J Ind Microbiol Biotechnol (2012) 39:367–382
DOI 10.1007/s10295-011-1063-z

REVIEW

Role of peroxisomes in the biosynthesis and secretion of β-lactams and other secondary metabolites

Juan-Francisco Martín · Ricardo V. Ullán · Carlos García-Estrada

Received: 28 September 2011 / Accepted: 16 November 2011 / Published online: 11 December 2011

Abstract Peroxisomes are eukaryotic organelles surrounded by a single bilayer membrane, containing a variety of proteins depending on the organism; they mainly perform degradation reactions of toxic metabolites (detoxification), catabolism of linear and branched-chain fatty acids, and removal of H_2O_2 (formed in some oxidative processes) by catalase. Proteins named peroxins are involved in recruiting, transporting, and introducing the peroxisomal matrix proteins into the peroxisomes. The matrix proteins contain the peroxisomal targeting signals PTS1 and/or PTS2 that are recognized by the peroxins Pex5 and Pex7, respectively. Initial evidence indicated that the penicillin biosynthetic enzyme isopenicillin N acyltransferase (IAT) of *Penicillium chrysogenum* is located inside peroxisomes. There is now solid evidence (based on electron microscopy and/or biochemical data) confirming that IAT and the phenylacetic acid- and fatty acid-activating enzymes are also located in peroxisomes. Similarly, the *Acremonium chrysogenum* CefD1 and CefD2 proteins that perform the central reactions (activation and epimerization of isopenicillin N) of the cephalosporin pathway are targeted to peroxisomes. Growing evidence supports the conclusion that some enzymes involved in the biosynthesis of mycotoxins (e.g., AK-toxin), and the biosynthesis of signaling molecules in plants (e.g., jasmonic acid or auxins) occur in peroxisomes. The high concentration of substrates (in many cases toxic to the cytoplasm) and enzymes inside the peroxisomes allows efficient synthesis of metabolites with interesting biological or pharmacological activities. This compartmentalization poses additional challenges to the cell due to the need to import the substrates into the peroxisomes and to export the final products; the transporters involved in these processes are still very poorly known. This article focuses on new aspects of the metabolic processes occurring in peroxisomes, namely the degradation and detoxification processes that lead to the biosynthesis and secretion of secondary metabolites.

Keywords Peroxisomes · β-Lactam biosynthesis · Peroxins · Mycotoxins · Bile acid metabolism

J.-F. Martín (✉)
Área de Microbiología, Departamento de Biología Molecular, Facultad de CC. Biológicas y Ambientales, Universidad de León, Campus de Vegazana s/n, 24071 León, Spain
e-mail: jf.martin@unileon.es

J.-F. Martín · R. V. Ullán · C. García-Estrada
Instituto de Biotecnología de León (INBIOTEC), Av. Real, 1, 24006 León, Spain

Introduction: peroxisomes and fungal metabolism

Peroxisomes are ubiquitous eukaryotic organelles surrounded by a single bilayer membrane. Peroxisomal proteins, termed peroxins, participate in matrix protein import, targeting and peroxisomal membrane proteins, inheritance, and regulation of peroxisomal size and abundance. Peroxisomal matrix proteins are targeted and imported into peroxisomes due to the presence of a conserved peroxisomal targeting signal (PTS), and once inside these organelles, they play a variety of essential roles for the cellular physiology, such as β-oxidation of fatty acids, oxidation and epimerization of branched-chain fatty acids, metabolism of reactive compounds, and polyamine catabolism. In filamentous fungi, the biosynthesis of β-lactam antibiotics is compartmentalized [27, 75, 85] and peroxisomes play a crucial role harboring some steps of the penicillin and cephalosporin

biosynthetic pathways. In this review we focus on peroxisomal proteins and enzymes, especially those involved in detoxification of toxic organic compounds, penicillin/cephalosporin biosynthesis, and multidrug resistance-mediated secretion of metabolites from peroxisomes.

Peroxisomes and related organelles

Peroxisomes, also known as microbodies, represent a class of morphologically plastic organelles (ranging from 0.1 to 1 μm in diameter) found in virtually all eukaryotic cells [34]. Peroxisomes occur in mammalian kidney and liver cells, plants (especially in leaf photosynthetic cells closely associated with chloroplasts and mitochondria), fungi, yeasts, and certain protozoans. In contrast to other organelles, such as the nucleus, mitochondria, or chloroplasts, peroxisomes do not contain DNA. Although these organelles were first described in 1954, they were identified and designated as peroxisomes (due to the typical presence of oxidases and catalases) a decade later [15].

Peroxisomes are surrounded by a thin single bilayer membrane (about 7 nm) with a low protein-to-lipid ratio that, due to the toxic or hazardous nature of some metabolites accumulated inside this organelle, acts as a barrier protecting the cytosol from those compounds. In this context, the peroxisomes act as a waste furnace of toxic organic compounds, where they are oxidized, coupled to carrier molecules, and finally secreted out of the cells (see below). The peroxisomal membrane is a dynamic structure modified according to environmental needs, being formed mainly from the endoplasmic reticulum, although mitochondria are also involved in the biogenesis of the peroxisome membrane [92].

Peroxisome biogenesis and abundance

The formation of peroxisomes is dependent on the proteins encoded by the PEX genes (over 30 different proteins described so far and collectively known as peroxins) that are involved in matrix protein import, targeting and peroxisomal membrane proteins, inheritance, and regulation of peroxisomal number and size [47, 60]. Peroxisome matrix proteins are believed to be synthesized in free ribosomes in the cytosol prior to import. Depending on the conserved peroxisomal targeting signal (PTS) included either at their C-terminus (PTS1) or near the N-terminus (PTS2) [60], these proteins are recognized by peroxins Pex5 and Pex7, respectively [66, 88], which bind them and release them inside the peroxisome.

The dynamism of peroxisomes is achieved through the molecular balance between biogenesis (augmentation) and degradation (autophagy). Basically, de novo formation of peroxisomes from the endoplasmic reticulum seems to be associated with the Pex3 protein, which plays also a role in peroxisome inheritance [86]. This peroxin is suggested to be essential for the formation of initial vesicular compartments termed pre-peroxisomes, which grow by recruiting newly synthesized peroxisomal membrane proteins and matrix proteins to form a mature peroxisome that multiplies by fission. Peroxisomal fission involves several, partially overlapping, consecutive steps, for some of which the most abundant peroxisomal membrane protein Pex11 is crucial [113]. The mechanism of action of Pex11 involves induction of membrane asymmetry, resulting in membrane curvature during peroxisome fission [93]. Besides Pex11, a second class of proteins is the family of dynamin-like proteins, which are large GTPases involved in membrane fusion and fission events [61, 92, 102, 113].

To maintain the peroxisomal metabolism according to the environmental and developmental requirements, degradation of peroxisomes (pexophagy) in addition to biogenesis controls the number of peroxisomes. Therefore, peroxisome abundance can be rapidly decreased through autophagic pathways, which selectively degrade peroxisomes by fusing them to lysosomes or vacuoles. Components of two ubiquitin-like conjugation systems and peroxins Pex3 and Pex14 are involved in selective organelle degradation [91, 110].

Peroxisome-related organelles

Certain organisms possess microscopic organelles related to peroxisomes that carry out additional specific functions. These organelles are represented by the glycosomes of trypanosomatids, Woronin bodies of filamentous fungi, and glyoxysomes of plants and filamentous fungi, although the distinction between glyoxysomes and peroxisomes is currently controversial.

Glycosomes, like peroxisomes, are surrounded by a single membrane and have a protein-dense matrix, which mainly contains glycolytic enzymes [45]. Glycosomes have been proposed to be related to peroxisomes since some pathways or enzymes commonly found in peroxisomes are also present in the glycosomes of some species [94, 146]. In addition, the significant sequence similarity existing between peroxisomal and glycosomal proteins required for matrix protein import in trypanosomatids, yeast, and humans suggests that these organelles have a close evolutionary relationship [31, 97]. The fact that the Embden–Meyerhof segment of glycolysis takes place inside glycosomes makes these organelles unique.

Woronin bodies are peroxisome-derived dense organelles that are unique to filamentous fungi. They have a double membrane and localize near the septae, where they play

an important role in sealing the septal pore after hyphal injury, thus preventing cytoplasmic bleeding [71]. The major component of the Woronin body is the HEX1 protein, which bears a PTS1 signal and localizes inside the peroxisomal matrix [54, 70], where it self-assembles to produce a solid micrometer-scale protein assembly [54, 152]. Although these organelles are formed from peroxisomes, their biogenesis is poorly understood [67].

Glyoxysomes are organelles found in plants (oil-storing tissues) and filamentous fungi [101, 143], playing a role in lipid mobilization to sugars through the glyoxylate cycle. Classification of the glyoxysomes, unlike the above-mentioned glycosomes and Woronin bodies, as being distinct from peroxisomes organelles is controversial due to the trivial differences displayed with respect to other peroxisomes. Glyoxysomes, in addition to having peroxisomal functions, were initially believed to possess the five key enzymes of the glyoxylate cycle: citrate synthase, aconitase, isocitrate lyase, malate synthase, and malate dehydrogenase. Currently, it is well documented that the only two unique glyoxylate cycle enzymes potentially distinguishing glyoxysomes from other peroxisomes are isocitrate lyase and malate synthase. These observations, together with functional genomics and cell biology studies, suggest that the term "glyoxysome" is not adequate and that glyoxysomes and peroxisomes should be unified under the same organelle class [101].

Peroxisomal matrix proteins

The presence of a large number of proteins in the peroxisomal matrix entails that several metabolic functions, which vary with the organism, the developmental stage, and the environmental conditions, take place inside these organelles (Table 1). In general, these functions are related to β-oxidation of fatty acids, metabolism of hydrogen peroxide, reactive oxygen and reactive nitrogen species signaling, cellular aging, antiviral innate immunity, and metabolism of polyamines [18, 34, 129, 141]. This wide set of functions might be more specific in either mammals, plants, or fungi. In mammalian cells, peroxisomes are also involved in the biosynthesis of plasmalogen, the most abundant phospholipid in myelin [141], in the formation of bile acids [30], and in the pentose phosphate pathway. Other known peroxisomal functions include the metabolism of glyoxylate (glyoxysomes) [64], photorespiration in plants, glycolysis (glycosomes) in trypanosomatids, the metabolism of unusual carbon and nitrogen sources that may be toxic to the cells in some yeasts (methanol, oleic acid, D-amino acids, and purines) [137], and biosynthesis of mycotoxins or β-lactam antibiotics in some filamentous fungi that contain the β-lactam biosynthesis genes [75, 85, 122] (Table 1).

Table 1 Main degradative and biosynthetic processes that take place in peroxisomes

Fatty-acid β-oxidation
3-Methyl fatty acid α-oxidation
Ether phospholipid biosynthesis
Glyoxylate detoxification
Bile acid biosynthesis
Phenyl (phenoxy) acetic acid detoxification (penicillin biosynthesis)
Mycotoxin biosynthesis (e.g., AK-toxin)
Degradation of purines
Degradation of polyamines
Degradation of pipecolic acid
Catabolism of leukotrienes
Methanol utilization
Acetate utilization/glyoxylate cycle

Targeting of peroxisomal matrix proteins

As indicated above, several enzymatic activities take place in peroxisomes. These activities are performed by different proteins that have to be imported inside peroxisomes from the cytosol [149]. Some of the proteins that are found in the peroxisomal matrix are peroxisome specific, whereas others have multiple subcellular localizations due to the presence of several targeting signals within the same protein, as in the case of 3-hydroxymethylglutaryl-coenzyme A (CoA) lyase [5] and α-methylacyl-CoA racemase [63]. In addition, the peroxisomal localization of a certain protein may be species specific. Peroxisomes can import folded monomeric and even oligomeric proteins [38]. The isopenicillin N acyltransferase (a peroxisomal enzyme catalyzing the last step of penicillin biosynthesis) is imported into peroxisomes irrespective of the processing state; this protein can enter peroxisomes as an α–β heterodimer or as an unprocessed monomer [35].

Peroxisome targeting sequences

The main mechanism determining the peroxisomal location of a protein is the presence of a consensus peroxisomal targeting sequence (PTS) in the amino acid sequence. The presence of a PTS, however, does not necessarily mean that the protein is truly peroxisomal, as has been confirmed for the human phosphomevalonate kinase, which bears a PTS1 but is usually located in the cytosol. This might indicate that other sequences apart from the PTS1 targeting signal are important for subcellular localization.

The peroxisome targeting sequences are divided into two types (PTS1 and PTS2) according to the amino acid residues that constitute them and the position that they take

within the protein. PTS1 is present in the C-terminal region of the protein and consists of the consensus sequence SKL or variations thereof (S/A/C)-(K/R/H/N)-(L/I/M), whereas PTS2 is located near the N-terminal region of the protein and is represented by the consensus sequence (R/K)-(L/I/V)-X_5-(Q/H)-(L/A) [16, 105, 123]. The receptor peroxins Pex5 and Pex7 recognize PTS1 and PTS2 signals, respectively [66, 88]. The residues surrounding a putative PTS are also important for targeting, since they may increase the affinity between the protein and its receptor [79, 82, 83, 103, 124]. PTS1-containing proteins are more abundant than those containing a PTS2 sequence.

In addition to PTS1 and PTS2, it has also been reported that in some proteins other internal targeting sequences can also serve as PTS, and several peroxisomal matrix proteins lacking the canonical PTS1 are sorted to peroxisomes via Pex5 [138]. Another way to import proteins into the peroxisomal matrix is by piggyback transport, where proteins lacking a PTS can be sorted to peroxisomes in a Pex5-dependent manner by forming a complex with a PTS-containing protein [52, 138].

As was briefly mentioned above, import of PTS1- and PTS2-containing peroxisomal proteins is mediated by peroxins Pex5 and Pex7, respectively. Both cargo-loaded receptors dock on the same peroxisome translocon components, followed by cargo release and receptor recycling, as part of the complete translocation process between the cytosol and the peroxisome [14, 87].

During the import cycle, the PTS1-bearing protein is recognized and bound in the cytosol by Pex5. Binding is mediated by seven tetratricopeptide repeats (each consisting of a degenerate 34-amino-acid motif forming two helical bundles), which are present in the C-terminal half of the receptor protein [11, 32]. In contrast, in those proteins that have internal peroxisomal targeting sequences, interaction is mediated by the N-terminal half of Pex5 [149]. Once the cargo protein and the receptor are linked, this complex binds to the docking complex, which consists of peroxins Pex13, Pex14, and Pex17 (the latter only identified in yeasts) [89, 149] located at the peroxisomal membrane, where the cargo is released into the peroxisomal matrix. This translocation process requires the RING-finger ubiquitin ligases Pex2, Pex10, Pex12 [28, 50, 100, 117, 120, 148], and Pex8 (the latter only identified in yeasts) [1]. Then, Pex5 is released from the membrane in an adenosine triphosphate (ATP)- and ubiquitin-dependent manner (mediated by the Pex4 ubiquitin-conjugating enzyme in yeasts or UbcH5a/b/c in humans) [40, 99, 147] to the cytosol, where it is either degraded or recycled for a new import round [14, 19, 87]. Release of the receptor from the membrane is performed by the ATPase peroxins Pex1 and Pex6, which are anchored to the peroxisome membrane, by Pex15 in yeasts or by Pex26 in mammals [23, 37, 56, 76, 154].

PTS2-containing proteins are imported into peroxisomes by the Pex7 receptor, which requires additional protein components for peroxisome targeting, thus functioning as a PTS2 co-receptor [115]. Pex7-mediated import requires Pex5 in several organisms [10, 46, 77, 89, 95, 106, 150]. However, fungal Pex7 does not require Pex5 for peroxisomal protein import. Instead, it functions in concert with other auxiliary proteins, such as Pex18 and Pex21 in *Saccharomyces cerevisiae* [104] and Pex20 in *Neurospora crassa* [118], *Yarrowia lipolytica* [22], *Pichia pastoris*, and *Hansenula polymorpha* [96].

Although the background knowledge about the import machinery is wide, the mechanism of protein translocation across the peroxisomal membrane is still obscure. Different processes, such as membrane invagination events (similar to endocytosis), vesicle fusion processes, or dynamic transient pore formation, have been proposed as models for protein translocation [26, 80]. According to the transient pore hypothesis, Pex5 and Pex14 form a dynamic gated ion-conducting channel of about 9 nm induced by the cytosolic receptor–cargo complex [80]. In relation to this, it has also been suggested that the minimum translocation machinery required for matrix protein import in *P. pastoris* is represented by the PTS receptors and Pex14 [69].

Peroxisomal membrane proteins

Peroxisomal membrane proteins (PMPs) are encoded by nuclear genes, translated on cytoplasmic ribosomes, and imported posttranslationally into the peroxisomal membrane. The import machinery of PMPs is different from that of the peroxisomal matrix proteins since most PEX mutations that disrupt matrix protein import still showed functional integration of PMPs [13, 25, 39]. In general, two import pathways have been proposed for the targeting of PMPs [134]. In the first pathway, hydrophobic proteins are directly inserted into existing peroxisomal membranes after being synthesized in the cytosol [121]. Alternatively, in the second pathway [125, 126], PMPs can be synthesized on the rough endoplasmic reticulum, where they concentrate in pre-peroxisomal vesicles. These vesicles function as an origin for de novo formation of peroxisomes or are fused with the membrane of existing peroxisomes.

As a consequence of these two incorporation pathways PMPs can be divided into two classes named class I (for the first pathway) and class II (for the second one) [109]. Whereas class I corresponds to PMPs that are imported to peroxisomal membranes via a Pex19p-dependent pathway, class II PMPs are targeted independent of Pex19p to peroxisome membranes [55]. The peroxisomal membrane protein targeting signals (mPTS) were identified for several PMPs

and contain a basic amino acid sequence in combination with at least one transmembrane region [21, 49].

The class I PMPs possess an mPTS which is recognized in the cytosol by the peroxin Pex19p. This peroxin is a farnesylated protein mostly localized in the cytosol and partly associated with the peroxisomal membrane. Pex19p works as an import receptor and/or chaperone required for stabilization of PMPs at the peroxisomal membrane [78]. Once the mPTS motif of the PMP is recognized, Pex19p directs the Pex19p–PMP complex to the peroxisomal membrane by docking to its membrane-anchored binding partner Pex3p [29]. Next, the PMP is inserted into the peroxisomal membrane, probably with the assistance of Pex19p and Pex3p. Finally, Pex19p returns to the cytosol, where the PMP importation process is restarted [109].

A minor proportion of PMPs belong to the class II PMPs, which are sorted indirectly to peroxisomes via the endoplasmic reticulum. The mPTS of this class is located in the N-terminal region and contains a transmembrane region but not a Pex19p binding site [44, 119]. It is known that Pex3p, Pex16p, and Pex22p belong to the class II PMPs [33, 44]. Nevertheless, the mechanisms responsible for targeting to the peroxisomal membrane and incorporation into the endoplasmic reticulum are still rather poorly understood [44, 98, 126, 128].

Metabolite degrading and converting pathways in peroxisomes

The diversity of enzymes and metabolic processes located in peroxisomes is impressive. In all cases, these metabolic processes have in common the oxidation and/or degradation of organic compounds and the removal of H_2O_2 (formed in some oxidative reactions) by catalase (an enzyme present very frequently in peroxisomes). Not all enzymes known to be targeted to peroxisomes are present simultaneously in these organelles. The cells have the ability to dedicate "specialized" forms of peroxisomes to specific metabolic processes by concentrating in these organelles specific sets of enzymes depending upon the metabolic needs of the cells.

Some enzymes located in peroxisomes, such as the phenylacetyl-CoA ligase (PhlA) that activates phenylacetic or phenoxyacetyl acid, are clearly involved in detoxification of these organic acids by binding them to the amino group of 6-aminopenicillanic acid (6-APA). The aromatic organic acids used as substrates for penicillin biosynthesis are formed in nature by degradation of phenylpropanoid molecules that in turn are produced by catabolism of lignin or from aromatic amino acids (phenylalanine, tyrosine).

Phenylacetic and phenoxyacetic acids are toxic for *P. chrysogenum*, preventing growth at concentrations above 2.0 g/l [62, 65]. The laboratory strain Wis54-1255 resists up to 2.0 g/l phenylacetic acid in solid medium, whereas two different mutants disrupted in the *phlA* gene failed to grow at phenylacetic acid (or phenoxyacetic acid) concentration of 1.5 g/l. It is noteworthy that a transformant overexpressing the *phlA* gene (containing eightfold phenylacetyl-CoA ligase enzyme activity as compared with the control Wis54-1255 strain) resists up to 3.0 g/l phenylacetic acid. Indeed, resistance to phenylacetic or phenoxyacetic acid is one of the classical methods of strain selection following random mutagenesis [8].

Lamas-Maceiras et al. [65] observed that the *phlA*-disrupted mutants still contain 46% phenylacetyl-CoA ligase activity and provided evidence for the presence of a second gene homologous to *phlA*. Indeed, when the *P. chrysogenum* genome was sequenced, other enzymes annotated as coumaroyl-CoA ligases were observed [135]. Some of these coumaroyl-CoA-ligase-like proteins indeed have phenylacetyl-CoA ligase activity (see below).

Another example of toxic compounds that are removed in peroxisomes by binding to β-lactam intermediates is that of branched-chain fatty acids in *A. chrysogenum* [75] (see below). Some degradation pathways may have been recruited and integrated into secondary metabolite biosynthetic pathways.

Peroxisomal conversion of cholesterol to bile acids

Another good example of conversions performed by peroxisomal enzymes is the transformation of cholesterol to bile acids in the liver of mammals. We will refer here only to the so-called classical bile acid pathway that represents 75–90% of total bile acid formation [30]. These reactions include modifications of the ring structure of cholesterol, oxidation and shortening of the branched side-chain, and finally conjugation of the bile acid with an amino acid (glycine or taurine).

The initial ring modifications are catalyzed by microsomal P450 monooxygenases and related enzymes that convert cholesterol into 3α,7α-dihydroxycholestanoic acid (DHCA) and 3α,7α,12α-trihydroxycholestanoic acid (THCA) that are transported into peroxisomes for further conversion into bile acids. The first two reactions in the peroxisomes are similar to those performed by the proteins CefD1 and CefD2 in the cephalosporin biosynthesis pathway (Fig. 1) [132].

The first peroxisomal reaction in bile acid synthesis is the activation of DHCA (or THCA) as CoA esters that introduce these compounds into the peroxisomes. This reaction is catalyzed by an acyl-CoA ligase belonging to the very long acyl-CoA synthetases (VLCS) family. A related enzyme named bile acid acyl-CoA synthetase is exclusively located in the endoplasmic reticulum of

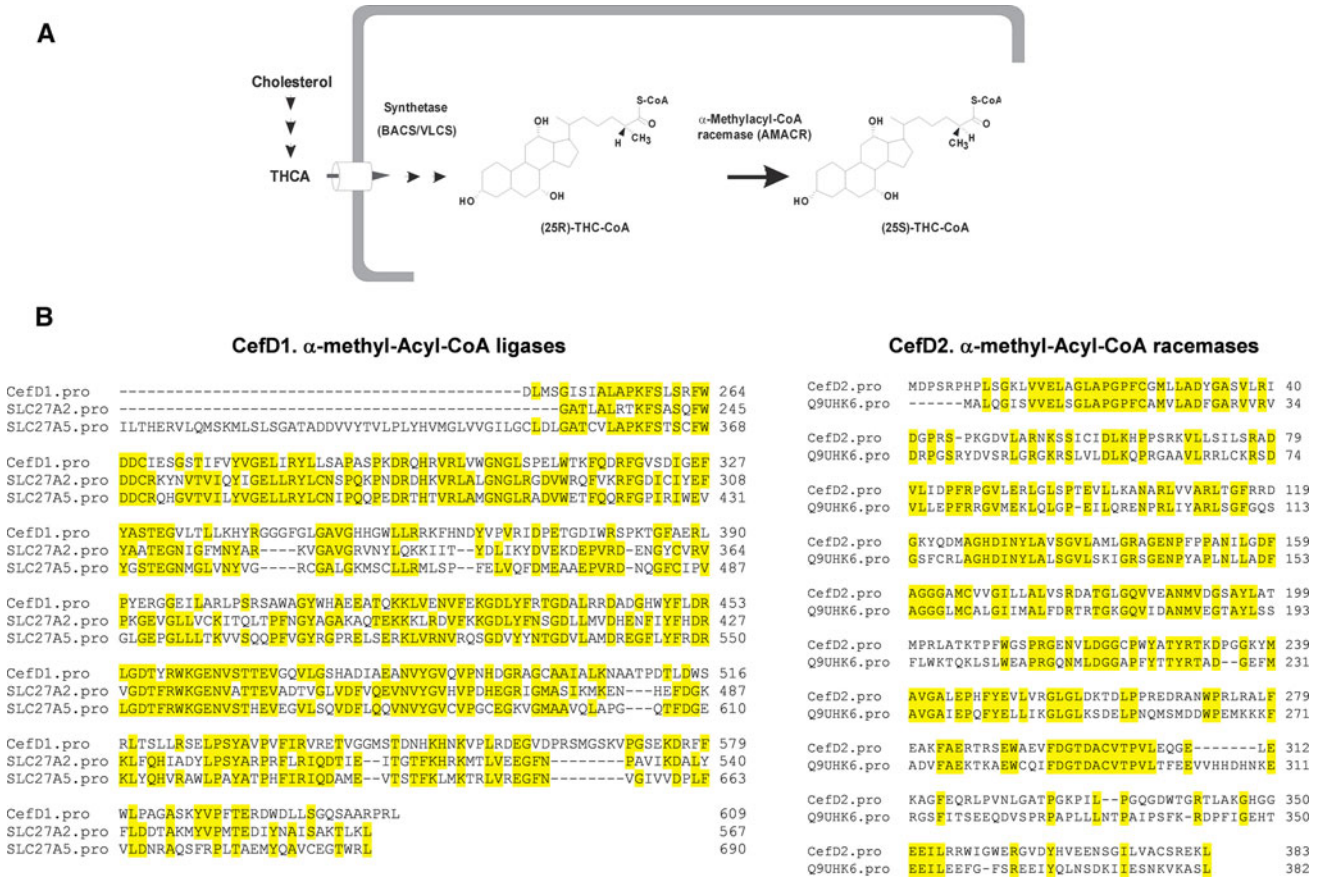

Fig. 1 **a** Peroxisomal reactions involved in the conversion of cholesterol to bile acids, and **b** similarity to the peroxisomal enzymes CefD1 and CefD2 involved in cephalosporin biosynthesis. Only the alignment of the C-terminal sequence of the CefD1 protein is represented. Conserved amino acids are shown in *yellow*

mammalian cells. The CoA-activated DHCA or THCA is then oxidized, and the side-chain is shortened by a series of well-known reactions similar to those involved in isomerization and degradation of branched-chain α-methyl fatty acids. These reactions are well documented and take place in liver peroxisomes [30]. The second peroxisomal enzyme α-methylacyl-CoA racemase is involved in the conversion of DHC-CoA or THC-CoA to chenodeoxycholoyl-CoA (CDC-CoA) or choloyl-CoA (CA-CoA), respectively. This racemase is required because the β-oxidation enzymes acting on the next reactions only accept (25S)-isomers whereas DHCA and THCA contain the (25R) side-chain configuration. In humans, the same enzyme is able to oxidize pristanic acid (with previous activation to pristanoyl-CoA) that has also a methyl group at the carbon 2-position [144].

The similarity of these enzymes that activate and racemize the bile acid intermediates to those enzymes involved in cephalosporin biosynthesis (CefD1 and CefD2) and their peroxisomal location is striking. It seems that these enzymes that originally were involved in degradation of branched-chain fatty acids may have been adapted for the biosynthesis of bile acids and secondary metabolites that involve conversions of branched acyl side-chains. These peroxisomal enzymes may still be used for nutritional degradation of branched-chain fatty acids, a typical peroxisomal process.

Peroxisomal enzymes involved in penicillin biosynthesis

Phenylacetyl-CoA ligases

For many years it has been known that *P. chrysogenum* is able to synthesize several penicillins with different side-chains at the C6-amino group. These include penicillin K (containing a caprylic acid side-chain), penicillin F (containing *trans*-3-hexenoic acid), and penicillin H (containing heptanoic acid), in addition to the better known penicillin G (phenylacetic acid side-chain) and penicillin V (phenoxyacetic acid side-chain). It was initially believed that the formation of diverse penicillins was due to the broad substrate specificity of the isopenicillin N acyltransferase (IAT) [3]. There is only one functional IAT in *P. chrysogenum* [36]. However, the discovery of the requirement for previous

Table 2 Acyl-CoA ligases related to PhlA encoded in the *P. chrysogenum* genome

Gene	PTS1 targeting sequence (C-terminal)	Enzyme	References
Pc22g14900 Phl (*phl*A)	SKI	Phenylacetate CoA ligase—*P. chrysogenum*	[65]
Pc22g20270 (*phl*B)	AKL	Acyl-CoA ligase (linear fatty acids) —*P. chrysogenum*	[62, 142]
Pc13g12270 (*phl*C)	AKL	Acyl-CoA (adipyl-CoA) ligase—*P. chrysogenum*	[151]
Pc 21g07810	Lacks PTS1	Similar to 4-coumarate CoA ligase—*A. thaliana*	[135]
Pc22g24780	AKL	Similar to 4-coumarate CoA ligase—*A. thaliana*	[135]
Pc21g22010	SKL	Similar to 4-coumarate CoA ligase—*A. thaliana*	[135]
Pc06g01160	TKI	Similar to 4-coumarate CoA ligase—*A. thaliana*	[135]
Pc21g20650	ARL	Similar to 4-coumarate CoA ligase—*A. thaliana*	[135]

Data from Lamas-Maceiras et al. [62], Wang et al. [142], van den Berg et al. [135], Koetsier et al. [62], and Yu et al. [151]

activation of precursor fatty acids and aromatic acids as thioesters revealed a much more complex situation. A phenylacetyl-CoA ligase (Phl) was initially cloned by Lamas-Maceiras et al. [65]. This enzyme was shown to activate phenylacetic acid and phenoxyacetic acid as substrates. Disruption of the *phl* gene (now known as *phlA*) resulted in a considerable decrease of phenylacetyl-CoA ligase activity and of penicillin production, but still about 40% residual enzyme activity was retained. We proposed that other phenylacetyl-CoA ligases exist in this fungus. The genome sequence of *P. chrysogenum* [135] confirmed that there are eight related genes that contain a substrate binding pocket similar to that of *p*-coumaroyl-CoA ligases (Table 2) [65, 135]. More recently, two of those other acyl-CoA ligases have been identified by different research groups: a second acyl-CoA ligase (named PhlB or AclA) was shown to activate medium-sized fatty acids such as decanoic acid, caproic acid, and to a lesser extent, adipic acid [62, 142]. A detailed biochemical analysis of PhlB revealed that it does not activate phenylacetic acid to a significant extent [62]. Recently, Yu et al. [151] reported the isolation of a third acyl-CoA ligase gene (*phlC*) that encodes an enzyme that showed high substrate specificity for medium and long fatty acids (similarly to PhlB) but also has some phenylacetic acid-activating activity. The three enzymes contain PTS1 targeting sequences and they seem to be located in peroxisomes.

In summary, the presently available information suggests that in *P. chrysogenum* there are at least three but probably up to eight acyl-CoA ligases related to biosynthesis of different natural penicillins (Table 2). A variety of natural substrates including different types of fatty acids and aromatic acids are activated by these enzymes. Other branched-chain fatty acids or very long-chain fatty acids may be degraded by β-oxidation and α-oxidation processes, being used finally by the different Phl enzymes as side-chains for biosynthesis of natural penicillins.

Isopenicillin N acyltransferase

One of the best known examples of peroxisomal localization of secondary metabolite biosynthetic enzymes is that of the isopenicillin N acyltransferase (IAT), the last enzyme of the penicillin biosynthetic pathway. Penicillins are formed by cyclization of the tripeptide δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) that is formed by a nonribosomal peptide synthetase [2]. An internal cyclization that results in the formation of the β-lactam and thiazolidine rings of penicillin is catalyzed by the isopenicillin N synthase [73, 107]. In the last step of penicillin biosynthesis, the α-aminoadipyl side-chain of isopenicillin N (IPN) is removed and replaced by a phenylacetyl (or other acyl) group, previously activated as their CoA derivatives by the action of the acyl-CoA ligases PhlA, PhlB, or PhlC. This replacement reaction is catalyzed by the IAT [3, 4].

The IAT is formed as a 40-kDa precursor protein (pro-IAT) which undergoes an autocatalytic process between residues Gly102 and Cys103. The two resulting protein fragments form a heterodimer composed of the α (11 kDa) and β (29 kDa) subunits [7]. We found that the *P. chrysogenum* proIAT is rapidly and efficiently self-processed into the α and β subunits.

Unlike the first two penicillin biosynthesis enzymes (ACV synthetase and IPN synthase) that are located in the cytoplasm, the IAT contains a consensus PTS1 signal and was observed to be located in peroxisomes [84, 85]. Indeed, when the ARL PTS1 targeting sequence of IAT is removed, the IAT remains in the cytoplasm and the IAT activity and the penicillin biosynthesis capability of the strain containing this modified nonperoxisomal IAT are drastically reduced [85]. It is likely that the cytoplasmic mislocalization of this enzyme is not adequate for self-processing and the mutant IAT protein is rapidly degraded.

The early observations using cell extract fractions were confirmed by inmunoelectron microscopy. A control strain

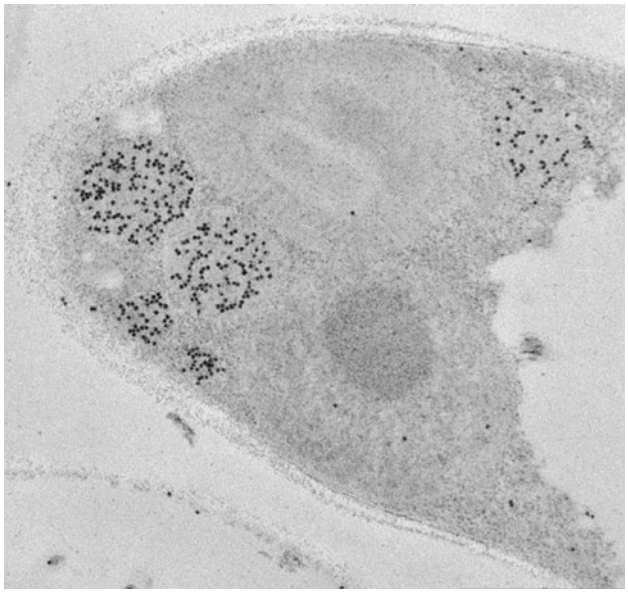

Fig. 2 Inmunoelectron microscopy photograph showing the localization of IAT in peroxisomes. The experimental procedures were those of García-Estrada et al. [35] (Photographs obtained by C. García-Estrada at the University of Groningen, The Netherlands)

lacking a functional IAT (*P. chrysogenum* npe6) [12] showed no immunodetectable IAT in the peroxisomes [84]. Mutants phenotypically similar to *P. chrysogenum* npe6 and showing reduced (or no) IAT activity arise with high frequency among survivors of induced mutagenesis of *P. chrysogenum* [12], suggesting that those mutants may have lost or reduced Pex-mediated efficiency of IAT targeting to the peroxisomal matrix. The peroxisomal localization of IAT has been confirmed by us using inmunoelectron microscopy [35] (Fig. 2). The targeting and transport of the IAT into peroxisomes is independent of the self-processing status of IAT, since we were able to prove that an unprocessable variant IAT^{C103S} is correctly targeted to peroxisomes in *P. chrysogenum* [35]. Coexpression of both the $penDE^{C103S}$ and wild-type *penDE* genes in *P. chrysogenum* led to a decrease in benzylpenicillin production. Changes in the wild-type IAT processing profile (quantified as β subunit formation) were observed in the Wis54-DE^{C103S} transformant, suggesting a regulatory role of the unprocessed IAT^{C103S} in the processing of the wild-type IAT. This was confirmed in *Escherichia coli*, where a delay in the processing of IAT in presence of the unprocessable IAT^{C103S} was observed. Our results indicate that IAT is posttranslationally regulated by its preprotein, which interferes with the self-processing [35].

In *A. nidulans*, another penicillin-producing fungus, the IAT is also targeted to peroxisomes, although the C-terminal ANI sequence of this protein [81] corresponding to the PTS1 targeting signal is somewhat abnormal [122]. Removal of this targeting signal resulted in mislocalization of the IAT to the cytoplasm. However, in contrast to what occurs in *P. chrysogenum*, this mislocalization does not fully block penicillin biosynthesis. Similar results were observed when penicillin production was studied in some *A. nidulans* mutants defective in peroxisomes, and the authors reported that in *A. nidulans* a small proportion of the IAT is located in the cytoplasm, which is different from what occurs in *P. chrysogenum* [122].

Important questions are why both the phenylacetyl-CoA ligases PhlA, PhlB, and PhlC [65] and the IAT are located in peroxisomes and the relevance of this compartmentalization for penicillin biosynthesis. The immunoelectron microscopy observations of Müller et al. [84, 85] and our own group [35] confirm that there is an elevated concentration of IAT molecules inside peroxisomes (Fig. 2). This elevated concentration of IAT and the peroxisome pH (slightly above 7.0) appear to be very favorable for IAT enzyme activity. Interestingly, an IAT-like enzyme (IAL) is encoded by a paralogous gene (*ial*) (Pc13g09140) located away from the penicillin gene cluster [36]. The IAL protein that is evolutively related to the IAT lacks the PTS1 signal at its C-terminal end, and is not targeted to peroxisomes. It does not contribute isopenicillin N acyltransferase activity for penicillin biosynthesis, and its physiological role in the cell remains unknown. Orthologous *ial* genes occur in many other ascomycetes.

Finally, the peroxisomal co-localization of the side-chain acyl CoA ligases (PhlA, PhlB, and PhlC) and the IAT provides a clear benefit for the coordination of the last step of penicillin biosynthesis. Due to the localization of these enzymes in peroxisomes, the import of IPN into these organelles is required and the secretion of benzylpenicillin, phenoxymethylpenicillin, and other natural penicillins from the peroxisomes to the culture medium may be limiting; this subject has been reviewed recently [75] and is not discussed here.

Role of *P. chrysogenum* peroxins in peroxisome stability and penicillin biosynthesis

Following the reports on the localization of the two enzymes of the penicillin biosynthesis pathway, attempts were made to clone and disrupt some of the *P. chrysogenum* peroxins, namely Pex1p, Pex6p [57], and Pex5p [58]. The *P. chrysogenum pex5* gene encodes a protein of 632 amino acids with molecular mass of 75 kDa similar to Pex5 proteins from other organisms. As indicated above, Pex5 acts as a receptor of PTS1-containing peroxisomal proteins.

Most of the *pex*5 disrupted clones showed slow growth and poor viability [58]. Although Pex5-defective mutants of yeasts (e.g., *H. polymorpha*) are viable, the

*P. chrysogenum pex*5 mutants may be more unstable than yeasts because peroxins are required for the formation of Woronin bodies, a special type of peroxisome that is essential to plug septa pores to prevent cytoplasm leakage following hypha damage. Similarly, attempts to obtain *pex*1 or *pex*6 null mutants were also unsuccessful [57]. The instability of the *P. chrysogenum pex*5 mutants has prevented, so far, detailed analysis of the role of the Pex5 protein in penicillin biosynthesis. However, evidence for the role of peroxins in penicillin biosynthesis was obtained by amplification of the peroxin gene *pex*11, which resulted in a twofold increase in penicillin production [59].

Peroxisomal enzymes involved in cephalosporin biosynthesis

Cephalosporins are broad-spectrum antibiotics produced by *A. chrysogenum* and a few other filamentous fungi that are widely and successfully used in medicine in the treatment of different bacterial infections. The cephalosporin biosynthetic pathway (Fig. 3a) begins with the nonribosomal condensation of the three precursor amino acids L-α-aminoadipic acid, L-cysteine, and L-valine to form the tripeptide ACV [2]. This reaction is carried out by the ACV synthetase [6] encoded by the *pcbAB* gene [41]. The tripeptide is cyclized to form IPN by the IPN synthase encoded by the *pcbC* gene [111]. The IPN, which has a weak antibacterial activity, may be secreted without passing through peroxisomes (thus ending a "penicillin pathway") or converted to cephalosporins in several steps, some of which take place in peroxisomes (the "cephalosporin pathway"). In the latter case, the IPN is isomerized to the D-configuration by enzymes encoded by two linked genes, *cefD1-cefD2*, giving rise to penicillin N (PenN) [74, 132], which is later converted to deacetoxycephalosporin C (DAOC) by a ring expansion step catalyzed by the deacetoxycephalosporin C synthase (encoded by the *cefEF* gene). This enzyme is also able to oxidize the methyl group at carbon 3′ of deacetoxycephalosporin C to give deacetylcephalosporin C (DAC) [20, 114]. Both activities are located in a single polypeptide that is encoded by the *cefEF* gene [112]. The last step of the cephalosporin biosynthesis pathway involves the conversion of DAC to cephalosporin C (CPC) by the DAC-acetyltransferase that is encoded by the *cef*G gene [42, 140] (Fig. 3a).

In *A. chrysogenum* strains, the genes for cephalosporin C biosynthesis are organized in two separate clusters located on different chromosomes (Fig. 3b) [43]. The "early" gene cluster, located on chromosome VII (4.6 Mb), contains the genes *pcbAB* and *pcbC* (encoding the enzymes for the first two steps of the pathway to form IPN) and *cefD1* and *cefD2*, responsible for the epimerization of IPN into PenN.

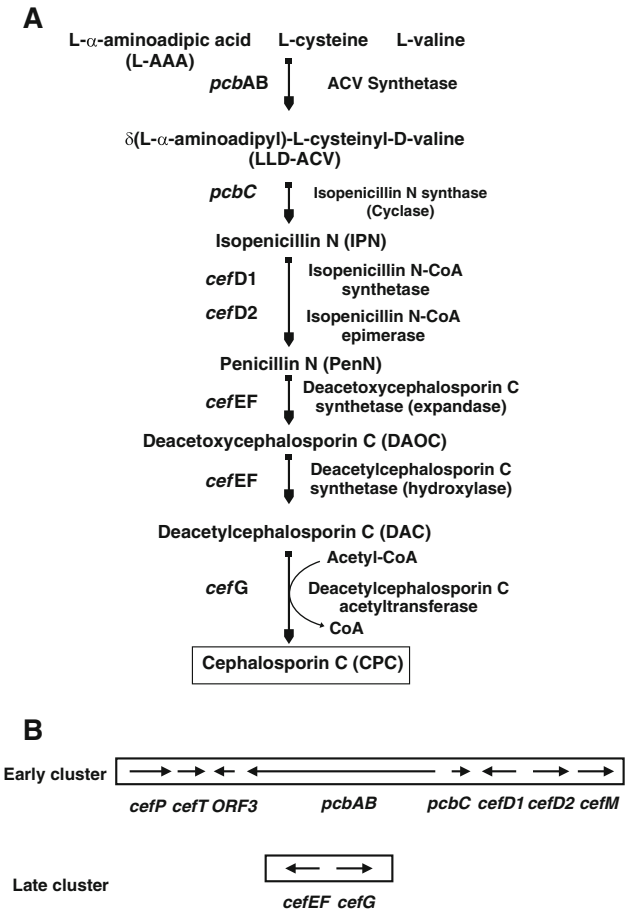

Fig. 3 Cephalosporin C biosynthetic pathway and organization of the two clusters of cephalosporin C biosynthesis genes in *A. chrysogenum*. **a** Biosynthesis pathway of cephalosporin C. **b** Cephalosporin C gene clusters. The "early" cluster includes the biosynthetic (*pcbAB*, *pcbC*, *cefD1*, and *cefD2*) and the transporter genes (*cefT*, *cefM*, and *cefP*). The "late" cluster includes *cefEF* and *cefG* genes

The "late" gene cluster is located on chromosome I (2.2 Mb) and contains the *cefEF* and *cefG* genes whose protein products are involved in the conversion of PenN to CPC.

In *A. chrysogenum*, it was initially reported that the enzymes of the cephalosporin biosynthesis pathway (ACV synthetase, IPN synthase, expandase/hydroxylase, and DAC acetyltransferase) have cytosolic location [27, 136, 139]. However, the central step of the cephalosporin C biosynthetic pathway (catalyzed by a two-component protein system encoded by the *cefD1* and *cefD2* genes) seems to be located in peroxisomes [132]. It is interesting that the epimerization step discriminates between the short "IPN pathway" resulting in secretion of IPN (a weak antibiotic) and the long "cephalosporin pathway" (that includes a continuation of the short pathway). Bioinformatic analysis of CefD1 and CefD2 amino acid sequences showed that both enzymes contain a peroxisomal targeting signal. CefD1 has

a C-terminal PTS1 (PRL), whereas CefD2 contains both PTS1 (EKL) and PTS2 (KLVVELAGL) signals [127]. Moreover, in early biochemical studies [68], it was reported that pH 7.0 is the optimum pH for conversion of IPN into PenN in *A. chrysogenum* cell-free extracts, which is coincident with the estimated pH of the peroxisomal lumen [139]. The presence of CefD1- and CefD2-related proteins (although not strict orthologs) in other fungi lacking the cephalosporin cluster suggests that *A. chrysogenum* has recruited and adapted these genes from other fungal functions or perhaps from nonfungal organisms by a horizontal gene transfer mechanism [43, 74].

The IPN epimerization system is similar to mammalian α-methylacyl racemases

The IPN epimerization enzymes CefD1 and CefD2 are homologous to enzymes involved in degradation of α-methyl fatty acids (Fig. 1) [74]. In animal cells, the racemases involved in the degradation of these branched fatty acids are used for their detoxification. In *A. chrysogenum*, they may act similarly, converting them to α-hydroxy-adipic acid, α-ketoadipic acid, and α-aminoadipic acid that are detoxified by binding them to 6-APA. The formation of adipyl- or glutaryl-6-APA is known in *β*-lactam-producing fungi, since these compounds are substrates for the ring-expanding enzymes in the in vivo biosynthesis of modified cephalosporins.

α-Methylacyl-CoA racemases are enzymes of branched-chain fatty acid degradation, some of which are involved in bile acid biosynthesis. They catalyze the racemization of a wide range of α-methyl branched-chain carboxylic acids activated as CoA-thioesters [144]. Physiological substrates of this enzyme are dietary branched fatty acids such as pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), which is an α-oxidation product of phytanic acid, itself derived from the degradation of chlorophyll in foods. The same enzyme is used for bile acid biosynthesis (see above). *β*-Oxidation of methyl branched-chain fatty acids and also the degradation of the cholesterol side-chain leading to bile acids occur in peroxisomes [30]. However, Kotti et al. [63] have shown that the same enzyme may be targeted to peroxisomes and mitochondria. The N-terminal sequence acts as a weak mitochondrial targeting sequence, whereas the C-terminal sequence contains a PTS1 signal.

Transport of cephalosporin intermediates into and out of peroxisomes

The epimerization of IPN to PenN in peroxisomes implies the presence of specific transport systems for both cephalosporin C biosynthetic intermediates across the peroxisomal membrane. On the basis of gene-clustering patterns for secondary metabolite biosynthesis [48] we identified in the early cephalosporin gene cluster some of these key metabolite transporters. Using this approach, the membrane proteins involved in transport of intermediates have been cloned and characterized.

Two peroxisomal transporters CefM and CefP in the cephalosporin cluster

We found two peroxisomal membrane transporters encoded by the *cefM* [127] and *cefP* genes [133]. These genes are located in the "early" cephalosporin gene cluster (Fig. 3b). Analysis of the CefM and CefP proteins by bioinformatic tools revealed the presence of a Pex19p binding domain in the amino acid sequences of both proteins. This domain is characteristic of the class I PMPs (see above) that are recruited by the Pex19 protein to be incorporated into the peroxisomal membrane [78, 108].

The *cefM* gene located upstream of *cefD1* (Fig. 3b) encodes an efflux pump protein (482 amino acids with a deduced molecular mass of 52.2 kDa) belonging to family 3 (drug efflux proteins) of the major facilitator superfamily (MFS) class of membrane proteins. A *cefM* knockout mutant showed a drastic reduction in extracellular PenN and CPC production, whereas it accumulated intracellular PenN. Confocal microscopy experiments with the GFP fluorescent CefM hybrid protein showed a probable microbody membrane location of the CefM protein [127].

The other key metabolite transporter gene known as *cefP* [133] is located upstream of the *cefT* gene [131] (Fig. 3b). The *cefP* gene encodes a protein of 866 amino acids with a deduced molecular mass of 99.2 kDa and with 11 putative transmembrane spanners. Targeted inactivation of the *cefP* gene resulted in a drastic reduction in the CPC and an accumulation of IPN in the culture broths. The lack of the CefP transporter in the disrupted strains blocks the conversion of IPN to PenN, indicating that the *cefP* gene is essential for cephalosporin C biosynthesis. In vivo fluorescence microscopy experiments using a functional DsRed–CefP hybrid protein indicated that the organelles initially described as microbodies [127] are authentic peroxisomes, since this hybrid protein co-localizes with the peroxisome-targeted reporter protein EGFP-SKL [133].

These results allowed a model for the transport of the intermediates IPN and PenN into and out of peroxisomes to be proposed (Fig. 4). This model starts with ACV and IPN synthesis in the cytosol by cytoplasmic ACV synthetase and IPN synthase, respectively [27]. The intermediate IPN is transported by the CefP protein from the cytosol to the peroxisomal matrix across the peroxisomal membrane, where it is converted to PenN by the two-protein (CefD1–CefD2) epimerization system [132, 133]. Later,

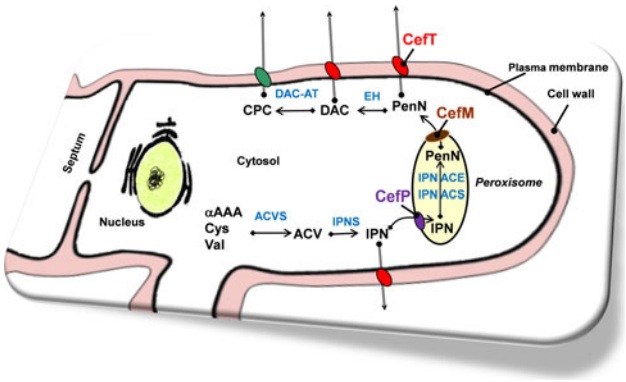

Fig. 4 Compartmentalization of the cephalosporin C biosynthetic pathway in *A. chrysogenum*. Proposed model describing the transport of intermediates into and out of peroxisomes, showing the localization of the CefT, CefM, and CefP transporters. Cys, L-cysteine, Val, L-valine; ACV, δ(L-α-aminoadipyl)-L-cysteinyl-D-valine; ACVS, ACV synthetase; 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, DAC acetyltransferase; CPC, cephalosporin C

the epimerization product PenN seems to be transported from the peroxisomal lumen to the cytosol by the CefM carrier [127]. Finally, the two last enzymes of the cephalosporin C biosynthetic pathway convert PenN to CPC in the cytosol [27, 136].

Peroxisomes and secondary metabolites in fungi and plants

Thousands of secondary metabolites are synthesized by diverse fungi and plants. Only in a few cases are there molecular and cell physiology studies on the localization of the biosynthetic enzymes. A few examples will be described here, although probably many others will appear in future scientific literature.

Peroxisomal enzymes involved in biosynthesis of mycotoxins in *Alternaria alternata*

Many fungal secondary metabolites are toxic to plants or animals (mycotoxins). The fungus *Alternaria alternata* produces plant toxins when it grows on pears, tangerines, and strawberries. The Japanese pear pathotype of *A. alternata* produces AK-toxin (epoxy-decatrienoic acid ester) that causes black spots in pears. Four genes involved in AK-toxin biosynthesis have been studied (*AK1*, *AK2*, *AK3*, and *AKR*). The first three genes encode proteins with PTS1 signals: SKL, SKI, and PKI, respectively. GFP-labeling studies showed that these proteins are targeted to peroxisomes [51]. A mutant of *A. alternata* defective in the peroxin Pex6 was found to be impaired in the localization of these enzymes to peroxisomes and failed to produce AK-toxin. This mutant was unable to utilize fatty acids and showed reduced conidiation and smaller appresoria. All these results confirmed that the biosynthesis of AK-toxin and the oxidation of fatty acids are parallel processes performed by peroxisomal enzymes.

Peroxisomal enzymes involved in jasmonic acid biosynthesis

Jasmonic acid [(1*R*,2*R*)-3-oxo-2-(2*Z*)-2-pentenyl-cyclopentaneacetic acid] is a well-known regulatory molecule of plant secondary metabolites synthesized by different plants [17]. It induces the biosynthesis of different secondary metabolites that are involved in plant tissue differentiation and in defense responses against bacterial infections or fungal attacks.

Jasmonic acid is synthesized either through the so-called octadecanoic acid pathway starting from linolenic acid or by the hexadecanoic acid pathway from hexadecatrienoic acid (C16:3) [130, 145]. In subsequent steps the initial precursors are converted to cyclopentenone 12-oxo-phytodienoic acid (OPDA) or dinor-OPDA, respectively.

For conversion to jasmonic acid, OPDA (or an OPDA derivative) has to be activated and transported into peroxisomes, where it is reduced to 3-oxo-2(2'-pentenyl)-cyclopentane-1-octanoic acid by the enzyme OPDA reductase-3. Schneider and coworkers [116] reported that in *Arabidopsis thaliana* a new type of acyl-CoA synthetase belonging to the *p*-coumaroyl-CoA ligase family has the capacity to activate the 12-oxo-phytodienoic acid intermediate of jasmonic acid (enzymes At4g05160 and At5g63380). Fluorescent labeling of these proteins showed that they are targeted to leaf peroxisomes. Therefore, these enzymes start the activation process that shortens by β-oxidation the side-chain of the fatty acid precursors leading to jasmonic acid formation. Interestingly, these enzymes are related to the phenylacetyl-CoA ligases of *P. chrysogenum* in their substrate binding pocket.

Peroxisomal enzymes shorten indolebutyric acid to indoleacetic acid during auxin biosynthesis in *Arabidopsis*

The plant hormones indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are two natural plant auxins that are involved in the control of plant growth including vascular development, apical dominance, root elongation, and lateral root initiation among other processes [24, 153]. IBA is often more effective than IAA in lateral root initiation, although IBA is less abundant than IAA in plants. The in vivo ratio of IBA to IAA is important for commercial applications in plant development.

For many years, the biosynthetic relationship between IAA and IBA remained obscure [153]. Chain-length derivatives of IAA with an even number of carbon atoms have auxin effects and they are shortened stepwise in two carbons by cell extracts of different plants, suggesting that IAA may be synthesized from intermediates containing fatty acid chains of even number of carbon atoms. This shortening mechanism was shown to be mediated by peroxisomal enzymes through a pathway similar to fatty acid β-oxidation [46].

Several peroxisome-defective mutants (*ped*) of *Arabidopsis* have been isolated by different authors. Zolman et al. [153] studied several of those mutants that were classified into four groups. They failed to convert IBA into IAA, and these authors proposed that IBA is converted in vivo to IAA by peroxisomal enzymes. One of the four mutant classes was shown to carry a mutation in the *pex5* gene encoding a peroxisomal-targeting protein. Therefore, IBA and perhaps analogs with side-chains of six (or more) carbon atoms are formed as intermediates of IAA in vivo, subsequently being shortened by peroxisomal enzymes to yield IAA.

Conversion of cinnamic acid to salicylic acid

Another putative secondary metabolism peroxisomal process is the conversion of cinnamic acid into salicylic acid during biosynthesis of the latter compound in *Arabidopsis*. Salicylic acid is a signaling metabolite involved in control of thermotolerance and temperature sensitivity, and in systemic acquired resistance in plants. It is formed from cinnamic acid by decarboxylation and shortening of the side-chain to benzoic acid followed by hydroxylation of benzoic acid at the *meta* position to form salicylic acid. *Trans*-cinnamic acid is previously formed by nonoxidative decarboxylation of phenylalanine.

It has been proposed that cinnamic acid is activated to cinnamoyl-CoA by one of the numerous peroxisomal *p*-coumaroyl-CoA ligase-like proteins existing in *A. thaliana* and that shortening of the side-chain occurs by β-oxidation in peroxisomes [90]. However, there is no biochemical or genetic evidence to support this proposal. Many aromatic acid derivatives similar to cinnamic acid or phenylacetic acid are precursors of fungal and plant secondary metabolites in nature, and at least some of them are likely to be partially synthesized or modified by peroxisomal enzymes.

Future outlook

In the last two decades, impressive progress has been made on the omics of the β-lactam-producing strain *P. chrysogenum*. The genome of *P. chrysogenum* Wis54-1255 was reported [135], and soon afterwards a comprehensive article was published on the proteomics of *P. chrysogenum* in relation to changes during strain improvement [53].

Moreover, there is good background knowledge on the classical genetics [9, 72] and biochemistry of the enzymes involved in penicillin biosynthesis (reviewed in [75]). However, the field of the cell biology of β-lactam-producing fungi has lagged behind the molecular biology advances. The compartmentalization of some steps of the biosynthesis of secondary metabolites, which are related to oxidation and degradation of toxic precursors, is documented in a few cases, but there is no information for the thousands of secondary metabolites synthesized by fungi and plants. The GFP-tagged protein targeting and inmunoelectron microscopy techniques are useful tools to clarify the localization of the different biosynthetic enzymes.

The compartmentalization of some secondary biosynthetic steps, but not the entire biosynthetic pathway, implies transport of biosynthetic intermediates into and out of the peroxisomes (or other types of microbodies). These transport systems need to be studied in more detail, since they are probably limiting steps for secondary metabolite biosynthesis and secretion. The tools for these studies (e.g., peroxisome isolation and separation from other organelles) need to be improved to progress in this field.

Acknowledgments This article was supported by grants from the European Union (Eurofungbase LSSG-CT-2005-018964) and EUROFUNGBASE. We thank M. Veenhuis and K. Sjollema (University of Groningen) for the microscopy studies and B. Martín, J. Merino, and A. Mulero for their excellent technical assistance.

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