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Minireview

Regulation and compartmentalization of β-lactam biosynthesis

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Summary

Penicillins and cephalosporins are β-lactam antibiotics widely used in human medicine. The biosynthesis of these compounds starts by the condensation of the amino acids L-α-aminoadipic acid, L-cysteine and L-valine to form the tripeptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine catalysed by the non-ribosomal peptide 'ACV synthetase'. Subsequently, this tripeptide is cyclized to isopenicillin N that in *Penicillium* is converted to hydrophobic penicillins, e.g. benzylpenicillin. In *Acremonium* and in streptomycetes, isopenicillin N is later isomerized to penicillin N and finally converted to cephalosporin. Expression of genes of the penicillin (*pcbAB*, *pcbC*, *pendDE*) and cephalosporin clusters (*pcbAB*, *pcbC*, *cefD1*, *cefD2*, *cefEF*, *cefG*) is controlled by pleitropic regulators including LaeA, a methylase involved in heterochromatin rearrangement. The enzymes catalysing the last two steps of penicillin biosynthesis (phenylacetyl-CoA ligase and isopenicillin N acyltransferase) are located in microbodies, as shown by immunoelectron microscopy and microbodies proteome analyses. Similarly, the *Acremonium* two-component CefD1–CefD2 epimerization system is also located in microbodies. This compartmentalization implies intracellular transport of isopenicillin N (in the penicillin pathway) or isopenicillin N and penicillin N in the cephalosporin route. Two transporters of the MFS family *cefT* and *cefM* are involved in transport of intermediates and/or secretion of cephalosporins. However, there is no known transporter of benzylpenicillin despite its large production in industrial strains.

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Introduction: the structure of β-lactams

β-Lactams, like many other secondary metabolites, have unusual chemical structures. All β-lactams contain a four-membered β-lactam ring closed by an amide bond (Fig. 1). Penicillins contain a bicyclic 'penam' nucleus formed by fused β-lactam and thiazolidine rings and an acyl side-chain bound to the amino group at C-6. They are produced by a few *Penicillium* and *Aspergillus* species (Aharonowitz *et al.*, 1992). Recently penicillins have been found to be produced by a few other deuteromycetes (Laich *et al.*, 1999; 2002; 2003). A second β-lactam compound, cephalosporin C, produced by the fungi *Acremonium chrysogenum*, *Paecilomyces persicinus* and *Kallichroma tethys* (Kim *et al.*, 2003) and some other deuteromycetes, contains the cephem nucleus (a six-membered dihydrothiazine ring fused to the β-lactam ring) (Fig. 1). Cephalosporin C has a D-α-aminoadipyl side-chain attached to the C-7 amino group, which is identical to that of hydrophilic penicillin N but differs from those of hydrophobic penicillins. Penicillins and cephalosporins are of great clinical interest as inhibitors of peptidoglycan biosynthesis in bacteria.

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

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

Penicillin, cephalosporin and cephamycin biosynthetic pathways

A brief description of the biochemical pathways leading to classical β-lactam antibiotic biosynthesis is made here.

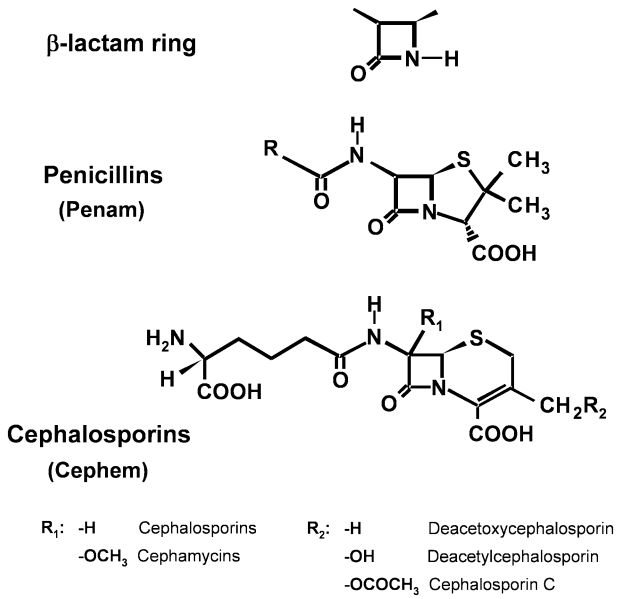

Fig. 1. Chemical structure of the β-lactam ring, the penicillins (penam nucleus) and cephalosporins (cephem nucleus). Different penicillins contain distinct hydrophobic or hydrophilic side-chains (R). The R_1 and R_2 groups of cephalosporins or cephamycins are indicated below the structure of cephalosporin.

More detailed information on the specific steps are given in other review articles (Aharonowitz *et al.*, 1992; Brakhage, 1998; Demain *et al.*, 1998; Martín *et al.*, 1999; Liras and Martín, 2009).

The biosynthesis of the β-lactam compounds proceeds through a series of sequential reactions including the formation of a linear tripeptide intermediate and cyclization of the tripeptide to form the penam nucleus (so-called 'early biosynthetic steps'), conversion of the penam to the cephem nucleus by ring expansion ('intermediate steps') and 'late (decoration) steps' (Fig. 2) involving modifications of the β-lactam nucleus.

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

In fungi, α-aminoadipic acid is an intermediate of the lysine biosynthesis pathway. In addition, lysine is catabolized to α-aminoadipic acid in *Penicillium chrysogenum* (i) by an ω-aminotransferase, encoded by the *oat1* gene, which is induced by lysine (Martín de Valmaseda *et al.*, 2005), and (ii) by a reversal of the lysine biosynthesis pathway catalysed by the enzymes saccharopine dehydrogenase/saccharopine reductase (Naranjo *et al.*, 2001; Martín de Valmaseda *et al.*, 2005).

Two early enzymatic steps are common to all the classical β-lactam producers, resulting in the formation of isopenicillin N (IPN), the first compound in the pathway with antibiotic activity. The first enzyme of the pathway is the ACV synthetase (ACVS), a non-ribosomal peptide synthetase. The ACV synthetases are very large multifunctional proteins (Mr in the order of 420 kDa) encoded by intron-free genes of 11 kb named *pcbAB* (Díez *et al.*, 1990; Gutiérrez *et al.*, 1991) which occur in the fungal and bacterial penicillin and cephalosporin (and cephamycin) clusters (Fig. 3). This enzyme sequentially activates the three substrates with ATP to form aminoacyl-adenylates, binds them to the enzyme as thioesters, epimerizes the L-valine to D-valine, links together the three amino acids to form the peptide L-δ(α-aminoadipyl)-L-cysteinyl-D-valine (hereafter named ACV) and, finally, releases this tripeptide from the enzyme by the action of an internal thioesterase activity. The ACV synthetases have three well conserved domains that activate each of the three amino acids respectively (Zhang and Demain, 1992; Aharonowitz *et al.*, 1993; Martín, 2000a).

The second enzyme in the pathway is the IPN synthase (IPNS, cyclase) encoded by the *pcbC* gene. The IPN synthases are intermolecular dioxygenases that require Fe^{2+}, molecular oxygen and ascorbate. They remove four hydrogen atoms from the ACV tripeptide forming the bicyclic structure (penam nucleus) of IPN. The cyclase of *P. chrysogenum* has been crystallized showing a bread roll-like structure (Roach *et al.*, 1995; 1997).

In addition to the *pcbAB* and *pcbC* genes common to filamentous fungi and bacteria, the producers of hydrophobic penicillins (i.e. *Penicillium*, *Aspergillus*) contain a third gene in the penicillin cluster, named *penDE*, of eukaryotic origin (it contains three introns at difference of *pcbAB* and *pcbC* that are of bacterial origin) which encodes an IPN acyltransferase (IAT). This enzyme hydrolyses the α-aminoadipic side-chain of IPN and introduces an acyl molecule activated as its acyl-CoA derivative to produce hydrophobic penicillins (e.g. benzylpenicillin). This gene is not present in cephalosporin C- or cephamycin-producing microorganisms.

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

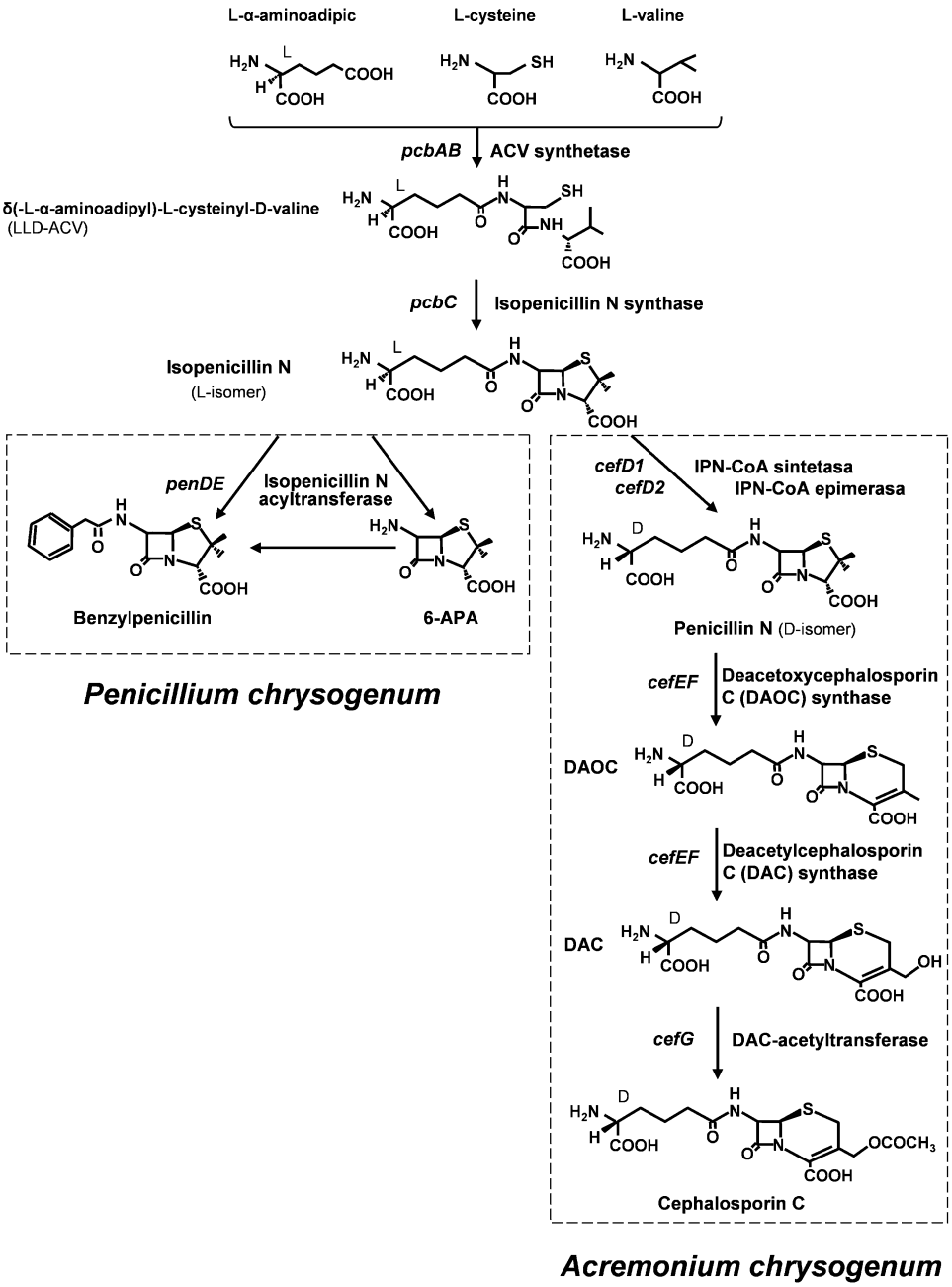

Fig. 2. Biosynthetic pathways of benzylpenicillin and cephalosporin in *Penicillium chrysogenum* (left) and *Acremonium chrysogenum* (right) respectively. The first two steps (upper part of the figure) are common to both pathways. The L- or D-configuration of the α-aminoadipic side-chains in each molecule is indicated by L or D on carbon 1 of this amino acid.

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

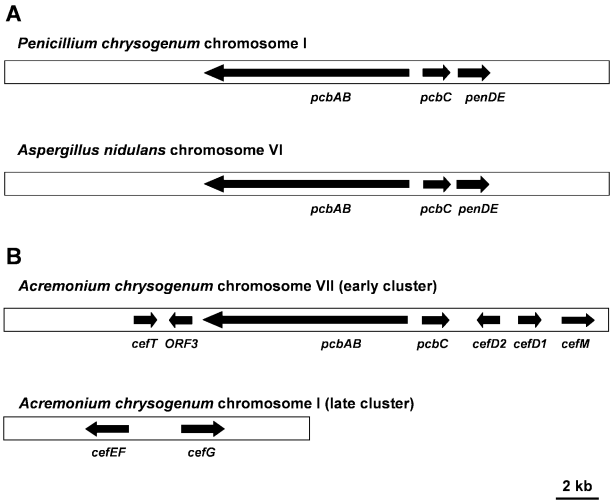

Fig. 3. A. Penicillin gene clusters in *Penicillium chrysogenum* and *Aspergillus nidulans*.
B. Cephalosporin gene clusters in *Acremonium chrysogenum*. The 'early cluster' contains seven genes encoding the biosynthetic steps up to penicillin N and the MFS transporters, and the 'late cluster' contains the two genes converting penicillin N to cephalosporin C (see text).

epimerase activity and accumulation of IPN in the culture (Ullán *et al*., 2002a). The proposed conversion includes three biochemical steps: CefD1 converts IPN into isopenicillinyl N-CoA; then CefD2 isomerizes the compound into penicillinyl N-CoA, which seems to be released from the enzyme by the third enzyme, a thioesterase (see Fig. 2).

The following step in the cephalosporin/cephamycin pathway is the enzymatic expansion of the five-membered thiazolidine ring of penicillin N to a six-membered dihydrothiazine ring. The enzyme responsible of this important conversion is the deacetoxycephalosporin C (DAOC) synthase. This protein is an intermolecular dioxygenase that requires Fe^{2+}, molecular oxygen and α-ketoglutarate to form DAOC and succinic acid. The DAOC synthase does not recognize (or does so very poorly) the isomer IPN, penicillin G or the deacylated 6-aminopenicillanic acid (6-APA) as substrates (Wu *et al*., 2005), although it recognizes adipyl- or glutaryl-6-APA derivatives. The DAOC synthase of *Streptomyces clavuligerus* has been crystallized (Öster *et al*., 2006) and the gene *cefE* has been introduced into *P. chrysogenum*, leading to the production of adipyl-7-aminodeacetoxycephalosporanic acid (ad-7-ADCA) and adipyl-7- aminocephalosporanic acid (ad-7-ACA), (reviewed in Díez *et al*., 1997). The production of adipyl derivatives requires addition of adipic acid to the fermentation. The DAOC synthase from *A. chrysogenum* is also able to catalyse the next step of the pathway, namely the hydroxylation at C-3-forming deacetylcephalosporin C (DAC), whereas in bacteria there is a separate C-3 hydroxylase encoded by *cefF* that performs this reaction.

The final step in cephalosporin C biosynthesis is the conversion of DAC to cephalosporin C by the DAC-acetyltransferase, which uses acetyl-CoA as donor of the acetyl group. This enzyme encoded by the *cefG* gene has an Mr of 49 kDa and is evolutionary similar to O-acetylhomoserine acetyl transferases (Gutiérrez *et al*., 1992; Velasco *et al*., 1999). The *cefG* gene contains two introns and is linked to the *cefEF* gene, but in the opposite orientation (Fig. 3).

Expression of the gene cluster for penicillin biosynthesis

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

Two of the penicillin biosynthetic genes, *pcbAB* and *pcbC*, are transcribed from divergent (bidirectional) promoter regions (Fig. 3). The expression of genes from a region containing two divergent promoters has been associated with reorganization of the chromatin structure that allows facilitated interaction of those promoter regions with the RNA polymerase II and the transcriptional factors (García *et al*., 2004; Ishida *et al*., 2006; Shwab *et al*., 2007).

Biosynthesis of hydrophobic penicillins in *P. chrysogenum* and *Aspergillus nidulans* is affected by several factors through complex regulatory processes (Chang *et al*., 1990; Aharonowitz *et al*., 1992; Feng *et al*., 1994; Martín, 2000b; Brakhage *et al*., 2004). Easily utilizable carbon, nitrogen and phosphorous sources dramatically affect the production of this antibiotic (Martín *et al*., 1999). The transcriptional regulation of the genes responsible for penicillin biosynthesis has been studied in detail. Regulatory elements have been identified (Chu *et al*., 1995; Feng *et al*., 1995; Haas and Marzluf, 1995), such as an enhancer region located in the divergent promoter *pcbAB–pcbC* region of *P. chrysogenum*, which binds a transcriptional activator named PTA1 (Kosalková *et al*., 2000; 2007). However, surprisingly, no penicillin pathway-specific regulatory genes have been found in the amplified region containing the three biosynthetic genes (Fierro *et al*., 2006; van den Berg *et al*., 2007), which indicates that penicillin biosynthesis might be controlled directly by global regulators (e.g. CreA, PacC, Nre) rather than by pathway-specific ones.

One of these regulators is the LaeA protein, which is a nuclear methyltransferase controlling expression of the

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

Organization and expression of the cephalosporin biosynthesis genes

While in *A. nidulans* and *P. chrysogenum* the penicillin biosynthesis genes are found in a single cluster, in *A. chrysogenum* the genes involved in cephalosporin biosynthesis are organized in at least two clusters located on different chromosomes. The *pcbAB*, *pcbC*, *cefD1* and *cefD2* genes are linked in the so-called 'early' cephalosporin cluster, while the 'late' cluster contains the *cefEF* and *cefG* genes (Fig. 3). These genes are involved in the last two steps, which are specific for cephalosporin C biosynthesis (Gutiérrez *et al*., 1992).

Expression of the cephalosporin biosynthesis genes in *A. chrysogenum* is controlled by several global regulators including the carbon catabolite repressor CreA (Jekosch and Kück, 2000), the pH regulator PacC (Schmitt *et al*., 2001; 2004a) and the winged helix transcriptional factor CPCR1 (Schmitt *et al*., 2004b).

Recently, the *velvet* gene of *A. chrysogenum veA* has been shown to control cephalosporin biosynthesis and arthrospore formation (Dreyer *et al*., 2007). *Acremonium chrysogenum* mutants disrupted in the *veA* gene show an 80% reduction in the production of cephalosporin C. Analyses of the transcripts of the cephalosporin C in the mutants indicated that the main effect of the *veA* mutation is on expression of the *cefEF* gene encoding the DAOC synthase/hydroxylase enzyme, which is reduced by 85% in the mutants as compared with the parental strain (Dreyer *et al*., 2007).

The VeA protein carries a putative nuclear localization signal. The *veA* gene is found in all ascomycetes studied so far (Kim *et al*., 2002; Li *et al*., 2006). Moreover, *VeA* of *A. chrysogenum* seems to be also involved in the developmentally dependent hyphal fragmentation to form arthrospores.

In addition to the effect of this global regulator, DL-methionine is a well-known inducer of cephalosporin biosynthesis (Martín and Demain, 2002). Exogenous D- (or DL-) methionine increases the level of mRNAs transcribed from *pcbAB*, *pcbC* and *cefEF* genes (Velasco *et al*., 1994). D-methionine (but not L-methionine) induces IPN synthase and deacetylcephalosporin-C acetyltransferase and also stimulates arthrospore formation (Velasco *et al*., 1994). The effect of methionine may be related to that of the recently described *veA*-encoded protein.

Compartmentalization of intermediates and enzymes in the biosynthesis of penicillins

The penicillin biosynthetic pathway occurs in different cellular compartments (reviewed by van de Kamp *et al*., 1999), which enables the spatial separation of precursors and enzymes, and the regulation and optimization of the processes involved. The distinct subcellular organization of penicillin biosynthesis implies transport of enzymes, precursors, intermediates and products through these compartments (Fig. 4). Therefore, every enzymatic step performed in an organelle has its own optimal environmental conditions.

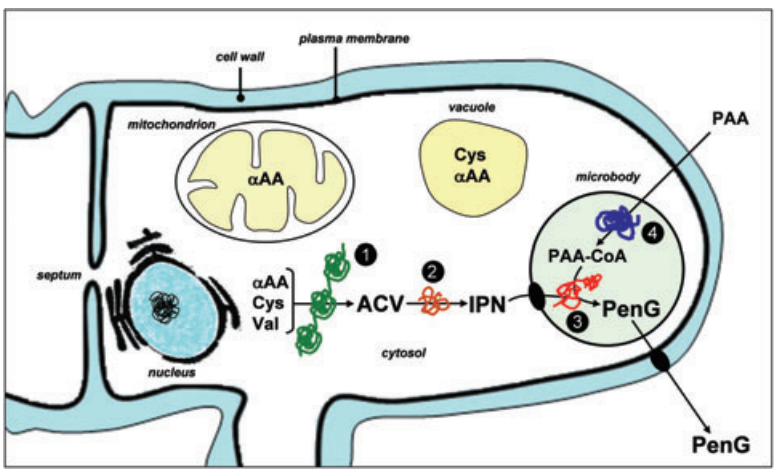

Fig. 4. Compartmentalization of the penicillin biosynthetic pathway. Schematic representation showing the enzymatic steps and organelles involved in the penicillin biosynthetic pathway. (1) ACV synthetase; (2) IPN synthase; (3) IPN acyltransferase; (4) Phenylacetyl-CoA (aryl-CoA) ligase. αAA, L-α-aminoadipic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

As indicated above, the penicillin biosynthetic pathway begins with the non-ribosomal condensation of the amino acids L-α-aminoadipate, L-cysteine and L-valine to form the tripeptide ACV. This step is catalysed by the 420 kDa ACV synthetase (Díez *et al*., 1990; Byford *et al*., 1997; Martín, 2000a). Initial studies on *P. chrysogenum* ACV synthetase associated this protein to membrane structures that were identified as Golgi-like organelles (Kurylowicz *et al*., 1987). Additional cell fractionation experiments located ACV synthetase attached to or inside vacuoles (Müller *et al*., 1991; Lendenfeld *et al*., 1993). However, the pH for the optimal *in vitro* ACV synthase activity (pH = 8.4, which was higher than that of the vacuolar pH), the co-factor requirement and protease sensitivity indicated that this enzyme is a cytosolic enzyme. Cytosolic localization of this enzyme was later confirmed by electron microscopy techniques (Van der Lende *et al*., 2002).

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

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

After the formation of ACV in the cytosol, this compound serves as substrate of the 38 kDa IPN synthase, which cyclizes the tripeptide to form IPN, an intermediate already containing the β-lactam nucleus (Ramos *et al*., 1985; Carr *et al*., 1986; Baldwin *et al*., 1987; 1991b; Roach *et al*., 1997). The IPN synthase behaves as a soluble enzyme (Abraham *et al*., 1981), although its activity in cell-free extracts seems to be stimulated by addition of Triton X-100 or sonication (Sawada *et al*., 1980). This protein colocalizes with ACV synthetase in the cytosol, as it was confirmed by electron microscopy (Müller *et al*., 1991; Van der Lende *et al*., 2002).

Peroxisomal location of the IATs

Unlike the cephalosporin C biosynthetic pathway, where IPN is epimerized to form penicillin N (see below), in the penicillin pathway the L-α-aminoadipyl side-chain of IPN is exchanged for a more hydrophobic side-chain by the acyl-CoA : IPN acyltransferase IAT (Álvarez *et al*., 1987; Barredo *et al*., 1989; Tobin *et al*., 1990; Whiteman *et al*., 1990).

The IAT is expressed as a 40 kDa precursor protein (proIAT) which undergoes an autocatalytic self-processing between residues Gly-102–Cys-103 in *P. chrysogenum*, thus constituting a heterodimer of two subunits; α (11 kDa, corresponding to the N-terminal fragment) and β (29 kDa, corresponding to the C-terminal region) (Barredo *et al*., 1989; Veenstra *et al*., 1989; Tobin *et al*., 1990; 1993; Whiteman *et al*., 1990). *In vivo*, IAT catalyses six different reactions (Álvarez *et al*., 1993) related to the cleavage of IPN (releasing 6-APA) and the replacement of the α-aminoadipyl side-chain of this intermediate by aromatic acids activated in the form of aryl-CoA derivatives (Álvarez *et al*., 1993). As a consequence of the side-chain substitution, hydrophobic penicillins (benzylpenicillin or phenoxyacetylpenicillin), which show a significant increase of antimicrobial activity as compared with that of IPN, are formed.

Isopenicillin N acyltransferases from *P. chrysogenum* and *A. nidulans* contain a functional peroxisomal targeting sequence PTS1 at the C-terminal end (ARL and ANI respectively). Electron microscopy immunodetection has shown that the acyltransferase of *P. chrysogenum* is located inside peroxisomes (microbodies) (Müller *et al*., 1991; 1992; 1995; García-Estrada *et al*., 2008b). In addition, transport of IAT inside the peroxisomal matrix is not dependent on the processing state of the protein, since

the unprocessed proIAT variant IAT^{C103S} is correctly targeted to peroxisomes, although it is not active (García-Estrada *et al.*, 2008b).

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

The peroxisomal colocalization of IAT and aryl CoA ligases indicates that the last two enzymes of the penicillin pathway form a peroxisomal functional complex, pointing to this organelle as a key compartment of the penicillin biosynthetic pathway. Peroxisomes (microbodies) are organelles that show a diameter of 200–800 nm and are surrounded by a single membrane (Müller *et al.*, 1991). They are involved in a variety of metabolic pathways like β-oxidation of fatty acids (Kionka and Kunau, 1985; Thieringer and Kunau, 1991a,b; Valenciano *et al.*, 1996; 1998; Kiel *et al.*, 2009) or karyogamy (Berteaux-Lecellier *et al.*, 1995). *Penicillium chrysogenum* microbody luminal pH has been estimated to be slightly alkaline, in the range of 7.0–7.5 (Van der Lende *et al.*, 2002), a pH range optimal for the aryl-CoA ligase and IAT (Álvarez *et al.*, 1987; 1993; Gledhill *et al.*, 1997).

The importance of microbodies in penicillin biosynthesis has been highlighted through the alteration of the number and shape of these organelles as a result of the overexpression of the Pc-Pex11p protein (a peroxin that is involved in microbody abundance): overexpression of this protein led to an increase in the production of penicillin. This positive effect is likely to be related to an increased transport of penicillin and/or its precursors across the microbody membrane (Kiel *et al.*, 2005), which evidences that transport events through the membrane of this organelle are very important. Because of the hydrophilic nature of IPN, the uptake of this precursor from cytosol is likely to occur through specific carriers. An active IPN transport system must be present in the peroxisomal membrane to assure an adequate pool of IPN inside microbodies. The 6-APA is a penicillin precursor that is originally formed into the microbody by the IAT; it has been shown that this compound is taken up very efficiently through the plasma and peroxisomal membranes, probably by passive diffusion (García-Estrada *et al.*, 2007). The difference in the transport efficiency between IPN and 6-APA may be due to differences in the polarity between these two compounds. The hydrophobic penicillins formed by the IAT might be released from the microbody through simple diffusion, but this hypothesis is very unlikely because the accumulated extracellular level is much higher than the intracellular one.

Are there active transport systems for large-scale secretion of penicillin or alternative vesicle transport?

Penicillin is secreted in very large quantities by the overproducing strains. In fed-batch cultures these industrial strains reach a biomass of about 25–30 g l^{-1} and they produce more than 40–50 g l^{-1} of penicillin. Since the last steps of penicillin biosynthesis (namely the activation of phenylacetic acid to phenylacetyl-CoA and the transacylation reaction by the IAT) are located in peroxisomes, the final product has to be transported out of the peroxisome, first into the cytoplasm and then into the culture medium.

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

After sequencing the *P. chrysogenum* genome, two approaches have been used to investigate if any of those ABC transporters has a role in penicillin secretion, namely (i) transcriptional upregulation following phenylacetic acid addition to the culture and (ii) overexpression in an industrial penicillin-producing strain when compared with the wild-type strain (van den Berg *et al.*, 2008).

Transcriptional studies following phenylacetic acid addition revealed two groups of MDR genes that were upregulated. However, many of those genes are probably related to phenylacetate transport and catabolism, which is known to affect penicillin biosynthesis (Rodríguez-Sáiz *et al*., 2001), rather than with penicillin secretion.

The second approach, namely comparison of expression of MDR genes in a laboratory strain and in the industrial producer, revealed that some of the ABC transporters had increased expression in the industrial strain (van den Berg *et al*., 2008). However, a detailed functional analysis is required to confirm if the disruption or amplification of these genes affect penicillin secretion.

A gene encoding an ABC transporter of *A. nidulans* putatively involved in penicillin secretion was reported by Andrade and colleagues (2000). However, a detailed analysis of the reported evidence suggests that this gene is likely involved in nutrient transport, affecting directly or indirectly penicillin biosynthesis.

The pexophagy phenomenon in filamentous fungi: a putative secretion through vesicles

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

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

Compartmentalization and internal transport systems in the biosynthesis of cephalosporins

The cephalosporin pathway is an example of a complex secondary metabolite pathway that appears to involve several internal transporters. Only two fungal carriers have been described to be directly or indirectly involved in the β-lactam antibiotic secretion: (i) the multidrug ABC transporter encoded by the *atr*D gene of *A. nidulans* (Andrade *et al*., 2000) that affects penicillin biosynthesis, and (ii) the *cefT* gene that is involved in transport of β-lactam compounds in *A. chrysogenum* (Ullán *et al*., 2002b). The *cefT* gene is located in the early cephalosporin gene cluster and encodes a multidrug efflux pump protein belonging to the MFS class. The CefT protein is a membrane protein that has 12 transmembrane spanners (TMS) and contains all characteristic motifs of the drug: H+ antiporter 12-TMS group of the major facilitator superfamily. Inactivation of the *cefT* does not reduce cephalosporin C biosynthesis, indicating that CefT is not the main transporter for cephalosporin C biosynthesis. However, when *cefT* was overexpresed in *A. chrysogenum*, it resulted in a twofold increase in total cephalosporin production (Ullán *et al*., 2002b).

Recently, heterologous expression of the *cefT* gene in the cephalosporin producer *P. chrysogenum* TA98, a strain carrying the cephalosporin biosynthesis genes (Ullán *et al*., 2007), revealed that the CefT protein is functional in *P. chrysogenum* acting as a hydrophilic β-lactam transporter involved in the secretion of hydrophilic β-lactams containing the α-aminoadipic acid side-chain (Ullán *et al*., 2008). *Penicillium chrysogenum* TA98 transformants showed an increase in the secretion of DAC and hydrophilic penicillins (IPN and PenN). In addition, when *cefT* was expressed in the parental *P. chrysogenum* Wis 54-1255, it resulted in an increased secretion of IPN and a drastic reduction of benzylpenicillin production. Similar results were obtained by Nijland and colleagues (2008) with the introduction of the *cefT* gene of *A. chrysogenum* in an adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (ad7-ACCCA)-producing *P. chrysogenum* strain. Expression of *cefT* in this industrial strain results in almost a twofold increase in cephalosporin (ad-7-ACCCA) production.

It is noteworthy that Southern and Northern analyses showed the presence of an endogenous *P. chrysogenum* gene similar to *cefT*. The exact role of the *cefT* analogue is not yet known.

In *A. chrysogenum* all publications reported that the enzymes ACV synthetase (Baldwin *et al*., 1990), IPN synthase (Samsom *et al*., 1985), DAOC synthase-hydroxylase (Scheidegger *et al*., 1984; Dotzlaf and Yeh, 1987; Samsom *et al*., 1987) and DAC acetyltransferase (Gutiérrez *et al*., 1992; Matsuda *et al*., 1992; Velasco *et al*., 1999) of the cephalosporin biosynthesis pathway have a cytosolic location (reviewed in van de Kamp *et al*., 1999; Evers *et al*., 2004).

The central step of the cephalosporin biosynthetic pathway is the conversion of isopenicillin N to penicillin N that is catalysed by the isopenicillinyl N-CoA synthetase and isopenicillin N-CoA epimerase proteins encoded by the *cefD1* and *cefD2* genes respectively (Ullán *et al*.,

2002a). Bioinformatic analysis of the CefD1 and CefD2 proteins revealed the presence of putative peroxisomal targeting signals (PTSs) characteristic of peroxisomal matrix proteins (Reumann, 2004). Targeting and import of the peroxisomal matrix proteins depends on the PTSs present in each protein. Peroxisomal targeting signals fall into two categories: PTS1 and PTS2 (Hettema *et al.*, 1999). The PTS1 signal is located at the C-terminal end of proteins (consensus sequence: SKL or its derivative S/C/A-K/R/H-L) (Purdue and Lazarow, 2001) whereas the PTS2 signal is located at the N-terminal region of proteins with a consensus sequence [(R/L)(L/V/I)-X5-(H/Q)(L/A)] (Rachubinski and Subramani, 1995; Reumann, 2004). CefD1 protein contains a putative PTS1 whereas CefD2 contains putative PST1 and PST2 targeting sequences. Moreover, the optimum pH for the *in vitro* conversion of IPN into PenN in *A. chrysogenum* cell-free extracts was 7.0 (Baldwin *et al.*, 1981; Jayatilake *et al.*, 1981; Lübbe *et al.*, 1986). This optimum pH for the PenN synthesis is the same that was estimated for the peroxisomal lumen (van der Lende *et al.*, 2002). Recently, the CefD1 and CefD2 homologue proteins of *P. chrysogenum* have been found in the peroxisome matrix (Kiel *et al.*, 2009). Therefore, the epimerization step seems to take place in peroxisomes and requires specific transport steps of precursors and intermediates across the peroxisomal membrane.

Our research group has identified some of these key metabolite transporters. Recently, we found the *cefM* gene (Teijeira *et al.*, 2009) that is located in the early cephalosporin cluster (chromosome VII) downstream from *cefD1* and encodes another efflux pump protein belonging to the MFS class of membrane proteins specifically to Family 3 (drug efflux proteins), the same family of the CefT protein (Ullán *et al.*, 2002b). Bioinformatic analysis of the CefM protein sequence revealed the presence of a Pex19p-binding domain (http://www.peroxisomedb.org/) located between amino acids 212–221. This domain is characteristic of proteins that are recruited by the Pex19 protein to be incorporated in the peroxisomal membrane (Rottensteiner *et al.*, 2004). Targeted inactivation of *cefM* gene was accomplished by the two marker strategy (Liu *et al.*, 2001; Ullán *et al.*, 2002a,b). The disrupted mutant (Teijeira *et al.*, 2009) showed a drastic reduction (more than 90%) in the extracellular penicillin N and cephalosporins production, and accumulated more intracellular penicillin N than the parental strain. Complementation *in trans* with the *cefM* gene cloned in integrative vectors restored the intracellular penicillin N levels, and the secreted levels of the cephalosporin intermediates and cephalosporin C.

Localization of the CefM transporter

cefM–GFP fusions were used to study the *in vivo* localization of the CefM protein. The confocal microscopy analysis revealed a microbody localization of this protein (Teijeira *et al.*, 2009). Taken together, these results suggest that the CefM protein is involved in the penicillin N secretion from the microbody lumen to the cytosol in *A. chrysogenum* and reveals a compartmentalization of the cephalosporin C biosynthetic pathway.

Based on these results, we propose a hypothetical model of compartmentalization in the biosynthesis of cephalosporins (Fig. 5). This compartmentalization model starts with the ACV and IPN synthesis in the cytosol by cytoplasmic ACV synthetase and IPN synthase (Evers *et al.*, 2004). Later the IPN is transported to the peroxisome where it is converted in PenN by the two-protein (CefD1–CefD2) epimerization system (Ullán *et al.*, 2002a). Afterwards, the penicillin N is transported to the cytosol, by means of the CefM carrier (Teijeira *et al.*, 2009), where the two last enzymes of the cephalosporin pathway synthesize cephalosporin C (van de Kamp *et al.*, 1999; Evers *et al.*, 2004). On the other hand, the secretion of intermediates of the cephalosporin C biosynthetic

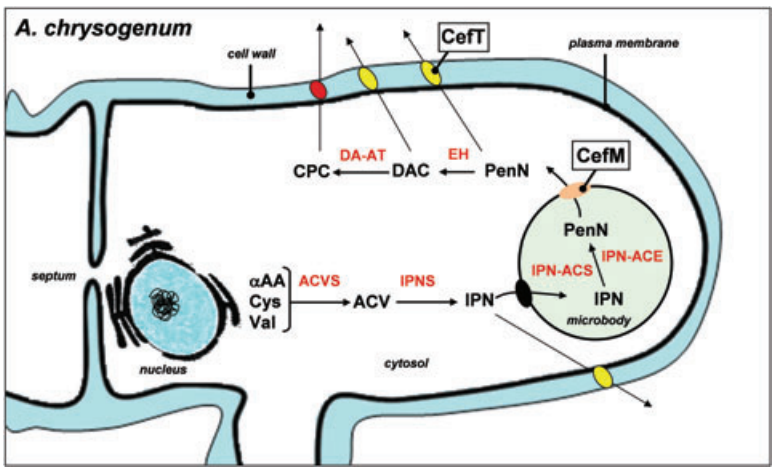

Fig. 5. Proposed model describing the compartmentalization of the cephalosporin C biosynthetic pathway in *A. chrysogenum* showing the localization of the CefT and CefM transporters. ACVS, δ-L-α-aminoadipyl-L-cysteinyl-D-valine synthetase; ACV, L-δ(α-aminoadipyl)-L-cysteinyl-D-valine; IPNS isopenicillin N synthase; IPN, isopenicillin N; IPN-ACS isopenicillin N-CoA synthetase; IPN-ACE; isopenicillin N-CoA epimerase; PenN, penicillin N; EH, deacetoxycephalosporin synthase (expandase/hydroxylase); DAC, deacetylcephalosporin C; DAC-AT, deacetylcephalosporin acetyltransferase; CPC, cephalosporin C.

pathway in *A. chrysogenum* appears to be performed by the CefT trasporter (Fig. 5). Although the CefT protein has not been yet localized in *A. chrysogenum* using fluorescent dyes, heterologous expression of the CefT–GFP fusion in *P. chrysogenum* indicates that it is located in the plasma membrane (Nijland *et al*., 2008).

Summary and future outlook

In summary, peroxisomes play an important role in β-lactams synthesis as several of the key biosynthetic steps take place in this organelle. The biosynthetic pathway of cephalosporin C in *A. chrysogenum* is compartmentalized and takes place in the cytosol and the peroxisomes, like the biosynthetic pathway of penicillin in *P. chrysogenum*. The CefM protein seems to act as a transporter between the peroxisomal lumen and the cytosol to provide the penicillin N molecules for the subsequent expandase/hydroxylase and acetyl-CoA : DAC acetyltransferase reactions. More detailed experimental work is still required to confirm the role of each of these transporters, and how to take advantage of the genetic modification of these genes to increase transport of the intermediates and secretion of the final product. Significant progress has been recently made on the proteomics of peroxisome biosynthesis (Kiel *et al*., 2009). The 'omics' studies will allow a global view of the role of organelles in fungal physiology and secondary metabolite biosynthesis. Our present knowledge on transport of biosynthetic intermediates and secretion of the final products is only sketchy and further progress will depend upon scientific advance on the knowledge of secondary metabolite transporters.

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References

Abraham, E.P., Huddlestone, J.A., Jayatilake, G.S., O'Sullivan, J., and White, R.L. (1981) Conversion of δ(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N in cell-free extracts of *Cephalosporium acremonium*. In *Recent Advances in the Chemistry of β-Lactam Antibiotics*. Gregory, G.I. (ed.). London: Royal Society of Chemistry, pp. 125–134.

Aharonowitz, Y., Cohen, G., and Martín, J.F. (1992) Penicillin and cephalosporin biosynthetic genes: structure, regulation, and evolution. *Annu Rev Microbiol* **46:** 461–495.

Aharonowitz, Y., Bergmeyer, J., Cantoral, J.M., Cohen, G., Demain, A.L., Fink, U., *et al.* (1993) Delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase, the multienzyme integrating the four primary reactions in beta-lactam biosynthesis, as a model peptide synthetase. *Biotechnology* **11:** 807–810.

Álvarez, E., Cantoral, J.M., Barredo, J.L., Díez, B., and Martín, J.F. (1987) Purification to homogeneity and characterization of the acyl-CoA: 6-APA acyltransferase of *Penicillium chrysogenum*. *Antimicrob Agents Chemother* **31:** 1675–1682.

Álvarez, E., Meesschaert, B., Montenegro, E., Gutiérrez, S., Díez, B., Barredo, J.L., and Martín, J.F. (1993) The isopenicillin N acyltransferase of *Penicillium chrysogenum* has isopenicillin N amidohydrolase, 6-aminopenicillanic acid acyltransferase and penicillin amidase activities, all of which are encoded by the single *penDE* gene. *Eur J Biochem* **215:** 323–332.

Andrade, A.C., Van Nistelrooy, J.G., Peery, R.B., Skatrud, P.L., and De Waard, M.A. (2000) The role of ABC transporters from *Aspergillus nidulans* in protection against cytotoxic agents and in antibiotic production. *Mol Gen Genet* **263:** 966–977.

Baldwin, J.E., Keeping, J.W., Singh, P.D., and Vallejo, C.A. (1981) Cell free conversion of isopenicillin N into deacetoxycephalosporin C by *Cephalosporium acremonium* mutant M-0198. *Biochem J* **194:** 649–651.

Baldwin, J.E., Killin, S.J., Pratt, A.J., Sutherland, J.D., Turner, N.J., Crabbe, J.C., *et al.* (1987) Purification and characterization of cloned isopenicillin N synthetase. *J Antibiot* **40:** 652–659.

Baldwin, J.E., Bird, J.W., Field, R.A., O'Callaghan, N.M., and Schofield, C.J. (1990) Isolation and partial characterisation of ACV synthetase from *Cephalosporium acremonium* and *Streptomyces clavuligerus*. *J Antibiot* **43:** 1055–1057.

Baldwin, J.E., Bird, J.W., Field, R.A., O'Callaghan, N.M., Schofield, C.J., and Willis, A.C. (1991a) Isolation and partial purification of ACV synthetase from *Cephalosporium acremonium* and *Streptomyces clavuligerus*: evidence for the presence of phosphopantothenate in ACV synthetase. *J Antibiot* **44:** 241–248.

Baldwin, J.E., Lynch, G.P., and Schofield, C.J. (1991b) Isopenicillin N synthase: a new mode of reactivity. *J Chem Soc Chem Commun* **1:** 736–738.

Bañuelos, O., Casqueiro, J., Fierro, F., Hijarrubia, M.J., Gutiérrez, S., and Martín, J.F. (1999) Characterization and lysine control of expression of the *lys1* gene of *Penicillium chrysogenum* encoding homocitrate synthase. *Gene* **226:** 51–59.

Bañuelos, O., Casqueiro, J., Gutiérrez, S., and Martín, J.F. (2000) Overexpression of the *lys1* gene in *Penicillium chrysogenum*: homocitrate synthase levels, alpha-aminoadipic acid pool and penicillin production. *Appl Microbiol Biotechnol* **54:** 69–77.

Barredo, J.L., van Solingen, P., Díez, B., Álvarez, E., Cantoral, J.M., Kattevilder, A., *et al.* (1989) Cloning and characterization of the acyl-coenzyme A: 6-aminopenicillanic-acid-acyltransferase gene of *Penicillium chrysogenum*. *Gene* **83:** 291–300.

van den Berg, M.A. (2001) Method for enhancing secretion of beta-lactam transport. PCT patent WO 2001/32904.

van den Berg, M.A., Westerlaken, I., Leeflang, C., Kerkman, R., and Bovenberg, R.A.L. (2007) Functional characterization of the penicillin biosynthetic gene cluster of *Penicillium chrysogenum* Wisconsin 54-1255. *Fungal Genet Biol* **44:** 830–844.

Berteaux-Lecellier, V., Picard, M., Thompson-Coffe, C., Zickler, D., Panvier-Adoutte, A., and Simonet, J.M. (1995) A nonmammalian homolog of the PAF1 gene (Zellweger syndrome) discovered as a gene involved in caryogamy in the fungus *Podospora anserina*. *Cell* **81:** 1043–1051.

Bhattacharjee, J.K. (1985) Alpha-aminoadipate pathway for the biosynthesis of lysine in lower eukaryotes. *Crit Rev Microbiol* **12:** 131–151.

Bok, J.W., and Keller, N.P. (2004) LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot Cell* **3:** 527–535.

Bok, J.W., Balajee, S.A., Marr, K.A., Andes, D., Nielsen, K.F., Frisvad, J.C., and Keller, N.P. (2005) LaeA, a regulator of morphogenetic fungal virulence factors. *Eukaryot Cell* **4:** 1574–1582.

Bok, J.W., Noordermeer, D., Kale, S.P., and Keller, N.P. (2006) Secondary metabolic gene cluster silencing in *Aspergillus nidulans*. *Mol Microbiol* **61:** 1636–1645.

Brakhage, A.A. (1998) Molecular regulation of beta-lactam biosynthesis in filamentous fungi. *Microbiol Mol Biol Rev* **62:** 547–585.

Brakhage, A.A., Spröte, P., Al-Abdallah, Q., Gehrke, A., Plattner, H., and Tüncher, A. (2004) Regulation of penicillin biosynthesis in filamentous fungi. *Adv Biochem Eng Biotechnol* **88:** 45–90.

Byford, M.F., Baldwin, J.E., Shiau, C.Y., and Schofield, C.J. (1997) The mechanisms of ACV synthetase. *Chem Rev* **97:** 2631–2650.

Carr, L.G., Skatrud, P.L., Scheetz, M.E., Queener, S.W., and Ingolia, T.D. (1986) Cloning and expression of the isopenicillin N synthetase gene from *Penicillium chrysogenum*. *Gene* **48:** 257–266.

Casqueiro, J., Gutiérrez, S., Bañuelos, O., Fierro, F., Velasco, J., and Martín, J.F. (1998) Characterization of the *lys2* gene of *Penicillium chrysogenum* encoding alpha-aminoadipic acid reductase. *Mol Gen Genet* **259:** 549–556.

Chang, L.T., McGrory, E.L., and Elander, R.P. (1990) Penicillin production by glucose-derepressed mutants of *Penicillium chrysogenum*. *J Ind Microbiol* **6:** 165–169.

Chu, Y.W., Renno, D., and Saunders, G. (1995) Detection of a protein which binds specifically to the upstream region of the *pcbAB* gene in *Penicillium chrysogenum*. *Curr Genet* **28:** 184–186.

Coque, J.J.R., Liras, P., Láiz, L., and Martín, J.F. (1991) A gene encoding lysine 6-aminotransferase, which forms the β-lactam precursor α-aminoadipic acid, is located in the cluster of cephamycin biosynthetic genes in *Nocardia lactamdurans*. *J Bacteriol* **173:** 6258–6264.

Demain, A.L., Martín, J.F., and Elander, R.P. (1998) Penicillin biochemistry and genetics. In *Penicillin, a Paradigm for Biotechnology*. Mateles, R.I. (ed.). Chicago, IL, USA: Candida, pp. 93–113.

Díez, B., Gutiérrez, S., Barredo, J.L., van Solingen, P., van der Voort, L.H.M., and Martín, J.F. (1990) The cluster of penicillin biosynthetic genes. *J Biol Chem* **265:** 16358–16365.

Díez, B., Mellado, E., Rodríguez, M., Fouces, R., and Barredo, J.L. (1997) Recombinant microorganisms for industrial production of antibiotics. *Biotechnol Bioeng* **55:** 216–226.

Dotzlaf, J.E., and Yeh, W.K. (1987) Copurification and characterization of deacetoxycephalosporin C synthetase/hydroxylase from *Cephalosporium acremonium*. *J Bacteriol* **169:** 1611–1618.

Dreyer, J., Eichhorn, H., Friedlin, E., Kürnsteiner, H., and Kück, U. (2007) A homologue of the *Aspergillus velvet* gene regulates both cephalosporin C biosynthesis and hyphal fragmentation in *Acremonium chrysogenum*. *Appl Environ Microbiol* **73:** 3412–3422.

Ehmann, D.E., Gehring, A.M., and Walsh, C.T. (1999) Lysine biosynthesis in *Saccharomyces cerevisiae*: mechanism of alpha-aminoadipate reductase (Lys2) involves posttranslational phosphopantetheinylation by Lys5. *Biochemistry* **38:** 6171–6177.

Evers, M.E., Trip, H., van den Berg, M.A., Bovenberg, R.A.L., and Driessen, A.J. (2004) Compartmentalization and transport in beta-lactam antibiotics biosynthesis. *Adv Biochem Eng Biotechnol* **88:** 111–135.

Feng, B., Friedlin, E., and Marzluf, G.A. (1994) A reporter gene analysis of penicillin biosynthesis gene expression in *Penicillium chrysogenum* and its regulation by nitrogen and glucose catabolite repression. *Appl Environ Microbiol* **60:** 4432–4439.

Feng, B., Friedlin, E., and Marzluf, G.A. (1995) Nuclear DNA-binding proteins which recognize the intergenic control region of penicillin biosynthetic genes. *Curr Genet* **27:** 351–358.

Fierro, F., Barredo, J.L., Díez, B., Gutiérrez, S., Fernández, F.J., and Martín, J.F. (1995) The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences. *Proc Natl Acad Sci USA* **92:** 6200–6204.

Fierro, F., García-Estrada, C., Castillo, N.I., Rodríguez, R., Velasco-Conde, T., and Martín, J.F. (2006) Transcriptional and bioinformatic analysis of the 56.8 kb DNA region amplified in tandem repeats containing the penicillin gene cluster in *Penicillium chrysogenum*. *Fungal Genet Biol* **43:** 618–629.

de la Fuente, J.L., Rumbero, A., Martín, J.A., and Liras, P. (1997) Δ-1-Piperideine-6-carboxylate dehydrogenase, a new enzyme that forms α-aminoadipate in *Streptomyces clavuligerus* and other cephamycin C-producing actinomycetes. *Biochem J* **327:** 59–64.

García, I., Gonzalez, R., Gómez, D., and Scazzocchio, C. (2004) Chromatin rearrangements in the *prnD–prnB* bidirectional promoter: dependence on transcription factors. *Eukaryot Cell* **3:** 144–156.

García-Estrada, C., Vaca, I., Lamas-Maceiras, M., and Martín, J.F. (2007) *In vivo* transport of the intermediates of the penicillin biosynthetic pathway in tailored strains of *Penicillium chrysogenum*. *Appl Microbiol Biotechnol* **76:** 169–182.

García-Estrada, C., Ullán, R.V., Velasco-Conde, T., Godio, R.P., Teijeira, F., Vaca, I., *et al.* (2008a) Post-translational enzyme modification by the phosphopantetheinyl transferase is required for lysine and penicillin biosynthesis but

not for roquefortine or fatty acid formation in *Penicillium chrysogenum*. *Biochem J* **415:** 317–324.

García-Estrada, C., Vaca, I., Fierro, F., Sjollema, K., Veenhuis, M., and Martín, J.F. (2008b) The unprocessed preprotein form IAT^{C103S} of the isopenicillin N acyltransferase is transported inside peroxisomes and regulates its self-processing. *Fungal Genet Biol* **45:** 1043–1052.

Gledhill, L., Greaves, P.A., and Griffin, J.P. (1997) Phenylacetyl-CoA ligase from *Penicillium chrysogenum*. Patent IPN WO97/02349, Smithkline Beecham UK.

Guo, S., Evans, S.A., Wilkes, M.B., and Bhattacharjee, J.K. (2001) Novel posttranslational activation of the LYS2-encoded alpha-aminoadipate reductase for biosynthesis of lysine and site-directed mutational analysis of conserved amino acid residues in the activation domain of *Candida albicans*. *J Bacteriol* **183:** 7120–7125.

Gutiérrez, S., Díez, B., Montenegro, E., and Martín, J.F. (1991) Characterization of the *Cephalosporium acremonium pcbAB* gene encoding α-aminoadiyl-cysteinyl-valine synthetase, a large multidomain peptide synthetase: linkage to the *pcbC* gene as a cluster of early cephalosporin-biosynthetic genes and evidence of multiple functional domains. *J Bacteriol* **173:** 2354–2365.

Gutiérrez, S., Velasco, J., Fernández, F.J., and Martín, J.F. (1992) The *cefG* gene of *Cephalosporium acremonium* is linked to the *cefEF* gene and encodes a deacetylcephalosporin C acetyltransferase closely related to homoserine O-acetyltransferase. *J Bacteriol* **174:** 3056–3064.

Haas, H., and Marzluf, G.A. (1995) NRE, the major nitrogen regulatory protein of *Penicillium chrysogenum*, binds specifically to elements in the intergenic promoter regions of nitrate assimilation and penicillin biosynthetic gene clusters. *Curr Genet* **28:** 177–183.

Hettema, E.H., Distel, B., and Tabak, H.F. (1999) Import of proteins into peroxisomes. *Biochim Biophys Acta* **1451:** 17–34.

Ishida, C., Aranda, C., Valenzuela, L., Riego, L., Deluna, A., Recillas-Targa, F., *et al.* (2006) The UGA3-GLT1 intergenic region constitutes a promoter whose bidirectional nature is determined by chromatin organization in *Saccharomyces cerevisiae*. *Mol Microbiol* **59:** 1790–1806.

Jaklitsch, W.M., and Kubicek, C.P. (1990) Homocitrate synthase from *Penicillium chrysogenum*. Localization, purification of the cytosolic isoenzyme, and sensitivity to lysine. *Biochem J* **269:** 247–253.

Jayatilake, S., Huddleston, J.A., and Abraham, E.P. (1981) Conversion of isopenicillin N into penicillin N in cell-free extracts of *Cephalosporium acremonium*. *Biochem J* **194:** 645–647.

Jekosch, K., and Kück, U. (2000) Loss of glucose repression in an *Acremonium chrysogenum* beta-lactam producer strain and its restoration by multiple copies of the *cre1* gene. *Appl Microbiol Biotechnol* **54:** 556–563.

van de Kamp, M., Driessen, A.J., and Konings, W.N. (1999) Compartmentalization and transport in beta-lactam antibiotic biosynthesis by filamentous fungi. *Antonie Van Leeuwenhoek* **75:** 41–78.

Keller, N.P., Turner, G., and Bennett, J.W. (2005) Fungal secondary metabolism – from biochemistry to genomics. *Nat Rev Microbiol* **3:** 937–947.

Keszenman-Pereyra, D., Lawrence, S., Twfieg, M.E., Price, J., and Turner, G. (2003) The *npgA*/*cfwA* gene encodes a putative 4′-phosphopantetheinyl transferase which is essential for penicillin biosynthesis in *Aspergillus nidulans*. *Curr Genet* **43:** 186–190.

Kiel, J.A, and van der Klei, I.J. (2009) Proteins involved in microbody biogenesis and degradation in *Aspergillus nidulans*. *Fungal Genet Biol* **46:** S62–71.

Kiel, J.A., van der Klei, I.J., van den Berg, M.A., Bovenberg, R.A.L., and Veenhuis, M. (2005) Overproduction of a single protein, Pc-Pex11p, results in 2-fold enhanced penicillin production by *Penicillium chrysogenum*. *Fungal Genet Biol* **42:** 154–164.

Kiel, J.A., van den Berg, M.A., Fusetti, F., Poolman, B., Bovenberg, R.A.L., Veenhuis, M., and van der Klei, I.J. (2009) Matching the proteome to the genome: the microbody of penicillin-producing *Penicillium chrysogenum* cells. *Funct Integr Genomics* **9:** 167–184.

Kikuma, T., Ohneda, M., Arioka, M., and Kitamoto, K. (2006) Functional analysis of the ATG8 homologue Aoatg8 and role of autophagy in differentiation and germination in *Aspergillus oryzae*. *Eukaryot Cell* **5:** 1328–1336.

Kim, C.F., Lee, S.K., Price, J., Jack, R.W., Turner, G., and Kong, R.Y. (2003) Cloning and expression analysis of the *pcbAB–pcbC* beta-lactam genes in the marine fungus *Kallichroma tethys*. *Appl Environ Microbiol* **69:** 1308–1314.

Kim, H., Han, K., Kim, K., Han, D., Jahng, K., and Chae, K. (2002) The *veA* gene activates sexual development in *Aspergillus nidulans*. *Fungal Genet Biol* **37:** 72–80.

Kionka, C., and Kunau, W.H. (1985) Inducible beta-oxidation pathway in *Neurospora crassa*. *J Bacteriol* **161:** 153–157.

Klionsky, D.J., Herman, P.K., and Emr, S.D. (1990) The fungal vacuole: composition, function, and biogenesis. *Microbiol Rev* **54:** 266–292.

Klionsky, D.J., Cregg, J.M., Dunn, W.A., Jr, Emr, S.D., Sakai, Y., Sandoval, I.V., *et al.* (2003) A unified nomenclature for yeast autophagy-related genes. *Dev Cell* **5:** 539–545.

Kosalková, K., Marcos, A.T., Fierro, F., Hernando-Rico, V., Gutiérrez, S., and Martín, J.F. (2000) A novel heptameric sequence (TTAGTAA) is the binding site for a protein required for high level expression of *pcbAB*, the first gene of the penicillin biosynthesis in *Penicillium chrysogenum*. *J Biol Chem* **275:** 2423–2430.

Kosalková, K., Rodríguez-Sáiz, M., Barredo, J.L., and Martín, J.F. (2007) Binding of the PTA1 transcriptional activator to the divergent promoter region of the first two genes of the penicillin pathway in different penicillium species. *Curr Genet* **52:** 229–237.

Kosalková, K., García-Estrada, C., Ullán, R.V., Godio, R.P., Feltrer, R., Teijeira, F., *et al.* (2009) The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. *Biochimie* **91:** 214–225.

Kubicek, C.P., Hönlinger, C.H., Jaklitsch, W.M., Affenzeller, K., Mach, R., Gerngross, T.U., and Ying, L. (1990) Regulation of lysine biosynthesis in the fungus *Penicillium chrysogenum*. In *Amino Acids: Chemistry, Biology and Medicine*. Lubec, G., and Rozenthal, G.A. (eds). Leiden, the Netherlands: ESCOM, Science Publishers B.V., pp. 1029–1034.

Kubicek-Pranz, E.M., and Kubicek, C.P. (1991) Production and biosynthesis of amino acids by fungi. In *Handbook of Applied Mycology Vol. 4: Fungal Biotechnology*. Arora, D.K., Elander, R.P., and Mukerji, K.G. (eds). New York: Marcel Dekker, pp. 313–356.

Kurylowicz, W., Kurzatkowski, W., and Kurzatkowski, J. (1987) Biosynthesis of benzylpenicillin by *Penicillium chrysogenum* and its Golgi apparatus. *Arch Immunol Ther Exp* **35:** 699–724.

Laich, F., Fierro, F., Cardoza, R.E., and Martín, J.F. (1999) Organization of the gene cluster for biosynthesis of penicillin in *Penicillium nalgiovense* and antibiotic production in cured dry sausages. *Appl Environ Microbiol* **65:** 1236–1240.

Laich, F., Fierro, F., and Martín, J.F. (2002) Production of penicillin by fungi growing on food products: identification of a complete penicillin gene cluster in *Penicillium griseofulvum* and a truncated cluster in *Penicillium verrucosum*. *Appl Environ Microbiol* **68:** 1211–1219.

Laich, F., Fierro, F., and Martín, J.F. (2003) Isolation of *Penicillium nalgiovense* strains impaired in penicillin production by disruption of the *pcbAB* gene and application as starters on cured meat products. *Mycol Res* **107:** 1–10.

Lamas-Maceiras, M., Vaca, I., Rodríguez, E., Casqueiro, J., and Martín, J.F. (2006) Amplification and disruption of the phenylacetyl-CoA ligase gene of *Penicillium chrysogenum* encoding an aryl-capping enzyme that supplies phenylacetic acid to the isopenicillin N acyltransferase. *Biochem J* **395:** 147–155.

Lambalot, R.H., Gehring, A.M., Flugel, R.S., Zuber, P., LaCelle, M., Marahiel, M.A., *et al.* (1996) A new enzyme superfamily – the phosphopantetheinyl transferases. *Chem Biol* **3:** 923–936.

van der Lende, T.R., van de Kamp, M., Berg, M., Sjollema, K., Bovenberg, R.A.L., Veenhuis, M., *et al.* (2002) Delta-(L-alpha-Aminoadipyl)-L-cysteinyl-D-valine synthetase, that mediates the first committed step in penicillin biosynthesis, is a cytosolic enzyme. *Fungal Genet Biol* **37:** 49–55.

Lendenfeld, T., Ghali, D., Wolschek, M., Kubicek-Pranz, E.M., and Kubicek, C.P. (1993) Subcellular compartmentation of penicillin biosynthesis in *Penicillium chrysogenum*. The amino acid precursors are derived from the vacuole. *J Biol Chem* **268:** 665–671.

Li, S., Myung, K., Guse, D., Donkin, B., Proctor, R.H., Grayburn, W.S., and Calvo, A.M. (2006) FvVE1 regulates filamentous growth, the ratio of microconidia to macroconidia and cell wall formation in *Fusarium verticillioides*. *Mol Microbiol* **62:** 1418–1432.

Liras, P., and Martín, J.F. (2009) β-Lactam antibiotics. In *Encyclopedia of Microbiology*, 3rd edn. Schaechter, M. (ed). Oxford, UK: Elsevier, pp. 274–289.

Liras, P., Rodríguez-García, A., and Martín, J.F. (1998) Evolution of the clusters of genes for β-lactam antibiotics: a model for evolutive combinatorial assembling of new β-lactams. *Int Microbiol* **1:** 271–278.

Liu, G., Casqueiro, J., Bañuelos, O., Cardoza, R.E., Gutiérrez, S., and Martín, J.F. (2001) Targeted inactivation of the *mecIB* gene, encoding cystathionine-gamma-lyase, shows that the reverse transsulfuration pathway is required for high-level cephalosporin biosynthesis in *Acremonium chrysogenum* C10 but not for methionine induction of the cephalosporin genes. *J Bacteriol* **183:** 1765–1772.

Lübbe, C., Wolfe, S., and Demain, A.L. (1986) Isopenicillin N epimerase activity in a high cephalosporin-producing strain of *Cephalosporium acremonium*. *Appl Microbiol Biotechnol* **23:** 367–368.

Marquez-Fernandez, O., Trigos, A., Ramos-Balderas, J.L., Viniegra-Gonzalez, G., Deising, H.B., and Aguirre, J. (2007) Phosphopantetheinyl transferase CfwA/NpgA is required for *Aspergillus nidulans* secondary metabolism and asexual development. *Eukaryot Cell* **6:** 710–720.

Martín, J.F. (2000a) α-Aminoadipyl-cysteinyl-valine synthetases in β-lactam producing organisms. From Abraham's discoveries to novel concepts of non-ribosomal peptide synthesis. *J Antibiot* **53:** 1008–1021.

Martín, J.F. (2000b) Molecular control of expression of penicillin biosynthesis genes in fungi: regulatory proteins interact with a bidirectional promoter region. *J Bacteriol* **182:** 2355–2362.

Martín, J.F., and Demain, A.L. (2002) Unraveling the methionine-cephalosporin puzzle in *Acremonium chrysogenum*. *Trends Biotechnol* **20:** 12502–12507.

Martín, J.F., Casqueiro, J., Kosalková, K., Marcos, A.T., and Gutiérrez, S. (1999) Penicillin and cephalosporin biosynthesis: mechanism of carbon catabolite regulation of penicillin production. *Antonie Van Leeuwenhoek* **75:** 21–31.

Martín de Valmaseda, E.M., Campoy, S., Naranjo, L., Casqueiro, J., and Martín, J.F. (2005) Lysine is catabolized to 2-aminoadipic acid in *Penicillium chrysogenum* by an omega-aminotransferase and to saccharopine by a lysine 2-ketoglutarate reductase. Characterization of the omega-aminotransferase. *Mol Genet Genomics* **274:** 272–282.

Matsuda, A., Sugiura, H., Matsuyama, K., Matsumoto, H., Ichikawa, S., and Komatsu, K. (1992) Cloning and disruption of the *cefG* gene encoding acetyl coenzyme A: deacetylcephalosporin C o-acetyltransferase from *Acremonium chrysogenum*. *Biochem Biophys Res Commun* **186:** 40–46.

Matsuyama, A., Arai, R., Yashiroda, Y., Shirai, A., Kamata, A., Sekido, S., *et al.* (2006) ORFeome cloning and global analysis of protein localization in the fission yeast *Schizosaccharomyces pombe*. *Nat Biotechnol* **24:** 841–847.

Müller, W.H., van der Krift, T.P., Krouwer, A.J., Wösten, H.A., van der Voort, L.H., Smaal, E.B., and Verkleij, A.J. (1991) Localization of the pathway of the penicillin biosynthesis in *Penicillium chrysogenum*. *EMBO J* **10:** 489–495.

Müller, W.H., Bovenberg, R.A.L., Groothuis, M.H., Kattevilder, F., Smaal, E.B., Van der Voort, L.H., and Verkleij, A.J. (1992) Involvement of microbodies in penicillin biosynthesis. *Biochim Biophys Acta* **1116:** 210–213.

Müller, W.H., Essers, J., Humbel, B.M., and Verkleij, A.J. (1995) Enrichment of *Penicillium chrysogenum* microbodies by isopycnic centrifugation in nycodenz as visualized with immuno-electron microscopy. *Biochim Biophys Acta* **1245:** 215–220.

Naranjo, L., Martín de Valmaseda, E., Bañuelos, O., López, P., Riaño, J., Casqueiro, J., and Martín, J.F. (2001) The conversion of pipecolic acid into lysine in *Penicillium chrysogenum* requires pipecolate oxidase and saccha-

ropine reductase: characterization of the *lys7* gene encoding saccharopine reductase. *J Bacteriol* **183:** 7165–7172.
Nijland, J.G., Kovalchuk, A., van den Berg, M.A., Bovenberg, R.A.L., and Driessen, A.J. (2008) Expression of the transporter encoded by the *cefT* gene of *Acremonium chrysogenum* increases cephalosporin production in *Penicillium chrysogenum*. *Fungal Genet Biol* **45:** 1415–1421.
Nishida, H., and Nishiyama, M. (2000) What is characteristic of fungal lysine synthesis through the alpha-aminoadipate pathway? *J Mol Evol* **51:** 299–302.
Öster, L.M., Lester, D.R., Terwisscha van Scheltinga, A., Svenda, M., van Lun, M., Généreux, C., and Andersson, I. (2006) Insights into cephamycin biosynthesis: the crystal structure of CmcI from *Streptomyces clavuligerus*. *J Mol Biol* **358:** 546–558.
Pinan-Lucarré, B., Paoletti, M., Dementhon, K., Coulary-Salin, B., and Clavé, C. (2003) Autophagy is induced during cell death by incompatibility and is essential for differentiation in the filamentous fungus *Podospora anserina*. *Mol Microbiol* **47:** 321–333.
Pinan-Lucarré, B., Balguerie, A., and Clavé, C. (2005) Accelerated cell death in *Podospora* autophagy mutants. *Eukaryot Cell* **4:** 1765–1774.
Purdue, P.E., and Lazarow, P.B. (2001) Peroxisome biogenesis. *Annu Rev Cell Dev Biol* **17:** 701–752.
Rachubinski, R.A., and Subramani, S. (1995) How proteins penetrate peroxisomes. *Cell* **83:** 525–528.
Ramos, F.R., López-Nieto, M.J., and Martín, J.F. (1985) Isopenicillin N synthetase of *Penicillium chrysogenum*, an enzyme that converts delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N. *Antimicrob Agents Chemother* **27:** 380–387.
Reumann, S. (2004) Specification of the peroxisome targeting signals type 1 and type 2 of plant peroxisomes by bioinformatics analyses. *Plant Physiol* **135:** 783–800.
Richie, D.L., Fuller, K.K., Fortwendel, J., Miley, M.D., McCarthy, J.W., Feldmesser, M., *et al.* (2007) Unexpected link between metal ion deficiency and autophagy in *Aspergillus fumigatus*. *Eukaryot Cell* **6:** 2437–2447.
Roach, P.L., Clifton, I.J., Fülöp, V., Harlos, K., Barton, G.J., Hajdu, J., *et al.* (1995) Crystal structure of isopenicillin N synthase is the first from a new structural family of enzymes. *Nature* **375:** 700–704.
Roach, P.L., Clifton, I.J., Hensgens, C.M., Shibata, N., Schofield, C.J., Hajdu, J., and Baldwin, J.E. (1997) Structure of isopenicillin N synthase complexed with substrate and the mechanism of penicillin formation. *Nature* **387:** 827–830.
Rodríguez-Sáiz, M., Barredo, J.L., Moreno, M.A., Fernández-Cañón, J.M., Peñalva, M.A., and Díez, B. (2001) Reduced function of a phenylacetate-oxidizing cytochrome p450 caused strong genetic improvement in early phylogeny of penicillin-producing strains. *J Bacteriol* **183:** 5465–5471.
Rottensteiner, H., Kramer, A., Lorenzen, S., Stein, K., Landgraf, C., Volkmer-Engert, R., and Erdmann, R. (2004) Peroxisomal membrane proteins contain common Pex19p-binding sites that are an integral part of their targeting signals. *Mol Biol Cell* **15:** 3406–3417.
Sakai, Y., Oku, M., van der Klei, I.J., and Kiel, J.A. (2006) Pexophagy: autophagic degradation of peroxisomes. *Biochim Biophys Acta* **1763:** 1767–1775.
Samsom, S.M., Belagaje, R., Blankenship, D.T., Chapman, J.L., Perry, D., Skatrud, P.L., *et al.* (1985) Isolation, sequence determination and expression in *Escherichia coli* of the isopenicillin N synthetase gene from *Cephalosporium acremonium*. *Nature* **318:** 191–194.
Samsom, S.M., Dotzlaf, J.F., Slisz, M.L., Becker, G.W., van Frank, R.M., Veal, L.E., *et al.* (1987) Cloning and expression of the fungal expandase/hydroxylase gene involved in cephalosporin biosynthesis. *Biotechnology* **5:** 1207–1214.
Sawada, Y., Baldwin, J.E., Singh, P.D., Solomon, N.A., and Demain, A.L. (1980) Cell-free cyclization of delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N. *Antimicrob Agents Chemother* **18:** 465–470.
Scheidegger, A., Küenzi, M.T., and Nüesch, J. (1984) Partial purification and catalytic properties of a bifunctional enzyme in the biosynthetic pathway of beta-lactams in *Cephalosporium acremonium*. *J Antibiot* **37:** 522–531.
Schmitt, E.K., Kempken, R., and Kück, U. (2001) Functional analysis of promoter sequences of cephalosporin C biosynthesis genes from *Acremonium chrysogenum*: specific DNA–protein interactions and characterization of the transcription factor PACC. *Mol Genet Genomics* **265:** 508–518.
Schmitt, E.K., Bunse, A., Janus, D., Hoff, B., Friedlin, E., Kürnsteiner, H., and Kück, U. (2004a) Winged helix transcription factor CPCR1 is involved in regulation of beta-lactam biosynthesis in the fungus *Acremonium chrysogenum*. *Eukaryot Cell* **3:** 121–134.
Schmitt, E.K., Hoff, B., and Kück, U. (2004b) Regulation of cephalosporin biosynthesis. *Adv Biochem Eng Biotechnol* **88:** 1–43.
Shwab, E.K., Bok, J.W., Tribus, M., Galehr, J., Graessle, S., and Keller, N.P. (2007) Histone deacetylase activity regulates chemical diversity in *Aspergillus*. *Eukaryot Cell* **6:** 1656–1664.
Sugui, J.A., Pardo, J., Chang, Y.C., Müllbacher, A., Zarember, K.A., Galvez, E.M., *et al.* (2007) Role of *laeA* in the regulation of *alb1*, *gliP*, conidial morphology, and virulence in *Aspergillus fumigatus*. *Eukaryot Cell* **6:** 1552–1561.
Teijeira, F., Ullán, R.V., Guerra, S.M., García-Estrada, C., Vaca, I., Casqueiro, J., and Martín, J.F. (2009) The transporter CefM involved in translocation of biosynthetic intermediates is essential for cephalosporin production. *Biochem J* **418:** 113–124.
Thieringer, R., and Kunau, W.H. (1991a) Beta-oxidation system of the filamentous fungus *Neurospora crassa*. Structural characterization of the trifunctional protein. *J Biol Chem* **266:** 13118–13123.
Thieringer, R., and Kunau, W.H. (1991b) The beta-oxidation system in catalase-free microbodies of the filamentous fungus *Neurospora crassa*. Purification of a multifunctional protein possessing 2-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase activities. *J Biol Chem* **266:** 13110–13117.
Tobin, M.B., Fleming, M.D., Skatrud, P.L., and Miller, J.R. (1990) Molecular characterization of the acyl-coenzyme A: isopenicillin N acyltransferase gene (*penDE*) from *Penicillium chrysogenum* and *Aspergillus nidulans* and activity of recombinant enzyme in *E. coli*. *J Bacteriol* **172:** 5908–5914.
Tobin, M.B., Baldwin, J.E., Cole, S.C.J., Miller, J.R., Skatrud, P.L., and Sutherland, J.D. (1993) The requirement for subunit interaction in the production of *Penicillium*

chrysogenum acyl-conenzyme A: isopenicillin N acyltransferase in *Escherichia coli*. *Gene* **132:** 199–206.

Trip, H., Evers, M.E., Kiel, J.A., and Driessen, A.J. (2004) Uptake of the beta-lactam precursor alpha-aminoadipic acid in *Penicillium chrysogenum* is mediated by the acidic and the general amino acid permease. *Appl Environ Microbiol* **70:** 4775–4783.

Turgay, K., Krause, M., and Marahiel, M.A. (1992) Four homologous domains in the primary structure of GrsB are related to domains in a superfamily of adenylate forming enzymes. *Mol Microbiol* **6:** 529–546.

Ullán, R.V., Casqueiro, J., Bañuelos, O., Fernández, F.J., Gutiérrez, S., and Martín, J.F. (2002a) A novel epimerization system in fungal secondary metabolism involved in the conversion of isopenicillin N into penicillin N in *Acremonium chrysogenum*. *J Biol Chem* **277:** 46216–46225.

Ullán, R.V., Liu, G., Casqueiro, J., Gutiérrez, S., Bañuelos, O., and Martín, J.F. (2002b) The *cefT* gene of *Acremonium chrysogenum* C10 encodes a multidrug efflux pump protein that significantly increases cephalosporin C production. *Mol Genet Genomics* **267:** 673–683.

Ullán, R.V., Campoy, S., Casqueiro, J., Fernández, F.J., and Martín, J.F. (2007) Deacetylcephalosporin C production in *Penicillium chrysogenum* by expression of the isopenicillin N epimerization, ring expansion, and acetylation genes. *Chem Biol* **14:** 329–339.

Ullán, R.V., Teijeira, F., and Martín, J.F. (2008) Expression of the *Acremonium chrysogenum cefT* gene in *Penicillium chrysogenum* indicates that it encodes an hydrophilic beta-lactam transporter. *Curr Genet* **54:** 153–161.

Valenciano, S., Lucas, J.R., Pedregosa, A., Monistrol, I.F., and Laborda, F. (1996) Induction of beta-oxidation enzymes and microbody proliferation in *Aspergillus nidulans*. *Arch Microbiol* **166:** 336–341.

Valenciano, S., De Lucas, J.R., Van der Klei, I., Veenhuis, M., and Laborda, F. (1998) Characterization of *Aspergillus nidulans* peroxisomes by immunoelectron microscopy. *Arch Microbiol* **170:** 370–376.

Van den Berg, M.A., Albang, R., Albermann, K., Badger, J.H., Daran, J.M., Driessen, A.J.M., *et al.* (2008) Genome sequencing and analysis of the filamentous fungus *Penicillium chrysogenum*. *Nat Biotechnol* **26:** 1161–1168.

Veenstra, A.E., van Solingen, P., Huininga-Muurling, H., Koekman, B.P., Groenen, M.A.M., Smaal, E.B., *et al.* (1989) Cloning of penicillin biosynthesic genes. In *Genetics and Molecular Biology of Industrial Microorganisms*. Hershberger, C.L., Queener, S.W., and Hegeman, G. (eds). Washington, DC, USA: American Society for Microbiology, pp. 262–269.

Velasco, J., Gutierrez, S., Fernández, F.J., Marcos, A.T., Arenos, C., and Martín, J.F. (1994) Exogenous methionine increases levels of mRNAs transcribed from *pcbAB*, *pcbC*, and *cefEF* genes, encoding enzymes of the cephalosporin biosynthetic pathway, in *Acremonium chrysogenum*. *J Bacteriol* **176:** 985–991.

Velasco, J., Gutiérrez, S., Campoy, S., and Martín, J.F. (1999) Molecular characterization of the *Acremonium chrysogenum cefG* gene product: the native deacetylcephalosporin C acetyltransferase is not processed into subunits. *Biochem J* **337:** 379–385.

Wang, F.Q., Liu, J., Dai, M., Ren, Z.H., Su, C.Y., and He, J.G. (2007) Molecular cloning and functional identification of a novel phenylacetyl-CoA ligase gene from *Penicillium chrysogenum*. *Biochem Biophys Res Commun* **360:** 453–458.

Whiteman, P.A., Abraham, E.P., Baldwin, J.E., Fleming, M.D., Schofield, C.J., Sutherland, J.D., and Willis, A.C. (1990) Acyl coenzyme A: 6-aminopenicillanic acid acyltransferase from *Penicillium chrysogenum* and *Aspergillus nidulans*. *FEBS Lett* **262:** 342–344.

Wu, X.B., Fan, K.Q., Wang, Q.H., and Yang, K.Q. (2005) C-terminus mutations of *Acremonium chrysogenum* deacetoxy/deacetylcephalosporin C synthase with improved activity toward penicillin analogs. *FEMS Microbiol Lett* **246:** 103–110.

Yorimitsu, T., and Klionsky, D.J. (2005) Autophagy: molecular machinery for self-eating. *Cell Death Differ* **2:** 1542–1552.

Zabriskie, T.M., and Jackson, M.D. (2000) Lysine biosynthesis and metabolism in fungi. *Nat Prod Rep* **17:** 85–97.

Zhang, J., and Demain, A.L. (1992) ACV synthetase. *Crit Rev Biotechnol* **12:** 245–260.

