisomal targeting sequences. This would imply that, as it was indicated for *P. chrysogenum*, an active IPN transport system must be present in the peroxisomal membrane to ensure an adequate pool of IPN inside microbodies.

After isomerization, PenN must be transported out of the peroxisome to serve as substrate of the next reactions in the cephalosporin C biosynthetic pathway. Once in the cytosol, the five-membered thiazolidine ring of PenN is oxidized and opened forming a six-membered dihydrothiazine ring after reclosure. This reaction is catalyzed by deacetoxycephalosporin C (DAOC) synthase (expandase), which is encoded by the *cefE* gene in bacteria and by the *cefEF* gene in *A. chrysogenum* (Liras, 1999). In the next step of the pathway, the methyl group at C-3 of DAOC is hydroxylated, forming deacetylcephalosporin C (DAC). This reaction is carried out by the C-3 hydroxylase, encoded by the *cefF* gene in *S. clavuligerus* and by the *cefEF* gene in *A. chrysogenum*. Therefore, unlike what occurs in bacteria, both reactions are catalyzed by the same *cefEF*-encoded enzyme DAOC synthase (expandase)/DAC synthase (hydroxylase) in *A. chrysogenum*.

Taking into account that IPN is synthesized in the cytosol, epimerization occurs in the peroxisomal matrix and expansion and hydroxylation seem to take place in the cytosol (Martín *et al*., 2010), specific transport systems for both biosynthetic intermediates (IPN and PenN) across the peroxisomal membrane must be present. In fact, two peroxisomal membrane transporters (CefP and CefM) have been recently identified in *A. chrysogenum*. CefP, which is encoded by the *cefP* gene, is a peroxisomal membrane protein of 866 amino acids with a deduced molecular mass of 99.2 kDa probably involved in the import of IPN into the peroxisomes (Ullán *et al*., 2010). The *cefM* gene encodes CefM, which is an efflux pump protein of 482 amino acids with a deduced molecular mass of 52.2 kDa likely involved in the translocation of PenN from the peroxisome (or peroxisome-like microbodies) to the cytosol, where it is further converted into cephalosporin C (Teijeira *et al*., 2009).

4.3. Late Enzymatic Steps

The late (and final) step in cephalosporin C biosynthesis is the conversion of DAC into cephalosporin C (Fig. 2). This reaction is catalyzed by the 49-kDa DAC acetyltransferase, which transfers an acetyl group from acetyl-CoA to the DAC molecule (Aharonowitz *et al*., 1992; Martín and Gutiérrez, 1995). This enzyme is encoded by the *cefG* gene, which contains two introns. After biosynthesis, cephalosporin C has to be transported out of the cell. It has been reported that another transporter

(CefT encoded by the *cefT* gene) may be involved in this process. Although this transporter participates in the secretion of cephalosporin C (Ullán *et al*., 2002b), it is not the main transporter for this antibiotic and is involved in the secretion of hydrophilic beta-lactams containing the L-α-AAA acid side chain (IPN and PenN) (Ullán *et al*., 2008; Nijland *et al*., 2008).

In cephamycin-producing actinomycetes, DAC undergoes carbamoylation, followed by hydroxylation and transfer of a methyl group to the hydroxyl present at C-7 (Liras, 1999) (Fig. 2).

5. ORGANIZATION OF THE PENICILLIN AND CEPHALOSPORIN C BIOSYNTHETIC GENES

Penicillin and cephalosporin C biosynthetic genes are organized in clusters (Fig. 3). This gene arrangement is typical from beta-lactam producer microorganisms, either fungi or bacteria (Brakhage *et al*., 2009).

In *P. chrysogenum* and *A. nidulans*, the *pcbAB–pcbC* (*acvA–ipnA*) genes are arranged in a divergent orientation and share a bidirectional promoter, whereas the *penDE* (*aatA*) gene is located downstream of the *pcbC* (*ipnA*) gene (Liras & Martín, 2006). The penicillin gene cluster is located on chromosome I in *P. chrysogenum* (Fierro *et al*., 1993) and in chromosome VI in *A. nidulans* (Montenegro *et al*., 1992). In penicillin high-producing strains of *P. chrysogenum*, the region containing the biosynthetic genes undergoes amplification in tandem repeats, which contributes to increase productivity (Fierro *et al*., 1995).

As it was indicated before, the *A. nidulans* genome contains another gene involved in the last step of the penicillin biosynthetic pathway, the *aatB* gene, which is not clustered with the rest of the penicillin genes (Fig. 3).

In the cephalosporin producer *A. chrysogenum*, two separate chromosomes contain the beta-lactam biosynthetic genes, which are clustered with transporter genes. In *A. chrysogenum* ATCC 48272, the biosynthetic genes *pcbAB*, *pcbC*, *cefD1* and *cefD2* are located on chromosome VII together with the secretion/translocation genes *cefT*, *cefM* and *cefP* (Gutiérrez *et al*., 1999; Ullán *et al*., 2002a; b; 2010; Teijeira *et al*., 2009). This is the so-called "early" cluster, which contains all the genetic information for the biosynthesis and secretion of IPN and PenN (Martín *et al*., 2012). The rest of the genes involved in cephalosporin biosynthesis, *cefEF* and *cefG*, are included in the "late cluster", which is located on chromosome I (Gutiérrez *et al*., 1999) (Fig. 3).

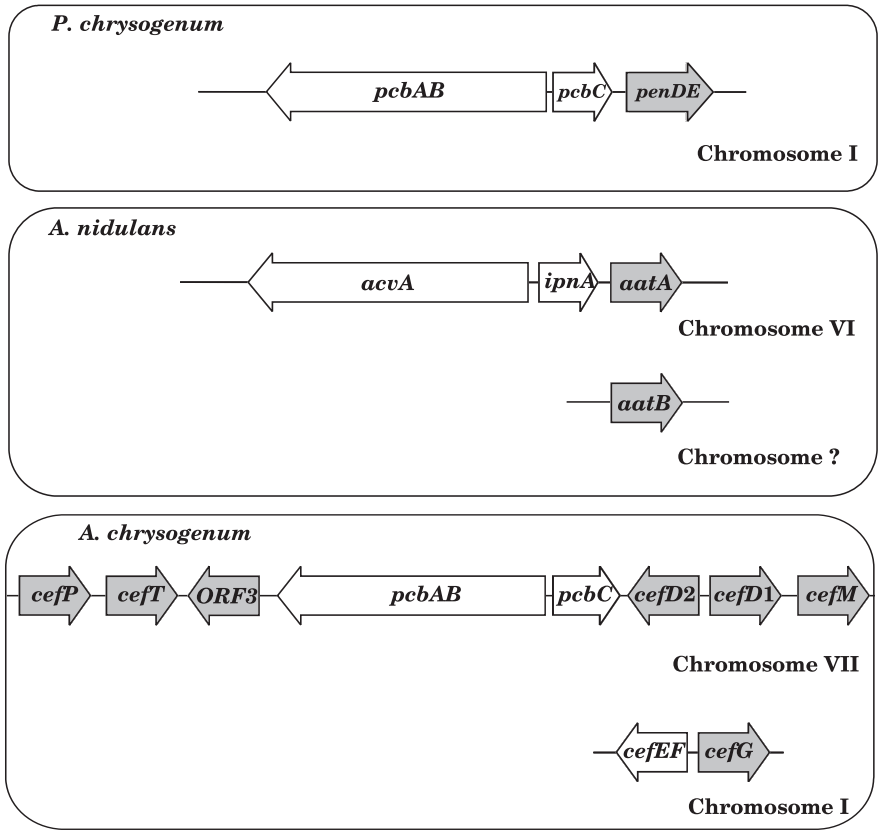

Fig. 3: Gene clusters for the biosynthesis of penicillin and cephalosporin in filamentous fungi. Genes of prokaryotic origin (lack introns) are depicted in white, whereas genes of eukaryotic origin (contain introns) are shaded.

6. CONTROL AND REGULATION OF THE BIOSYNTHESIS OF PENICILLINS AND CEPHALOSPORINS IN *P. CHRYSOGENUM* AND *A. CRHYSOGENUM*

Penicillin and cephalosporin biosynthesis is subjected to complex regulatory processes controlled by different transcription factors (Brakhage, 1998; Martín *et al.*, 1999; 2010; Martín, 2000b; Brakhage *et al.*, 2004). Some of these processes have been studied in detail and 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). This indicates that penicillin biosynthesis is controlled directly by global regulators (*e.g.* CreA, PacC, Nre) rather than by pathway-specific ones.

In *A. chrysogenum*, the "early" cephalosporin cluster contains an ORF encoding a nuclear regulatory protein (CefR), which is the first

modulator of beta-lactam intermediate transporters in *A. chrysogenum*, since it produces a small stimulatory effect on the *cefEF* gene expression and acts as a repressor of the exporter *cefT* gene (Teijeira *et al.*, 2011).

The main mechanisms and factors that are involved in penicillin and cephalosporin regulation are indicated below.

6.1. Nutrition and Environmental Conditions Controlling Beta-Lactam Biosynthesis

6.1.1. *Carbon catabolite regulation*

Penicillin biosynthesis in *P. chrysogenum* is strongly regulated by glucose and sucrose and to lower extent by maltose, fructose and galactose, but not by lactose. Therefore, the negative effect that glucose exerts on penicillin production can be overcome using lactose or feeding cultures with subrepressing doses of glucose. Penicillin production appears to be favoured by suboptimal growth conditions, since the fungus grows better with glucose than with lactose. Carbon regulation is exerted at different levels of the penicillin biosynthesis: flux of L-α-AAA; uptake and activation of side chain precursors; transcriptional and post-transcriptional regulation of the penicillin biosynthetic gene cluster.

Glucose reduces the L-α-AAA pool, likely reducing the flux to ACV as well, a phenomenon that is not observed with lactose. The formation of ACV and IPN are also repressed by high glucose concentrations (Revilla *et al.*, 1986) in *P. chrysogenum* AS-P-78, a penicillin high-producing strain. In accordance with this finding is the observation that the expression of both *pcbAB* and *pbcC* genes is repressed by glucose in another strain of *P. chrysogenum* (Q176), (Feng *et al.*, 1994). Nevertheless, Renno *et al.* (1992) claimed that steady-state mRNA levels of the *P. chrysogenum* penicillin biosynthetic genes are higher during rapid growth, when high amounts of glucose are present. These data display that measurement of carbon regulation depends, at least in part, on the experimental approach used.

In *A. nidulans*, glucose regulation of *ipnA* takes place, at least in part, at the transcriptional level, with a reduction in the IPN synthase activity when the cultures were grown in the presence of glucose. The effect of glucose on the *aatA* gene was posttranscriptionally mediated and the IAT specific activity in both *A. nidulans* and the wild-type strain of *P. chrysogenum* (NRRL, 1951) was reduced in cultures grown in the presence of glucose (Brakhage *et al.*, 1992), which is in contrast to the results provided by Revilla *et al.* (1986) in the AS-P-78 strain of

P. chrysogenum. This indicates that in this penicillin high-producing strain glucose-mediated regulation may have undergone partial modifications, at least in the control of IAT activity.

Glucose was also seen to have a negative effect on the *P. chrysogenum* acetyl-CoA synthetase, which is different from the PhlA, PhlB and PhlC CoA-ligases and is able to catalyse the activation of some of the side chain precursors required for the production of several penicillins *in vitro* (Martínez-Blanco *et al.*, 1992). In *P. chrysogenum*, the uptake of phenylacetic acid is repressed by different carbon sources and certain amino acids and is induced by phenylacetate (Ozcengiz and Demain, 2013).

The nutritional signal (high or low glucose levels) is transduced to the penicillin biosynthetic gene cluster by a carbon regulatory protein, which appears to be formed immediately after the inoculation of the penicillin fermentation (the repressive effect is clearly lower when glucose is added after 12 to 24 hours of incubation). In *A. nidulans*, carbon catabolite regulation of primary metabolism is mediated by a transcriptional regulatory protein encoded by the *creA* gene (Arst and MacDonald, 1975; Bailey and Arst, 1975; Dowzer and Kelly, 1991; Kulmburg *et al.*, 1993). CreA is a zinc finger transcription factor of the Cys2-His2-type that contains two zinc fingers, an alanine-rich region and frequent S(T)PXX motifs (Dowzer and Kelly, 1991). This protein recognizes the consensus binding sequence SYGGRG (Cubero and Scazzocchio, 1994; Cubero *et al.*, 2000; Kulmburg *et al.*, 1993).

However, *A. nidulans creA* mutants still exhibit glucose-mediated repression of *ipnA* transcript levels (Espeso and Peñalva, 1992). In addition, the deletion of a 29 bp sequence, which is protected by the CreA protein, did not change sucrose repression (Espeso *et al.*, 1993) and mutations in *creB* (it encodes a member of the family of deubiquitinating enzymes (Lockington and Kelly, 2001)) and *creC* (it encodes a protein with a proline-rich region, a putative nuclear localization region and five WD40-repeat mofits (Todd *et al.*, 2000)) had very low effect on carbon regulation of penicillin biosynthesis. These results suggest that in *A. nidulans* a second CreA-independent mechanism of carbon repression is involved in the control of penicillin biosynthesis.

In the bidirectional *pcbAB-pcbC* promoter of *P. chrysogenum* there are six SYGGRG motifs, whereas seven putative CreA binding sites have been identified in the promoter region of the *penDE* gene (van den Berg *et al.*, 2008). However, it is not yet clear whether *P. chrysogenum* has a similar mechanism of carbon regulation mediated by CreA.

In *A. chrysogenum*, cephalosporin C production depends on the carbon source used (Demain, 1963). Those carbon sources leading to a faster growth (glucose or glycerol) have a negative effect on beta-lactam production. Glucose has a stronger negative effect on cephalosporin C than on PenN production, indicating that repression is more intense during the late steps of the pathway (Behmer and Demain, 1983). The enzyme activity of the *pcbAB*, *pcbC* and *cefEF* gene products is reduced in the presence of glucose and therefore, this carbon source reduces cephalosporin C production. The glucose effect on ACVS is exerted at post-transcriptional level, since the ACVS specific activity is severely inhibited by glucose and glycerol, unlike ACVS protein levels, which remains unaffected. This phenomenon is due to the depletion of the cofactor ATP via sugar metabolism (Zhang *et al.*, 1989). On the other hand, the glucose effect on *pcbC* and *cefEF* genes is mediated at transcriptional level. The *pcbC* and *cefEF* gene promoters contain four putative CRE1 binding sites and the *cre1* gene product controls the expression of *pcbC* and *cefEF* genes (Jekosch *et al.*, 2000a). This control mechanism was deregulated during the strain improvement program (Jekosch *et al.*, 2000b).

6.1.2. *pH regulation*

Beta-lactam antibiotic production is regulated by the external pH of the medium (Espeso *et al.*, 1993). This pH regulation is mediated in filamentous fungi by the transcriptional activator PacC (Tilburn *et al.*, 1995). PacC is a transcription factor of the Cys2-His2-type that contains three zinc fingers (Espeso *et al.*, 1997). PacC activates transcription by binding to the consensus sequence 5′-GCCARG-3′ (Tilburn *et al.*, 1995). Penicillins and cephalosporins are produced at high levels under alkaline pH, since under these conditions, a proteolytic processing activates PacC. PacC activation occurs in response to a signal transduction provided by the six regulatory *pal* gene products at alkaline pH (Orejas *et al.*, 1995). The activated PacC protein functions as both activator and repressor, depending on the pH. PacC activates the transcription of genes expressed at alkaline pH, represses the transcription of genes expressed at acidic pH and probably exerts a strong autoregulation of its own gene because of the existence of putative PacC binding sites in the promoter of the *pacC* gene from *A. chrysogenum*, *P. chrysogenum* and *A. niger* (Schmitt *et al.*, 2001; Suárez and Peñalva, 1996; MacCabe *et al.*, 1996).

The use of repressing carbon sources (glucose or sucrose) causes acidification of the culture medium, whereas non-repressing carbon sources (lactose) produce alkalinisation. Therefore, although carbon source and pH have their own specific regulatory mechanisms, these two independent forms of regulation normally act in concert (Espeso

et al., 1993). While in *A. nidulans* the alkaline external pH is able to override the sucrose repression of the *ipnA* gene expression, in *P. chrysogenum* glucose repression appears to be stronger, since it is still active at alkaline pH (Suárez and Peñalva, 1996).

In *P. chrysogenum*, seven and eight putative PacC binding sites are present in the *pcbAB-pcbC* intergenic region and in the promoter region of *penDE*, respectively (Suárez and Peñalva, 1996). In *A. chrysogenum*, two putative PacC binding sites are present in each of the intergenic regions between *pcbAB* and *pcbC* and between c*efEF* and *cefG* (Schmitt *et al.*, 2001). These data suggest full pH regulation of the penicillin biosynthetic genes and partial pH regulation of some cephalosporin genes.

6.1.3. *Nitrogen source regulation*

The nitrogen source has a strong influence on beta-lactam antibiotic production by *P. chrysogenum* and *A. chrysogenum.*

An ammonium concentration higher than 100 mM strongly interfered with cephalosporin C production in *A. chrysogenum*. The addition of ammonium caused repression of DAOC synthase/ hydroxylase, but not of IPN synthase. L-arginine and L-asparagine were shown to be better nitrogen sources than ammonium for antibiotic production (Shen *et al.*, 1984).

In *P. chrysogenum*, the addition of 40 mM ammonium to lactose-grown mycelia caused the repression of expression from both *pcbAB* and *pcbC* promoters fused to gene *uidA* (Feng *et al.*, 1994).

Nitrogen regulation in fungi is mediated by AreA in *A. nidulans* and its homolog in *P. chrysogenum* NRE. These genes encode regulatory transcription factors with a single Cys-X_2-Cys-X_{17}-Cys-X_2-Cys-type zinc finger and an adjacent basic region that constitute a DNA binding domain. These transcription factors recognize the consensus sequences GATA (Marzluf, 1997). In *P. chrysogenum*, the bidirectional promoter region *pcbAB-pcbC*, contains a total of six GATA motifs. Only NRE strongly interacts *in vitro* with a site that contains two of these GATA motifs. In this binding site, the two GATA motifs, which are separated by 27 bp, are arranged in a head-to-head fashion (Haas and Marzluf, 1995).

In *A. nidulans*, there is only one GATA motif in the bidirectional *pcbAB-pcbC* promoter region. No evidence on a possible nitrogen-dependent regulation of penicillin biosynthesis in this fungus has been

reported so far. However, when the *pcbC* promoter from *P. chrysogenum* was introduced in *A. nidulans*, expression was sensitive to nitrogen regulation (Kolar *et al.*, 1991). This indicates that the same machinery mediating nitrogen repression of *pcbC* expression in *P. chrysogenum*, is present in *A. nidulans*.

In *A. chrysogenum*, the intergenic region of the corresponding *pcbAB-pcbC* genes contains 15 GATA motifs, which suggests that nitrogen repression of cephalosporin production may be also regulated by a GATA factor (Menne *et al.*, 1994).

6.1.4. *Phosphate source regulation*

An excess in phosphate enhances glucose repression during penicillin biosynthesis. In a phosphate-limited complex medium, the glucose repression of penicillin levels is about 13% when sugar is added at inoculation time, whereas it increases to 59% when the medium is supplemented with 100 mM inorganic phosphate (Martín *et al.*, 1999). Inorganic phosphate has no effect *per se* on penicillin production under non-repressing conditions.

In *A. chrysogenum*, a high phosphate concentration exerts a negative effect on cephalosporin production. In the absence of glucose, phosphate itself decreased the overall flux of formation of cephalosporin C. There is a direct negative effect on the formation of ACVS, IPN synthase and DAOC synthase (Zhang *et al.*, 1988). Phosphate probably acts on the IPN synthase and DAOC synthase/DAC synthase (hydroxylase) through the formation of a complex with iron. Iron is required for the activity of those enzymes and the phosphate inhibition can be reversed by the addition of iron as a ferrous salt (Lübbe *et al.*, 1984). However, this mechanism is not completely clear, since the inhibition of ACVS, which does not require iron as ferrous salt for its activity, is reversed by Fe^{2+} (Zhang *et al.*, 1989).

6.1.5. *Regulation by aeration conditions*

The availability of O_2 is important for penicillin production. A prerequisite for higher beta-lactam titters is a good aeration of mycelia with O_2 (Swartz, 1985; Hilgendorf *et al.*, 1987). Some enzymes, such as IPN synthase and DAOC synthase (expandase)/DAC synthase (hydroxylase), require oxygen for their activity. The possibility of increasing the cephalosporin production by genetic engineering and direct introduction of a bacterial oxygen binding protein in *A. chrysogenum* also supports the great importance that O_2 can have in

the production of these compounds. However, Renno *et al*. (1992) showed that the expression of *pcbAB* and *pcbC* in *P. chrysogenum* can also be induced in response to stress by the reduction of O_2 levels.

6.1.6. *Amino acid as mediators of regulation*

In *A. chrysogenum*, PenN and cephalosporin C production is stimulated by methionine, particularly the D-isomer (Komatsu *et al*., 1975; Zhang *et al*., 1987). The stimulatory effect of methionine during the cephalosporin C production in *A. chrysogenum* might be due to an increase in the *pcbAB*, *pcbC*, *cefEF* mRNA steady-state levels (and to a lesser extent in the *cefG* transcripts too) or to the supply of the sulphur atom of cysteine to cephalosporin C (Velasco *et al*., 1994). The presence of several consensus CANNTG sequences in the bidirectional *pcbAB-pcbC* promoter region in *A. chrysogenum*, prompted to suggest that methionine regulation may be mediated by a member of the basic region helix-loop-helix protein family. Some of these transcription factors are involved in the transcriptional control of the sulphur network in *S. cerevisiae* (Thomas *et al*., 1992).

The addition of L-lysine to the fermentation medium in *A. nidulans* and *P. chrysogenum* decreases penicillin production (Demain, 1957; Brakhage *et al*., 1992). In *A. chrysogenum*, high levels of L-lysine also interfere with cephalosporin C production (Mehta *et al*., 1979). The beta-lactam precursor L-α-AAA is an intermediate in the pathway that leads to L-lysine. L-lysine represses or inhibits several steps in the biosynthesis of L-lysine. The first enzyme of the L-lysine pathway, homocitrate synthase, is sensitive to lysine feedback inhibition in *P. chrysogenum* (Demain and Masurekar, 1974; Friedrich and Demain, 1977; Luengo *et al*., 1980). L-lysine also inhibits α-aminoadipate reductase at physiological concentrations (Affenzeller *et al*., 1989). Therefore, the effect of L-lysine on penicillin production is most likely exerted through the reduction of the L-α-AAA pool, through feedback inhibition and through repression of several L-lysine biosynthesis genes and enzymes. In *A. nidulans*, L-lysine represses the expression of reporter genes fused to the *acvA* and *ipnA* gene promoters, suggesting a more direct control of the expression of penicillin genes (Brakhage *et al*., 1992). In this fungus, differential effects on the expression of reporter genes fused to the promoters of the *acvA* and *ipnA* genes and penicillin production were measured in the presence of various amino acids (Then Bergh *et al*., 1998). L-threonine, L-aspartate, L-glutamate and L-cysteine led to increased *acvA* gene fusion expression, but had no effect on *ipnA* gene fusion expression. L-methionine (at concentrations above 10 mM), L-leucine, L-isoleucine, L-phenylalanine, L-valine,

L-histidine and L-lysine led to the repression of both *acvA* and *ipnA* gene fusion expression, which was dependent on the amino acid concentration. L-tyrosine, L-tryptophan, L-proline and L-α-AAA had no major effects on *acvA* gene fusion expression, but led to the repression of *ipnA* gene fusion expression. L-serine and L-arginine did not show any effect on the expression of either of these gene fusions at any concentration. The negative effects of L-histidine and L-valine were due to reduced activation by PacC under the acidic conditions caused by these amino acids. However, the repressive effects of L-lysine and L-methionine acted independently of PacC by unknown mechanisms.

6.1.7. *Regulation by polyamines*

Biosynthesis of penicillin G is stimulated by 1, 3-diaminopropane (1, 3-DAP) and spermidine in *P. chrysogenum*. Both polyamines, but not putrescine, produce a drastic increase in the transcription of penicillin biosynthetic genes *pcbAB*, *pcbC* and *penDE* (Martín *et al*., 2011). The effect of these polyamines is not due to a change in the mechanism that controls pH, since it does not affect the expression of *pacC*. The stimulatory effect of these polyamines, 1, 3-DAP and spermidine, is exerted, at least in part, through an increase in the expression of *laeA*, which encodes a global regulator that acts epigenetically on the expression of secondary metabolite genes by heterochromatin reorganization (see below) (Martín *et al*., 2012). The proteomics analysis of the effect of these two polyamines has revealed that both compounds promoted a deep reorganization of the proteome and increased the intracellular content of vesicles that derived to vacuoles in late stages (García-Estrada *et al*., 2013). Interestingly, 1, 3-DAP and spermidine induced the formation of a post-translational modification in the IAT (likely improving the activity of this enzyme and increasing penicillin production) and reduced the expression of enzymes degrading phenylacetic acid (a well-known precursor of benzylpenicillin). In addition, enzymes involved in the biosynthesis of valine and other precursors (*i.e.* CoA) of penicillin, were favoured by these polyamines.

6.1.8. *Regulation by corn steep liquor*

Corn steep liquor (CSL) is a by-product of the corn starch manufacturing process and has been used as a regular component of the microbiological culture media.

The addition of CSL to a production medium greatly increases penicillin yields in *P. chrysogenum* (Liggett and Koffler, 1948). In *A. nidulans*, penicillin production is undetectable unless corn

steep liquor is added to the production medium (Cove, 1966). One possible explanation of the basic properties of CSL is its composition. There is a high content of amino acids (including α-aminoadipic acid), polypeptides, minerals and lactic acid, which favours the biosynthesis of penicillin.

The most important result obtained after the addition of CSL in *A. nidulans* is a great increase in the expression of the penicillin biosynthetic gene cluster. The stimulatory effect still occurs even in the presence of repressing carbon sources of the penicillin biosynthesis process (MacCabe *et al*., 1990; Peñalva *et al*., 1991a; 1991b). However, the molecular mechanisms connecting the presence of CSL and the increased gene expression are still unknown.

6.1.9. *Pga1-mediated regulation*

Heterotrimeric GTP-binding proteins (G proteins) mediate various cellular processes in eukaryotic organisms in response to environmental and nutritional stimuli. G proteins comprise three subunits, α, β and γ, which remain inactive in the heterotrimeric state when GDP is bound to the α subunit (called Pga1 in *P.chrysogenum*). They are activated when GTP is bound to the α subunit, which dissociates from the βγ dimer and then will independently interact with downstream effectors (Hamm, 1998).

Pga1 regulates penicillin biosynthesis by controlling expression of the penicillin biosynthetic gene cluster. Mutants with the activating pga1G42R allele showed an increase in penicillin production and in the steady-state mRNA levels of biosynthetic genes (*pcbAB*, *pcbC* and *penDE*) (García-Rico *et al*., 2008a).

cAMP is a secondary messenger in the Pga1-mediated signal transduction pathway and its intracellular levels are clearly regulated by Pga1 (García-Rico *et al*., 2008b). cAMP levels are high during growth on lactose and decrease considerably when glucose or fructose are added in *P. chrysogenum* (Kozma *et al*., 1993). However, the regulation of penicillin biosynthesis by Pga1 may not be mediated by cAMP (García-Rico *et al*., 2008a).

6.2. Global Regulatory Factors

6.2.1. *LaeA*

LaeA has been identified as a global regulator of secondary metabolism the *Aspergillus* genus and in *P. chrysogenum* (PcLaeA).

LaeA is a nuclear protein with a methyltransferase domain (Bok and Keller, 2004) and it regulates gene clusters through chromatin remodelling. Namely, 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).

In *Aspergillus spp.*, the deletion of *laeA* blocks the expression of the penicillin gene cluster and overexpression of *laeA* triggers increased penicillin gene transcription and penicillin formation (Bok and Keller, 2004). Overexpression of PcLaeA gave rise to a 25% increase in penicillin production and *P. chrysogenum* knock-down mutants showed a drastic decrease in the expression of all genes belonging to the penicillin biosynthetic cluster, indicating that PcLaeA acts as an activator of penicillin production (Kosalková *et al.*, 2009).

PcLaeA is part of a velvet complex, containing at least 10 different proteins that coordinate secondary metabolism with fungal development (Hoff *et al.*, 2010).

6.2.2. *The velvet complex*

VeA, which is a component of the velvet complex, is encoded by a developmental gene first isolated in *A. nidulans* as a regulator of fungal morphogenesis and was later shown to affect the biosynthesis of different metabolites, such as penicillin. VeA represses the transcription of *ipnA* and is simultaneously necessary for the expression of *acvA* (Kato *et al.*, 2003). However, Spröte and Brakhage (2007) demonstrated that this protein mainly represses *acvA* transcription. These differences might be due to the composition of the velvet complex, where VeA interacts with other factors in a finely balanced stoichiometry to control secondary metabolite production. Therefore, the production of penicillin can be modified through the alteration in the VeA yield, a hypothesis that is supported by several investigations characterizing Velvet homologs as activators as well as repressors of secondary metabolism in filamentous fungi (Hoff *et al.*, 2010). In contrast, the disruption of the *A. chrysogenum veA* gene repressed the expression of six cephalosporin C biosynthetic gene (the most drastic effect was seen in *cefEF*) with the subsequent modification in the production of cephalosporin C (Dreyer *et al.*, 2007). This has also been described in *P. chrysogenum*, where PcVelA acts as a transcriptional activator of penicillin biosynthetic genes (*pcbC* and *penDE*) (Hoff *et al.*, 2010).

Recently, novel subunits of the velvet complex (PcVelB, PcVelC and PcVosA) have been characterized in *P. chrysogenum*. Velvet subunits

have opposing roles in the regulation of penicillin production (Kopke *et al*., 2013). PcVelC is a strong activator of penicillin biosynthesis and therefore, acts together with PcVelA and PcLaeA in *P. chrysogenum* controlling secondary metabolism production. PcVelB acts as a repressor of PcVelA and PcVelC and therefore, is a repressor of this process (Kopke *et al*., 2013). However, in *A. nidulans* the PcVelB homolog has an activating role (Bayram *et al*., 2008).

6.2.3. *CPCR1 (PcRFX1)–AcFKH1*

Another transcription factor has been identified in *A. chrysogenum* is CPCR1. This is the first member of the RFX family of transcription factors in filamentous fungi. RFX proteins are part of a subfamily of the winged-helix proteins, which are characterized by a nonconventional mode of DNA recognition (Gajiwala *et al*., 2000). This transcription factor only binds DNA in a dimeric state and is involved in the regulation of cephalosporin C biosynthesis by binding at least two sequences at the bidirectional promoter region *pcbAB-pcbC* (Schmitt and Kück, 2000). *cpcR1* gene knockout mutants showed reduced levels in the expression of *pcbC* and a reduction in PenN production, but not in cephalosporin C levels. Therefore, CPCR1 is not likely involved in the regulation of the late genes from the cephalosporin biosynthetic cluster (Schmitt *et al*., 2004a). CPCR1 interacts with AcFKH1, which is a member of the forkhead family of proteins and also belongs to the subfamily of winged helix transcription factors. AcFKH1 recognizes two forkhead binding sites in the promoter region of the *A. chrysogenum pcbAB* and *pcbC* genes (Schmitt *et al*., 2004b).

PcRFX1 (homolog of the *A. chrysogenum* CPCR1) has been recently identified and characterized in *P. chrysogenum* (Domínguez-Santos *et al*., 2012). The promoter region of the penicillin biosynthetic genes was analysed in search for putative PcRFX1 DNA binding sites. Combination of the DNA binding sequences previously reported for RFX and CPCR1 (Emery *et al*., 1996; Schmitt *et al*., 2004a) allowed the identification of one putative binding site in the promoter region of the *pcbC* gene. However, when the DNA binding sequence proposed for RfxA in *Penicillium marneffei* (Bugeja *et al*., 2010) was also considered, two other putative DNA binding sites, which overlap each other in four nucleotides, were also found in the promoter region of the *pcbAB* gene. Interestingly, in the promoter region of *penDE* gene no PcRFX1 binding site was found using those consensus sequences. However, one modification in the first nucleotide gave rise to the identification of a putative binding site in the *penDE* gene promoter region. In summary, two binding sites in *pcbAB*, one in *pcbC* and another in the promoter

region of *penDE* were found. The functionality of the putative PcRFX1 DNA binding sequences in the promoter regions of penicillin biosynthetic gene were verified using gene reporter assays and DNA binding assay (Domínguez-Santos *et al*., 2012).

Pcrfx1 knockdown mutants showed a reduction in IPN and penicillin G production, which was a consequence of a decrease in the expression of the penicillin biosynthetic genes. Therefore, PcRFX1 controls the whole penicillin biosynthetic process (Domínguez-Santos *et al*., 2012).

6.3. Other Transcription Factors

6.3.1. *CCAAT-binding complex AnCF*

The protein AnCF (*A. nidulans* CCAAT-binding factor), formerly known as PENR1 (Penicillin regulator 1), was shown to bind to a CCAAT-binding box (I) in the bidirectional region between the *acvA* and *ipnA* genes (box I located 409 bp upstream of the transcriptional start site of the *acvA* gene) (Bergh *et al*., 1996). It also binds to a CCAAT-binding box (II) in the promoter of the *A. nidulans aatA* gene (box II located about 250 bp upstream of the transcriptional start site of the *aatA* gene) (Litzka *et al*., 1996). Deletion of 4 bp within the box I led to an eight-fold increase of *acvA* expression and simultaneously to a reduction of *ipnA* expression to about 30% (Bergh *et al*., 1996). Moreover, replacement of the CCAAT core sequence by GATCC led to a four-fold reduction in the expression of an *aatA-lacZ* gene fusion (Litzka *et al*., 1996).

The first CCAAT-binding factor was discovered in *Saccharomyces cerevisiae* (named HAP complex) and consists of at least four subunits (Hap2, Hap3 and Hap5 form a heterotrimeric complex that is essential for DNA binding, whereas Hap4 is an acidic protein that acts as the transcriptional activation domain) (McNabb *et al*., 1995). In *A. nidulans*, AnCF is formed by subunits HapB, HapC and HapE, which are all necessary for DNA binding (Steidl *et al*., 1999). Considering that AnCF binds to CCAAT-containing sequences, which are present in the promoter regions of a large number of eukaryotic genes, it has been estimated that AnCF regulates more than 200 genes (Brakhage *et al*., 1999).

Consistent with the data obtained after the deletion of CCAAT binding boxes, the expression of both *ipnA* and *aatA* genes was reduced in the Δ*hapC* mutant. However, in the Δ*hapC* mutant, the expression of *acvA* was just slightly affected (Bergh *et al*., 1996). Thus, it appears

likely that, in addition to AnCF, a repressor protein binds closely to or overlaps the AnCF binding site. This would explain that the AnCF binding site exhibits a repressing effect on *acvA* expression in the wild type strain.

Weidner *et al*. (1997) showed that the lysine biosynthesis gene *lysF* of *A. nidulans* is regulated negatively by AnCF. In addition, AnCF is autoregulated negatively by repression of the *hapB* gene (Steidl *et al*., 2001). These results indicate that AnCF is able to act either as an activator or repressor of transcription in a gene-specific manner.

Several CCAAT binding boxes with high degree of similarity to the AnCF consensus binding sequence are present in the bidirectional promoter *pcbAB-pcbC* of both *P. chrysogenum* and *A. chrysogenum* as well as in the *penDE* promoter of *P. chrysogenum*. Moreover, these DNA fragments are able to bind *A. nidulans* AnCF protein (Bergh *et al*., 1996; Litzka *et al*., 1996), which suggests that homologous complexes may exist in both *P. chrysogenum* and *A. chrysogenum*. This is consistent with the fact that putative homolog subunits of HapB (Pc12g01590), HapC (Pc14g01630) and HapE (Pc12g04670) have been identified in the *P. chrysogenum* genome (van den Berg *et al*., 2008).

6.3.2. *AnBH*1

The AnBH1 protein (PENR2) belongs to the family of basic-region helix-loop-helix (bHLH) transcription factors and has been identified in *A. nidulans*. AnBH1 binds *in vitro* as a homodimer to an asymmetric E-box within the *aatA* promoter, which overlaps with the CCAAT-binding box II for AnCF. AnBH1 acts as a repressor of *aatA* gene expression and therefore, it counteracts the positive action of AnCF (Caruso *et al*., 2002).

One putative homolog of AnBH1 (Pc22g15870) has been identified in the *P. chrysogenum* genome (van den Berg *et al*., 2008).

6.3.3. *PTA1*

Sequential deletion of the *pcbAB* promoter region allowed the identification of three DNA regions, boxes A, B and C, which were involved in the expression of *pcbAB*. When the most distal of these boxes (box A) was incubated with protein extracts from mycelia grown with glucose as carbon source, a specific and defined retardation complex was observed. However, a less specific and defined retardation complex was visible when protein extracts from mycelia grown with lactose were

used. This complex was named PTA1 (Penicillin transcriptional activator 1), which interacted with the thymidines in a palindromic heptanucleotide TTAGTAA and positively regulates the expression of the *pcbAB* gene (Kosalková *et al.*, 2000).

7. CONCLUDING REMARKS

The biosynthesis of fungal beta-lactams is a good model to study the molecular mechanisms that control the expression of genes and the activity of enzymes involved in the production of secondary metabolites in fungi, because there is abundant information accumulated during the last four decades on enzymes, intermediates and regulation of penicillin and cephalosporin. However, as more information becomes available on the molecular genetics of beta-lactam biosynthesis, it is becoming evident that our knowledge of the control of beta-lactam genes is sketchy, with still many gaps to be filled.

7.1. Penicillin and Cephalosporin Biosynthetic Processes

It is generally believed that the enzymes and intermediates of penicillin biosynthesis are definitively known (as described above in this article). However, the exact origin of the non-proteinogenic amino acid α-aminoadipic acid is still obscure. The formation of α-aminoadipic acid has been proposed to proceed through α-ketoadipate (as in the lysine pathway in yeasts), but the transamination reaction converting α-ketoadipate to α-aminoadipic acid has not yet been identified. In addition, it is unknown which is the amino group donor or if it involves the introduction of an ammonium molecule by a NADPH-dependent α-ketoadipate reductase/aminase similar to the well-known glutamate dehydrogenase.

Still more intriguing is the conversion of the L-α-aminoadipic acid side chain of IPN to the D-configuration of PenN in *A. chrysogenum*. As described above, this step involves a novel eukaryote-type two-component system encoded by the *cefD1* and *cefD2* genes (Ullán *et al.*, 2002a; Martín *et al.*, 2004). The CefD1 and CefD2 proteins are bona fide peroxisomal enzymes and their primary role in other eukaryotes is the activation, isomerization and degradation of methyl-branched fatty acids (Martín *et al.*, 2012). The CefD1 and CefD2 enzymes are strictly required for PenN biosynthesis and therefore, for cephalosporin production. The late cephalosporin biosynthetic steps may use, alternatively, adipic acid or α-aminoadipic acid and is not clear whether the CefD1-CefD2 enzymes act on α-methyl fatty acids to provide the α-ketoadipate precursor of

D-α-aminoadipic acid or if these enzymes act directly on IPN changing the L-α-aminoadipic acid to the D-configuration of PenN.

7.2. Vesicle/Peroxisomal Location of Enzymes. Transport Mechanisms

The peroxisomal location of some beta-lactam biosynthetic enzymes is well documented in *P. chrysogenum* (particularly for the phenylacetyl-CoA ligase and the IAT). The compartmentalization of the late biosynthetic enzymes implies transport systems for the entry of IPN into the peroxisomes and for the secretion of benzylpenicillin. An inducible phenylacetic acid transporter of the major facilitator superfamily (MFS) has been recently identified (Fernández-Aguado *et al*., 2013), but the transporter of IPN and the system involved in penicillin secretion remain unknown (Martin *et al*., 2010).

Since antibiotics are toxic for the producer microorganisms, different types of secretion systems must be present to avoid suicide of the producer strains (Martín *et al*., 2005). As it was mentioned along this article, three MFS transporters have been identified in the "early" cephalosporin gene cluster. They are related to the peroxisomal steps required for the conversion of IPN to PenN and for the secretion of hydrophilic beta-lactam intermediates to the culture medium.

7.3. Regulatory Factors

The control of beta-lactam gene expression has been widely studied in *P. chrysogenum*, *A. nidulans* and *A. chrysogenum*. Overexpression of the beta-lactam biosynthetic genes is a straightforward approach for the industrial production of these antibiotics. As reviewed above, several broad spectrum regulatory genes have been identified in *P. chrysogenum*, *A. nidulans* and *A. chrysogenum*. Surprisingly, no specific regulatory genes have been identified within the penicillin biosynthetic cluster or in the genomic region that surrounds the penicillin cluster and undergoes tandem amplification in penicillin high-producing strains (Fierro *et al*., 2006; van den Berg *et al*., 2007). Only a regulatory gene (*cefR*) is located in the "early" cephalosporin gene cluster. CefR modulates the expression of cephalosporin biosynthetic and transporter genes (Teijeira *et al*., 2011).

Most of the best characterized regulatory genes (reviewed above) are common to both non-producing and beta-lactam producing fungi and act by modulating the expression of genes involved in development, differentiation and production of secondary metabolites; some of these

secondary metabolites have a strong implication in development (Palmer *et al*., 2013). Particular attention deserves the LaeA-VeA complex, which clearly modulates penicillin and cephalosporin in *P. chrysogenum*, *A. nidulans* and *A. chrysogenum*. Other proteins interact with these two components of the LaeA-VeA complex, modulating in a positive or negative form the expression of beta-lactam biosynthesis genes (Hoff *et al*., 2010).

As a concluding remark, all the evidence available indicates that the expression of the beta-lactam biosynthetic genes in the three best-known producer fungi is regulated by complex regulatory networks that also affect morphological development. Each of those transcriptional control systems is known to some extent, but an integrative view of the interactions among different regulatory systems is still missing. This integrative study is needed for a system biology understanding of the role of beta-lactams in the life cycle of these fungi.

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BIOTECHNOLOGY

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