Review

Juan-Francisco Martín*, Carlos García-Estrada and Ricardo V. Ullán

Transport of substrates into peroxisomes: the paradigm of β-lactam biosynthetic intermediates

Abstract: Peroxisomes are ubiquitous organelles that enclose catalases, fatty acid-oxidizing enzymes, and a variety of proteins involved in different cellular processes. Interestingly, the late enzymes involved in penicillin biosynthesis, and the isopenicillin N epimerization enzymes involved in cephalosporin biosynthesis are located inside peroxisomes in the producer fungi *Penicillium chrysogenum* and *Acremonium chrysogenum*. Peroxisome proteins are targeted to those organelles by peroxisomal targeting signals located at the C-terminus (PTS1) or near the N-terminal end (PTS2) of those proteins. Peroxisomal membrane proteins (PMPs) are largely recruited by the interaction with specific sequences in the Pex19 protein. The compartmentalization into peroxisomes of several steps of the biosynthesis of penicillin, cephalosporin, and other secondary metabolites raises the question of how the precursors and/or intermediates of the biosynthesis of β-lactam antibiotics are transported into peroxisomes and the mechanisms of secretion of the final products (penicillin or cephalosporin) from peroxisomes to the extracellular medium. Recent advances in peroxisome proteomics, immunoelectron microscopy, and fluorescence labeling have shown that the transport of these intermediates is mediated by membrane proteins of the major facilitator superfamily class (drug/H^+ antiporters) containing 12 transmembrane-spanning domains (TMS). In some cases, the transport of the substrates (e.g., fatty acids) or intermediates may be mediated by ATP-binding cassette (ABC) transporters. Knowledge on the transport and secretion mechanisms is of paramount importance to understand the complex mechanisms of cell differentiation and their crosstalk with the biosynthesis of different secondary metabolites that act as biochemical signals between the producer cells and also as communication signals with competing microorganisms (e.g., antimicrobial agents or plant elicitors).

Keywords: β-lactams; biosynthesis enzymes; drug/H^+ antiporters; major facilitator superfamily; peroxisomes; transport of intermediates.

***Corresponding author: Juan-Francisco Martín**, Area of Microbiology, Department of Molecular Biology, University of Leon, E-24071 León, Spain, e-mail: jf.martin@unileon.es

Carlos García-Estrada and Ricardo V. Ullán: INBIOTEC, Institute of Biotechnology of Leon, Avenida Real no. 1, Parque Científico de León, E-24006 León, Spain

Introduction

Peroxisomes, initially known as microbodies, are ubiquitous eukaryotic organelles with a diameter of 0.1–1 μm. They are found in mammalian kidney and liver cells, plants (especially in leaf photosynthetic cells closely associated with chloroplasts and mitochondria), filamentous fungi, yeasts, and certain protozoans. These organelles do not contain DNA and have a single bilayer membrane (about 7 nm) that surrounds a core matrix with peroxisomal proteins (1, 2). The latter play different essential metabolic functions, such as oxidation of fatty acids, oxidation and epimerization of branched-chain fatty acids, catabolism of D-amino acids and polyamines, metabolism of reactive compounds, bile acids synthesis in vertebrates, photorespiration in plants, or biosynthesis of some secondary metabolites (3–5). Their content in oxidases and catalases was the reason why they were designated as peroxisomes during the decade of the 1960s (6). As it can be deduced from the metabolic reactions that occur inside peroxisomes, toxic organic compounds and oxidized metabolites are transported into these organelles. There, these compounds are harmless to the rest of the cell, and to be eliminated, they are coupled to carrier molecules and finally secreted out of the cells (see below). In the case of branched or linear fatty acids, they are usually fully degraded to acetyl-CoA or propionyl-CoA, although in some cases, partially oxidized fatty acids are attached to other molecules and secreted.

In addition to peroxisomes, certain organisms possess peroxisome-related organelles, which carry out additional specific functions. This is the case of glycosomes, Woronin bodies, or glyoxysomes (the distinction between glyoxysomes and peroxisomes is currently controversial). Glycosomes are characteristic of trypanosomatids and

contain glycolytic enzymes for the Embden-Meyerhof segment of glycolysis (7). Woronin bodies are unique to filamentous fungi. They possess a double membrane and are localized near the septae, where they play an important role in sealing the septal pore after hyphal injury, thus preventing cytoplasmic bleeding (8). Peroxisome matrix proteins are synthesized in the free ribosomes in the cytosol before being imported into peroxisomes, which is mediated by a conserved peroxisomal targeting signal (PTS) included either at their C-terminus (PTS1) or near the N-terminus (PTS2) (9). In addition to peroxisomal matrix proteins, which play essential roles in different metabolic pathways, peroxisomes also contain other proteins termed peroxins. These peroxins (over 30 different proteins described so far) are encoded by the PEX genes and are involved in the peroxisomal matrix protein import, targeting and insertion of peroxisomal membrane proteins (PMPs), inheritance, and regulation of the peroxisomal number and size (1, 9).

Peroxisomes are dynamic organelles with plastic morphology that adjust their number and shape according to the environment-determined metabolic needs. This is favored by the properties of the peroxisomal membrane, which is formed mainly from the endoplasmic reticulum, although mitochondria are also involved in the biogenesis of the peroxisome membrane (2). This adaptation to the metabolic needs is mediated by a dynamic balance between peroxisomal biogenesis (augmentation) and degradation (autophagy).

Peroxisomal matrix proteins: recruiting, targeting, and import

Enzymatic activities occurring in peroxisomes, including those for the biosynthesis of fungal secondary metabolites, are catalyzed by different peroxisomal matrix proteins. They are usually peroxisome-specific, although some of these proteins can have two or more subcellular localizations due to the presence of several targeting signals within the same protein, e.g., 3-hydroxymethylglutary-CoA lyase (10) and α-methylacyl-CoA racemase (11). The β-oxidation pathway of fatty acids has a dual peroxisomal and mitochondrial localization in mammals and plants, but it is exclusively peroxisomal in yeasts (12) and filamentous fungi because mutants devoid of peroxisomes are unable to grow on oleate (13, 14). Proteins can be imported into peroxisomes either as folded monomers or as oligomers (15), irrespective of the processing state. For example, the isopenicillin N-acyltransferase (IAT, a peroxisomal enzyme catalyzing the last step of penicillin biosynthesis) can be transported inside peroxisomes either as the active heterodimer or as the unprocessed monomer (16).

Peroxisomal matrix proteins are not the only proteins present in peroxisomes, for they also include proteins integrated in the peroxisomal membrane. Both types of proteins are synthesized in cytoplasmic ribosomes, and therefore, they have to be post-translationally imported either into the peroxisomal matrix or into the membrane. The import machinery is specific for each of these two types of proteins.

The presence of a consensus PTS at the C-terminus of the amino acid sequence is the main mechanism determining the location of a peroxisomal matrix protein. However, not all proteins containing a PTS are necessarily sorted to peroxisomes, as it has been reported for the cytosolic human phosphomevalonate kinase (17). In addition, cryptic sequences inside proteins can also act as signals recognized by the peroxisome import machinery (see below).

Two types of PTSs (PTS1 and PTS2) have been described so far, the proteins containing a PTS1 being more abundant than those with a PTS2 sequence. PTSs differ in their amino acid composition and in their position within the protein. The PTS1 motif consists of the consensus sequence SKL or variations thereof (S/A/C)-(K/R/H/N)-(L/I/M) and is located on the C-terminal region of the protein, whereas PTS2 is represented by the consensus sequence (R/K)-(L/I/V)-X_5-(Q/H)-(L/A) and is present near the N-terminal region of the protein (18–20). It has been reported that amino acid residues around a PTS signal may increase the affinity between the protein and its receptor, and therefore, although not directly involved in sorting, they are also important for targeting (21–24).

Two different peroxins mediate the import of PTS1 and PTS2 peroxisomal proteins. PTS1 is recognized by peroxin Pex5, whereas PTS2 is detected by peroxin Pex7 (25, 26). 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 (27, 28). It has also been reported that internal cryptic targeting sequences can also serve as PTS, and therefore, some peroxisomal matrix proteins lacking the canonical PTS1 are sorted to peroxisomes *via* Pex5 (29). The piggyback transport is another way to import proteins to the peroxisomal matrix. By means of this system, those proteins that lack a PTS form a complex with a PTS-containing protein and are sorted to peroxisomes in a Pex5-dependent manner (29, 30).

Although the components of the import machinery are relatively well known, the molecular mechanism of protein translocation across the peroxisomal membrane is still poorly understood. Membrane invagination events (similar to endocytosis), vesicle fusion processes, or dynamic transient pore formation have been proposed as models for protein translocation, where Pex5 and Pex14 form a dynamic gated ion-conducting channel of about 9 nm induced by the cytosolic receptor-cargo complex (31). Once the PTS1-bearing protein has been recognized and bound in the cytosol by Pex5 (Pex5-protein complex), the resultant complex binds to PMPs Pex13, Pex14, and Pex17 (the latter only identified in yeasts) (32, 33), which constitute the docking complex. Then, the cargo is released into the peroxisomal matrix. This translocation process requires the RING-finger ubiquitin ligases Pex2, Pex10, Pex12 (34–39), and Pex8 (the latter only identified in yeasts) (40). Pex5 is released from the membrane in an ATP- and ubiquitin-dependent manner (mediated by the Pex4 ubiquitin-conjugating enzyme in yeasts or UbcH5a/b/c in humans) (41–43) to the cytosol, where it is either degraded or recycled for a new import round (27, 44). The 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 (45–49).

Peroxisomal membrane proteins

PMPs are encoded by nuclear genes, translated on cytoplasmic ribosomes, and imported post-translationally into the peroxisomal membrane. The import machinery of PMPs is distinct from the peroxisomal matrix proteins because most *pex* mutations that disrupt matrix protein import still showed a functional PMPs import (50–52). In general, two import pathways are proposed for the targeting of PMPs (53). In the first pathway, PMPs are directly inserted into existing peroxisomal membranes after being synthesized in the cytosol (54). Alternatively, in the second pathway (55, 56), PMPs can be synthesized on rough endoplasmic reticulum where they are concentrating in pre-peroxisomal vesicles. These vesicles function as an origin for *de novo* formation of peroxisomes or are fused with the peroxisomal membrane of existing peroxisomes.

As consequence of these two import pathways, PMPs can be divided into two classes (57) named class I (for the first pathway) and class II (for the second pathway). Whereas class I corresponds to PMPs that are imported to peroxisomes *via* a Pex19p-dependent pathway, PMPs of class II are targeted to peroxisomes independently of Pex19p (58).

The peroxisomal membrane protein targeting signals (mPTS) were identified for several PMPs and contain a basic amino acid sequence in conjunction with at least one transmembrane region (59, 60).

The class I PMPs possess a mPTS that is recognized in the cytosol by the peroxin Pex19p. This PMP-recruiting peroxin is a farnesylated protein mostly located in the cytosol and partly associated with the peroxisomal membrane. Pex19p works like an import receptor and/or chaperone required for stabilization of PMPs at the peroxisomal membrane (61).

Once recognized, the mPTS motif of the PMP, Pex19p directs the Pex19p-PMP complex to the peroxisomal membrane by docking to its membrane anchored binding partner Pex3p (62). Next, the PMP is inserted into the peroxisomal membrane probably with the assistance of Pex19p and Pex3p (the Pex16p collaboration is also necessary in some organisms). Finally, Pex19p returns to the cytosol where the PMPs importation process is restarted (57).

A minor proportion of the PMPs belong to the group of class II PMPs, proteins that 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 domain without a Pex19p binding site (63, 64).

Nowadays, it is known that Pex3p, Pex16p, and Pex22p belong to the class II PMPs (64, 65). Nevertheless, the molecular mechanisms responsible for the import of these proteins to the endoplasmic reticulum, and its later targeting to the peroxisomal membrane are still poorly understood (64, 66–68).

Transport of metabolites into peroxisomes

Two clearly different classes of transporters are known to introduce small metabolites into peroxisomes, namely the ATP-binding cassette (ABC) transporters, which rely on ATP hydrolysis to obtain energy for the transport, and the major facilitator superfamily (MFS) transporters (also known as secondary transporters), which translocate small solutes using the energy of the electrochemical gradient across the membrane (69).

The chemical nature of the metabolites that need to pass across the peroxisomal membrane can be predicted based on our current knowledge of the pathways that

occur in these organelles and the peroxisomal metabolism. The transport processes across this membrane is facilitated by several metabolite transporters. Indeed, an increasing number of transporters are being identified in the peroxisomal membrane of various species (70); this is surprising because the content of proteins in the peroxisomal membrane is limited, as shown by freeze-etch electron microscopy studies of fungal cell peroxisomes (4).

Peroxisomal ABC transporters

ABC transporters constitute an ubiquitous superfamily of integral membrane proteins that perform the ATP-powered translocation of a wide variety of substrates across extracellular and intracellular membranes (71). Typical ABC transporters consist of homologous halves, each containing a membrane-spanning domain (MSD), with multiple hydrophobic transmembrane helices, and a hydrophilic cytosolic nucleotide-binding fold (NBF), also known as the ATP binding domain. The NBF domain contains the highly conserved motifs: Walker A and Walker B (separated by about 90–120 amino acids) and the ABC signature motif situated upstream of the Walker B sequence (72, 73). A functional ABC protein typically comprises two MSDs containing several α-helices and two NBFs. In eukaryotic cells, these domains are generally organized as two half-size MSD transporters (with the topology MSD-NBF or NBF-MSD) or as full-size transporters (MSD-NBF-MSD-NBF or NBF-MSD-NBF-MSD) (72).

The development of genomic approaches has provided valuable information for the complete identification of ABC transporter genes in representative genomes of all major phyla. Based on the organization of domains and amino acid homology, the ABC transporter superfamily can be subdivided into seven subfamilies: ABC-A, ABC-B, ABC-C, ABC-D, ABC-E, ABC-F, and ABC-G (74).

Peroxisomal ABC transporters are mainly involved in the metabolism of lipids and formation of bioactive lipid metabolites (75). The ABC-D subfamily contains predominantly half-size transporters with the orientation MSD-NBF, which homodimerize and heterodimerize to form transporters of fatty acids (76). In mammals, it is thought that ABC-D1 functions as a transporter of very-long-chain fatty acids across the peroxisomal membrane, whereas, ABC-D2 has a certain functional redundancy with ABC-D1, but it performs other specific roles in lipid metabolism. Finally, in mammals, the most abundant PMP is the ABC-D3, which is capable of transporting various fatty acids (72). In yeasts, the ABCD-like genes pxa1 (also known as PTA1 or PAL1) and Pxa2 (also known as PAT2 or YKL741) are also involved in the oxidation of very-long-chain fatty acids (77).

In the plant kingdom, the transport of a variety of substrates such as fatty acids and plant hormone precursors into peroxisomes is mediated by PXA1 (peroxisomal ABC transporter 1), ACN2 (acetate nonutilizing 2) and AtPMP2 (*Arabidopsis thaliana* PMP2), AtABCD1 (also known as COMATOSE, or CTS), and PED3 (peroxisome defective 3) (76, 78).

Other ABC-D transporters have been characterized in other organisms, such as the fungus *Podospora anserina* (pABC1 and pABC2), the nematode *Caenorhabditis elegans* (pmp1, pmp2, pmp3, pmp4, and pmp5), and the protozoa *Trypanosoma brucei* (GAT1, GAT2, and GAT3) (78) and *Dictyostelium discoideum* (ABC-D.1, ABC-D.2, and ABC-D.3).

To understand the role of peroxisomal ABC transporters in cellular functions, it will be necessary to understand in more detail the transport of substrates across the peroxisomal membrane; this may be achieved by X-ray crystallographic studies together with a deeper knowledge of the regulation of these transporters by membrane proteomic approaches.

MFS transporters

The proteins of the MFS class are single-polypeptide secondary carriers that are capable of transporting small solutes and use the transmembrane electrochemical gradient of protons or Na^+ ions to drive the extrusion of drugs from the cell (69, 79). This superfamily of transport proteins currently consists of 74 families, each usually concerned with the transport of a certain type of substrate (80). MFS proteins possess mainly either 12 or 14 transmembrane spanning regions (TMS) (Figure 1) with capacity to transport a wide array of small molecules that include simple monosaccharides, oligosaccharides, drugs, amino acids, peptides, metabolites, enzyme cofactors, vitamins, nucleobases, nucleosides, nucleotides, iron chelating compounds (siderophores), and both inorganic and organic anions and cations (80).

Although the role of ABC transporters in the introduction of metabolites into peroxisomes has been studied in more detail (72), the involvement of MFS transporters has received less attention. However, several recent studies have found MFS proteins containing 12 TMS that are located in the peroxisomal membrane.

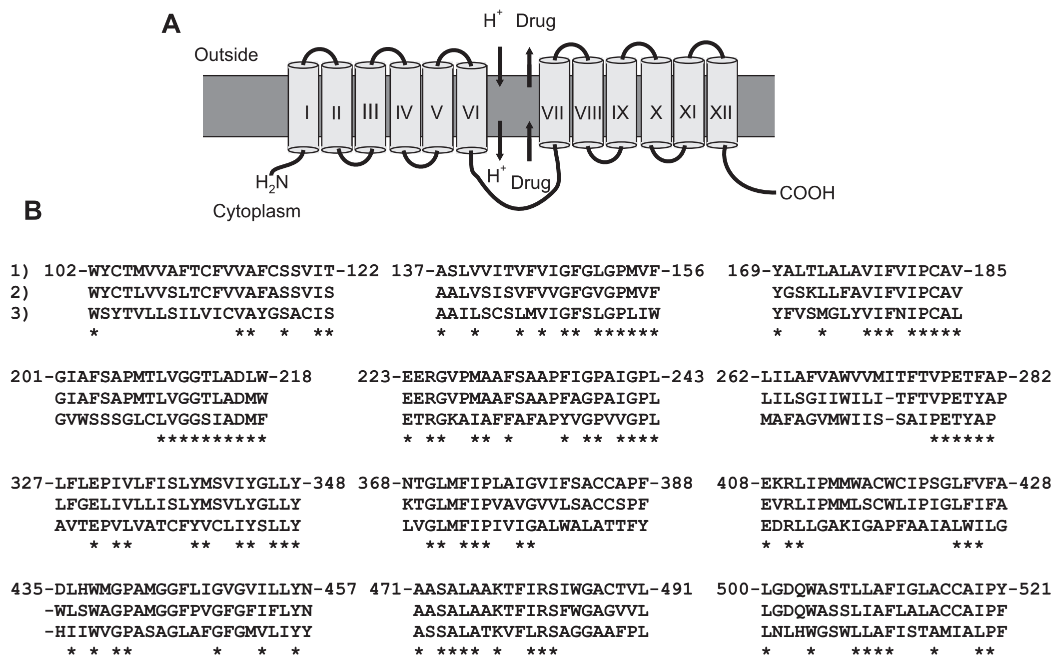

Figure 1 Model of the 12-TMS drug/H+ antiporters in cytoplasmic membranes.
(A) Note the cytoplasmic loop between TMS VI and VII that has been proposed to be involved in drug binding (101). (B) Alignment of the amino acids included in the 12 TMS of (1) PaaT of *P. chrysogenum*, (2) CefT of *A. chrysogenum*, and (3) TPO2 of *S. cerevisiae* (see text for details). The amino acid numbers correspond to the position in the PaaT protein of *P. chrysogenum*.

Drug/H+ antiporters: pumps for secondary metabolites

Many of the MFS transporters are classified as multiple drug resistance (MDR) proteins because they confer resistance to several drugs or components that are toxic for animal, plant, or microbial cells. These transporters that act as drug efflux pumps frequently confer resistance to chemically unrelated drugs. Their relevance has increased in the last decades due to the appearance of resistance to many antibiotics and other drugs used for the treatment of human and animal diseases (81). In yeasts, many drugs are excreted by MFS transporters that function by a drug/H+ antiporter mechanism.

An important question is what the role of drug/H+ antiporters in nature is. There is no doubt that these transporters are used to extrude toxic compounds from animal cells, although only some of the transported compounds are synthesized by animal cells. Most of them are plant or microbial secondary metabolites that enter the animal organisms within the food, sometimes as animal feed contaminants. Our human society uses many of these compounds in pharmacology for the treatment of a variety of diseases. Therefore, these drugs need to be removed from our tissues to avoid the toxic effect derived from prolonged action or from acute intoxications.

Light on the role of these transporters has been provided by the finding that many of these transporters that confer resistance to antibiotics are encoded by genes located on antibiotic biosynthesis clusters (82). With the advances in genomics, more and more transporters have been found in the gene clusters for antibiotics (69), mycotoxins (e.g., roquefortine C) (83), and other secondary metabolites. In antibiotic-producing bacteria that lack internal organelles, these MFS transporters (or in some cases ABC transporters) are located in the cell membrane and pump out the toxic antibiotics to avoid suicide of the producing cells (84). In filamentous fungi and plants that also produce a large number of diverse extracellular secondary metabolites, some of these transporters are located in the plasma membrane acting as authentic drug pumps releasing metabolites to the culture medium, but in some other cases, these MFS transporters are located in the peroxisomal membrane and serve to introduce biosynthetic intermediates into the peroxisomal lumen and/or to export the formed metabolites again to the cytoplasm for further conversion to the final products or directly to the culture medium.

An increasing number of secondary metabolites are partially synthesized or modified in peroxisomes (Table 1), and therefore, the implication of these transporters in the

context of peroxisome-located activities is acquiring great relevance.

The most advanced knowledge on molecular mechanisms of the drug/H+ antiporters is that of the yeast *Saccharomyces cerevisiae* (101). Although this yeast produces very few secondary metabolites (e.g., bisformyl-dityrosine) as compared with filamentous fungi (probably due to its adaptation to sugar-rich specific habitats with little nutritional variability), this yeast still contains a limited number of MFS transporters.

The MFS drug/H+ antiporters (DHAs) of yeasts are classified into two types: DHA1 and DHA2 (101). The DHA1 family includes 12 proteins of *S. cerevisiae* each containing 12 transmembrane spanners, whereas the 10 known protein members of the DHA2 family contain 14 transmembrane spanners.

All members of the yeast DHA1 and DHA2 families confer resistance to different antifungal drugs, but only in a few cases is the natural substrate of the protein known. For example, the class DHA1 member DTR1 transports bisformyldityrosine through the prospore membrane during the maturation of *S. cerevisiase* ascospores (102).

Several of the DHA2 protein family members are located in the vacuolar membrane (101), but it is unknown whether any of these proteins are initially targeted to peroxisomes because peroxisomal proteins are frequently engulfed into vacuoles by pexophagy processes.

Other members of the DHA1 family (TPO1, TPO2, TPO3, and TPO4) have been reported to be polyamine transporters, and a related MFS protein of *Penicillium chrysogenum* has been recently found to be located in the peroxisomal membrane in this fungus (103) (Figure 1). However, a note of caution has to be kept in mind because part of this protein (PaaT) may be degraded and targeted to vacuoles.

Peroxisomes provide an optimal microenvironment for penicillin (or other secondary metabolites) biosynthesis

The localization in peroxisomes of the enzyme systems for the biosynthesis of secondary metabolites derives from evolutive advantages of an adequate microenvironment provided by the following factors:

– Precursors for the biosynthesis of secondary metabolites, particularly those for polyketides (acetyl-CoA, malonyl-CoA, methylmalonyl-CoA) formed by catabolism of fatty acids are abundant in peroxisomes (104, 105). Also, the geranyl-geranyl-diphosphate for isoprenoid biosynthesis is formed in peroxisomes (96).
– The peroxisomal internal pH is optimal for some enzymes involved in secondary metabolism. The pH of the cytosol in filamentous fungi has been reported to be between 6.5 and 7.0 (106), whereas the internal pH of the peroxisomes was shown to be 7.5 (107). This peroxisomal pH is near the optimal pH for the IAT, which was found to be 7.5–8.0 (108, 109), for phenylacetyl-CoA ligase (86) and for the linear fatty acid CoA ligase (88).

In summary, it seems that evolution has adopted certain enzymes involved in biosynthesis of secondary metabolites to work in a controlled environment as is that of peroxisomes.

Table 1 Enzymes for secondary metabolite biosynthesis located in peroxisomes fungi, plants, and animal cells.

Secondary metabolite	Organism	Peroxisomal enzymes	References
Penicillin	*P. chrysogenum*	1. IAT	(16, 85)
		2. Phenylacetyl-CoA ligase (PhlA)	(86)
		3. Linear acyl-CoA ligase (PhlB)	(87, 88)
Cephalosporin C	*A. chrysogenum*	1. IPN-CoA ligase (CefD1)	(5, 89)
		2. IPN-CoA epimerase (CefD2)	(5, 89)
AK toxin	*Alternaria alternata*	AK1, AK2, AK3 Norsolorinic acid synthetase(s)	(90–92)
Aflatoxins	*Aspergillus*	Norsolorinic acid synthetase(s)	(93, 94)
Paxilline	*Penicillium paxilli*	Geranyl-geranyl-diphosphate synthase (PaxG)	(95, 96)
Jasmonic acid	*A. thaliana*	1. Cyclopentenone 12-oxo-phytodienoic acid (OPDA)-CoA ligase	(97)
		2. OPDA reductase	
Indoleacetic acid	*A. thaliana*	Indolebutyric acid β-oxidation enzymes	(49, 98)
Salicylic acid	*A. thaliana*	1. Cinnamoyl-CoA ligase	
		2. Cinnamoyl-CoA shortening enzymes	(99)
Bile acids	Animal cells (rats and humans)	Side-chain shortening and epimerizing enzymes	(100)

The last steps of penicillin biosynthesis are performed in peroxisomes

It is now well established that the last steps of penicillin biosynthesis performed by the IAT and the phenylacetyl-CoA ligase occur in peroxisomes (16, 85, 86, 110). These enzymes are synthesized in ribosomes in the cytoplasm and targeted to the peroxisomal matrix in a PTS1-dependent manner.

The early enzymes of the penicillin pathway α-aminoadipyl-cysteinyl-valine (ACV) synthetase and the β-lactam ring forming isopenicillin N (IPN) synthase are located in the cytoplasm (111), although the ACV synthetase may be associated to vacuoles (112). This means that transport of the intermediate IPN into the peroxisomes is a prerequisite for the last step to take place.

A small amount of IPN is secreted to the extracellular culture and is therefore lost for penicillin biosynthesis (113). At this time, it is unknown if the transporter that introduces IPN into perosixomes is the same one that secretes IPN out of the cell. The possibility that a small fraction of the IPN transporter is mistargeted to the secretory vesicles membrane or in the plasma membrane rather than to the peroxisomal membrane cannot be excluded. Another precursor of penicillin that has to be transported from the cytosol to the peroxisomal matrix is the penicillin side-chain precursor phenylacetic acid (PAA). A gene, *paaT*, encoding a phenylacetic acid transporter has been recently characterized. It encodes a drug/H^+ antiporter of 12 TMSs (Figure 1) that has been shown to be located in the peroxisomal membrane by fluoresce targeting microscopy (103). The same transporter is likely to introduce phenoxyacetic acid (POA) or related arylacetic acids into peroxisomes, whereas linear fatty acids (e.g., adipic acid, octanoic acid, decanoic acid, or dodecanoic acid) have transporters related to those of fatty acids (105).

Role of two peroxisomal membrane transporters in cephalosporin C production in *Acremonium chrysogenum*

Cephalosporin C and semisynthetic cephalosporins are widely and successfully used in medicine in the treatment of different bacterial infections. Cephalosporin C belongs to the class of broad-spectrum antibiotics obtained from cultures of the filamentous fungus *A. chrysogenum* (114, 115). The cephalosporin C biosynthetic pathway begins with the non-ribosomal condensation of the three precursor amino acids L-α-aminoadipic acid, L-cysteine, and L-valine to form the tripeptide ACV (116). This reaction is carried out by the ACV synthetase (117, 118), which is encoded by the *pcb*AB gene (119). The ACV tripeptide is cyclized to form IPN by the IPN synthase encoded by the *pcb*C gene (120). The IPN is then isomerized to the D-configuration by two linked genes, *cefD1-cefD2*, giving rise to penicillin N (PenN) (89, 121), which is later converted to deacetoxycephalosporin C (DAOC) by a ring expansion step catalyzed by the DAOC synthase (encoded by the *cefEF* gene). The latter enzyme is also able to oxidize the methyl group at carbon 3′ of DAOC to give deacetylcephalosporin C (DAC) (122, 123). Both activities are located in a single polypeptide that is encoded by the *cefEF* gene (124). The last step of the cephalosporin biosynthesis pathway involves the conversion of DAC to cephalosporin C by the DAC-acetyltransferase that is encoded by the *cef*G gene (125, 126).

In *A. chrysogenum*, the genes that encode β-lactam antibiotics biosynthesis and secretion are linked in two clusters that are located on separate chromosomes. In the *A. chrysogenum* ATCC 48272 strain, the *pcbAB*, *pcbC*, *cefD1*, and *cefD2* biosynthetic genes and the secretion/translocation genes *cefT*, *cefM*, and *cefP* are located on chromosome VII in the so-called early cluster (89, 127–130). This 'early' cluster contains all the genetic information for the biosynthesis of the IPN and PenN antibiotics (the latter is efficiently secreted), and therefore, it can be considered as a complete gene cluster for these penicillins (5, 116). The late cephalosporin biosynthetic genes *cefEF* and *cefG* are located in a second cluster, named 'late cluster', on chromosome I (127).

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. Bioinformatic analysis of the CefD1 and CefD2 amino acid sequences showed that both enzymes contain a peroxisomal targeting signal, i.e., CefD1 contains a PTS1, whereas CefD2 contains both PST1- and PST2-targeting motives. Moreover, several authors (131–133) determined that the optimum pH for the *in vitro* conversion of IPN into PenN in *A. chrysogenum* cell-free extracts was 7.0, near the estimated pH of the peroxisomal lumen (107). Additionally, the CefD1 and CefD2 homologous proteins have been identified in the peroxisome matrix of *P. chrysogenum* by mass spectrometry (134).

The epimerization of IPN to PenN in peroxisomes implies the need of specific transport systems for both biosynthetic intermediates (IPN and PenN) across the peroxisomal membrane. Based on the well-known gene-clustering patterns of genes for secondary metabolite biosynthesis (82, 135, 136), we identified in the 'early' cluster two genes, cefM and cefP, encoding peroxisomal membrane transporters (129, 130).

The analysis of the CefM and CefP proteins by bioinformatic tools revealed the presence of a Pex19p-binding domain in the amino acidic sequence of both proteins close to one of the transmembrane spanners. This kind of domain is characteristic of PMPs that are recruited by the Pex19 protein (137). The *cefM* gene (130) encodes an efflux pump protein (482 amino acids with a deduced molecular mass of 52.2 kDa) belonging to the Family 3 (drug efflux proteins) of the MFS class of proteins. A strain deleted in *cefM* showed a drastic reduction in extracellular PenN and cephalosporin production and accumulated intracellular PenN. The activity of the last two steps of the cephalosporin pathway (encoded by the *cefEF* and *cefG* genes) was not affected in this transformant, and therefore, the blockade in the cephalosporin pathway is before PenN formation. In summary, CefM is a membrane protein that affects the conversion of PenN to the following intermediate in the pathway. Confocal microscopy experiments with the EGFP fluorescent CefM hybrid protein showed a microbody membrane location of this protein. The fluorescence was located in microbodies of diverse size that were very abundant in swollen *A. chrysogenum* cells differentiating into arthrospores and also in the arthrospores themselves (130). These microbodies resemble the 'cargo vesicles' or endosomes of *Aspergillus parasiticus* (138). The differentiation into arthrospores is well known to be coincident with the intense cephalosporin production.

The other transporter gene located in the 'early' cephalosporin cluster, known as *cefP* (129), encodes a protein of 866 amino acids with a deduced molecular mass of 99.2 kDa and with 11 putative transmembrane spanners. A modified 12th TMS motif is probably present in this protein, although it is not recognized by the bioinformatic programs used to identify these domains. The targeted inactivation of the *cefP* gene resulted in a drastic reduction in the cephalosporin production and an accumulation of IPN in the culture broths, whereas IPN is not usually accumulated in cultures of the parental strain. However, CefP controls the expression of the neighbor regulatory gene *cefR* by an unknown mechanism, and therefore, the effect of CefP on cephalosporin may be due to a cascade mechanism because both *cefP* and *cefR* genes are required to complement the *cefP*-disrupted mutant. An alternative explanation is that the regulator affects not only CefP but also other transporters/enzymes that play a role in cephalosporin biosynthesis. *In vivo* fluorescence microscopy experiments using a functional DsRed-CefP hybrid protein indicated that this hybrid protein co-localizes with the EGFP-SKL peroxisome-targeted protein (129).

The amino acid sequences of CefP and CefM are rather different, and the exact role of these two proteins seems to be different (e.g., cefM may introduce IPN into 'cargo vesicles' that deliver its cargo to peroxisomes), although both of them affect the peroxisomal conversion of IPN to the first cephalosporin intermediate containing the cephem ring; this conversion proceeds through the epimerization of IPN to PenN, mediated by the proteins CefD1 and CefD2.

In summary, the IPN intermediate is synthesized in the cytosol in reactions catalyzed by the cytosolic enzymes ACV synthetase and IPN synthase (139) and needs to be transported to the peroxisomal matrix for its epimerization into PenN by the CefD1 and CefD2 proteins (89). A question that remains unanswered is the mechanism (or transporter system) by which the intermediates IPN and PenN are partially lost from the cytoplasm and accumulated into the culture broth, a phenomenon that goes in detriment of the second part of the pathway (conversion of PenN to cephalosporin C) because the secreted IPN or PenN are not reincorporated into the cells. The PenN export appears to be carried out by CefT, a third member of the MFS transporter family that is targeted to the cell membrane (128, 140, 141).

Although all published data suggest that MSF transporters play an important role in the transport of intermediates and precursors into peroxisomes, the biochemical evidence supporting the genetic studies and confocal fluorescence microscopy observations, is still scarce. Further fractionation studies and purification of different types of vesicles, combined with biochemical analyses of the MSF proteins and enzymes, are required to confirm the evidence currently available.

Expert opinion

It is clear from all available information that some steps of the biosynthesis of secondary metabolites are performed in the peroxisomal lumen (Table 1). These are frequently the middle or late steps in the pathway. The reason for this compartmentalization is essentially a more adequate environment (pH >7.0) and concentration of enzymes and

substrates. The compartmentalization requires the transport of early precursors from the cytosol, where they are formed by reactions of primary metabolism to the peroxisome lumen. In some well-studied cases, the transport of those precursors or intermediates is achieved by members of the MFS class, although ABC transporters might also be involved in some cases. Indeed, in bacteria, the secretion of the antibiotics and other secondary metabolites from the cells is performed by either MFS or ABC transporters, depending on the nature of the secondary metabolite (69). MFS transporters have been identified in the gene clusters of several fungal secondary metabolites, including cephalosporin (129, 130), cercosporin (142), and roquefortine C (83), among others. In these cases, it is very likely that the MFSs are involved in peroxisomal transport of intermediates as occurs with the cephalosporin intermediates described in this article.

Outlook

In the case of *P. chrysogenum*, a large number of MFS transporters (688 secondary transporters including 416 MFS transporters) have been identified (143), but unlike in *A. chrysogenum*, the specific MFS transporter that introduces IPN into the peroxisomes is still unknown. There are differences between the transport and epimerization mechanisms in *Acremonium* and *Penicillium*. Orthologous genes of *cefD1* and *cefD2* are absent from the penicillin gene cluster, and this absence explains why *Penicillium* lacks the ability to epimerize IPN to PenN. This has been confirmed experimentally; only when the *cefD1*, *cefD2*, *cefEF*, and *cefG* genes of *Acremonium* were introduced into *P. chrysogenum* the transformants acquired the ability to synthesize PenN and convert it to cephalosporins (144). Although genes related to *cefD1* and *cefD2* have been located in separate positions in the genome of *P. chrysogenum* (143) and other fungi, they are not strict orthologues of the cephalosporin biosynthetic genes and their natural role is probably related to the epimerization and degradation of methyl-branched fatty acids.

In the future, much more basic information is needed to understand the molecular mechanisms of transport of these intermediates. MFS transporters rely on the electrochemical gradient that is established between both sides of the membrane. This seems to be the case in the peroxisomal drug/H^+ antiporters, although the precise determinations of the pH and electrochemical gradients need to be made.

Another interesting question that remains to be clarified is the secretion mechanism of the final products of a peroxisomal pathway. The secretion of penicillin from the peroxisome to the medium is a matter of debate (5, 9, 116, 145). Authentic benzylpenicillin transporters in the peroxisomal or in the fungal membrane have not been scientifically confirmed. Alternatively, the fusion of peroxisomes with secretory vesicles that may unload the penicillin formed by an exocytosis mechanism has been proposed (94, 116, 138). This vesicle-mediated mechanism may be different from the known pexophagy because a *P. chrysogenum* mutant defective in pexophagy produces normal or even higher levels of penicillin due to the preservation of peroxisomes and the biosynthetic enzymes (4).

More information is also needed in the case of those secondary metabolites that involve two consecutive pathways located in different subcellular compartments. As exemplified by the cephalosporin pathway, the final product of the first pathway (PenN) may be secreted directly to the culture medium, where it remains as PenN, or from peroxisomes to the cytosol, where it is converted to the so-called late products (i.e., cephalosporins) and finally secreted to the external medium. The relationship between the transporters from peroxisome to cytosol and from cytosol to the medium requires additional studies.

Highlights

In recent years, solid evidence has confirmed that some enzymes involved in the biosynthesis of penicillins, cephalosporins, and other bioactive secondary metabolites are located in the lumen of peroxisomes of the producer fungi. Mislocalization of these enzymes in peroxisome-defective mutants causes a drastic decrease or the total lack of secondary metabolite biosynthesis.

This compartmentalization of the β-lactam biosynthetic enzymes requires the import into peroxisomes of early precursors or intermediates of the biosynthesis pathway and the secretion from peroxisomes to the extracellular medium of the final products of the pathways.

The targeting of peroxisomal luminal proteins to peroxisomes is mediated by well-known PTS. Indeed, enzymes of the β-lactam antibiotics rely on PTS to be located in perosixomes, but the transport of intermediates, cofactors, and other substrates of the peroxisomal enzymes is only beginning to be understood.

In general, the transport of biosynthetic intermediates into peroxisomes in fungi and plants is mediated by MFS transporters of the drug/H^+ antiporter family that contain 12 TMS. These transporters are similar to those conferring MDR in eukaryotic cells. However, the import of fatty acids into peroxisomes is mediated by ABC transporters.

Acknowledgments: This research is supported by Grants of the European Union (EUROFUNG and EUROFUNG-base). We acknowledge the excellent comments of Prof. Paloma Liras (University of León, Spain) to improve the manuscript.

Received November 9, 2012; accepted December 21, 2012

References

1. Heiland I, Erdmann R. Biogenesis of peroxisomes. Topogenesis of the peroxisomal membrane and matrix proteins. FEBS J 2005; 272: 2362–72.
2. Opalinski L, Veenhuis M, van der Klei I. Peroxisomes: membrane events accompanying peroxisome proliferation. Int J Biochem Cell Biol 2011; 43: 847–51.
3. Purdue PE, Lazarow PB. Peroxisome biogenesis. Annu Rev Cell Dev Biol 2001; 17: 701–52.
4. Bartoszewska M, Opalinski L, Veenhuis M, van der Klei IJ. The significance of peroxisomes in secondary metabolite biosynthesis in filamentous fungi. Biotechnol Lett 2011; 33: 1921–31.
5. Martin JF, Ullán RV, Garcia-Estrada C. Role of peroxisomes in the biosynthesis and secretion of beta-lactams and other secondary metabolites. J Ind Microbiol Biotechnol 2012; 39: 367–82.
6. De Duve C, Baudhuin P. Peroxisomes (microbodies and related particles). Physiol Rev 1966; 46: 323–57.
7. Hart DT, Misset O, Edwards SW, Opperdoes FR. Comparison of the glycosomes (microbodies) isolated from Trypanosoma brucei blood stream form and cultured procyclic trypomastigotes. Mol Biochem Parasitol 1984; 12: 25–35.
8. Markham P, Collinge AJ. Woronin bodies of filamentous fungi. FEMS Microbiol Rev 1987; 46: 1–11.
9. Kiel JA, Veenhuis M, van der Klei IJ. PEX genes in fungal genomes: common, rare or redundant. Traffic 2006; 7: 1291–303.
10. Ashmarina LI, Rusnak N, Miziorko HM, Mitchell GA. 3-Hydroxy-3-methylglutaryl-CoA lyase is present in mouse and human liver peroxisomes. J Biol Chem 1994; 269: 31929–32.
11. Kotti TJ, Savolainen K, Helander HM, Yagi A, Novikov DK, Kalkkinen N, Conzelmann E, Hiltunen JK, Schmitz W. In mouse alpha-methylacyl-CoA racemase, the same gene product is simultaneously located in mitochondria and peroxisomes. J Biol Chem 2000; 275: 20887–95.
12. Kunau WH, Dommes V, Schulz H. Beta-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress. Prog Lipid Res 1995; 34: 267–342.
13. Evers ME, Höhfeld J, Kunau WH, Harder W, Veenhuis M. Physiological studies on the utilization of oleic acid by Saccharomyces cerevisiae in relation to microbody development. FEMS Microbiol Lett 1991; 69: 73–8.
14. Opalinski L, Bartoszewska M, Fekken S, Liu H, de Boer R, van der Klei I, Veenhuis M, Kiel JA. De novo peroxisome biogenesis in Penicillium chrysogenum is not dependent on the Pex11 family members or Pex16. PLOS One 2012; 7: e35490.
15. Glover JR, Andrews DW, Rachubinski RA. Saccharomyces cerevisiae peroxisomal thiolase is imported as a dimer. Proc Natl Acad Sci USA 1994; 91: 10541–5.
16. García-Estrada C, Vaca I, Fierro F, Sjollema K, Veenhuis M, Martín JF. The unprocessed preprotein form IATC103S of the isopenicillin N acyltransferase is transported inside peroxisomes and regulates its self-processing. Fungal Genet Biol 2008; 45: 1043–52.
17. Hogenboom S, Tuyp JJ, Espeel M, Koster J, Wanders RJ, Waterham HR. Phosphomevalonate kinase is a cytosolic protein in humans. J Lipid Res 2004; 45: 697–705.
18. de Hoop MJ, Ab G. Import of proteins into peroxisomes and other microbodies. Biochem J 1992; 286: 657–69.
19. Subramani S. Protein import into peroxisomes and biogenesis of the organelle. Annu Rev Cell Biol 1993; 9: 445–78.
20. Rachubinski RA, Subramani S. How proteins penetrate peroxisomes? Cell 1995; 83: 525–8.
21. Swinkels BW, Gould SJ, Subramani S. Targeting efficiencies of various permutations of the consensus C-terminal tripeptide peroxisomal targeting signal. FEBS Lett 1992; 305: 133–6.
22. Purdue PE, Lazarow PB. Targeting of human catalase to peroxisomes is dependent upon a novel COOH-terminal peroxisomal targeting sequence. J Cell Biol 1996; 134: 849–62.
23. Mullen RT, Lee MS, Flynn CR, Trelease RN. Diverse amino acid residues function within the type 1 peroxisomal targeting signal. Implications for the role of accessory residues upstream of the type 1 peroxisomal targeting signal. Plant Physiol 1997; 115: 881–9.
24. Maynard EL, Gatto GJ Jr, Berg JM. Pex5p binding affinities for canonical and noncanonical PTS1 peptides. Proteins 2004; 55: 856–61.
25. Neuberger G, Maurer-Stroh S, Eisenhaber B, Hartig A, Eisenhaber F. Motif reWnement of the peroxisomal targeting signal 1 and evaluation of taxon-speciWc diVerences. J Mol Biol 2003; 328: 567–79.
26. Lazarow PB. The import receptor Pex7p and the PTS2 targeting sequence. Biochim Biophys Acta 2006; 1763: 1599–604.
27. Dammai V, Subramani S. The human peroxisomal targeting signal receptor, Pex5p, is translocated into the peroxisomal matrix and recycled to the cytosol. Cell 2001; 105: 187–96.
28. Nair DM, Purdue PE, Lazarow PB. Pex7p translocates in and out of peroxisomes in Saccharomyces cerevisiae. J Cell Biol 2004; 167: 599–604.

29. van der Klei IJ, Veenhuis M. Yeast and filamentous fungi as model organisms in microbody research. Biochim Biophys Acta 2006; 1763: 1364–73.
30. Islinger M, Li KW, Seitz J, Völkl A, Lüers GH. Hitchhiking of Cu/Zn superoxide dismutase to peroxisomes – evidence for a natural piggyback import mechanism in mammals. Traffic 2009; 10: 1711–21.
31. Meinecke M, Cizmowski C, Schliebs W, Krüger V, Beck S, Wagner R, Erdmann R. The peroxisomal importomer constitutes a large and highly dynamic pore. Nat Cell Biol 2012; 12: 273–7.
32. Nito K, Hayashi M, Nishimura M. Direct interaction and determination of binding domains among peroxisomal import factors in Arabidopsis thaliana. Plant Cell Physiol 2002; 43: 355–66.
33. Wolf J, Schliebs W, Erdmann R. Peroxisomes as dynamic organelles: peroxisomal matrix protein import. FEBS J 2010; 277: 3268–78.
34. Hu J, Aguirre M, Peto C, Alonso J, Ecker J, Chory J. A role for peroxisomes in photomorphogenesis and development of Arabidopsis. Science 2002; 297: 405–9.
35. Schumann U, Wanner G, Veenhuis M, Schmid M, Gietl C. AthPEX10, a nuclear gene essential for peroxisome and storage organelle formation during Arabidopsis embryogenesis. Proc Natl Acad Sci USA 2003; 100: 9626–31.
36. Sparkes IA, Brandizzi F, Slocombe SP, El-Shami M, Hawes C, Baker A. An Arabidopsis pex10 null mutant is embryo lethal, implicating peroxisomes in an essential role during plant embryogenesis. Plant Physiol 2003; 133: 1809–19.
37. Fan J, Quan S, Orth T, Awai C, Chory J, Hu J. The Arabidopsis PEX12 gene is required for peroxisome biogenesis and is essential for development. Plant Physiol 2005; 139: 231–9.
38. Williams C, van den Berg M, Geers E, Distel B. Pex10p functions as an E3 ligase for the Ubc4p-dependent ubiquitination of Pex5p. Biochem Biophys Res Commun 2008; 374: 620–4.
39. Platta HW, El Magraoui F, Bäumer BE, Schlee D, Girzalsky W, Erdmann R. Pex2 and pex12 function as protein-ubiquitin ligases in peroxisomal protein import. Mol Cell Biol 2009; 29: 5505–16.
40. Agne B, Meindl NM, NiederhoVK, Einwächter H, Rehling P, Sickmann A, Meyer HE, Girzalsky W, Kunau WH. Pex8p: an intraperoxisomal organizer of the peroxisomal import machinery. Mol Cell 2003; 11: 635–46.
41. Platta HW, El Magraoui F, Schlee D, Grunau S, Girzalsky W, Erdmann R. Ubiquitination of the peroxisomal import receptor Pex5p is required for its recycling. J Cell Biol 2007; 177: 197–204.
42. Williams C, van den Berg M, Sprenger RR, Distel B. A conserved cysteine is essential for Pex4p-dependent ubiquitination of the peroxisomal import receptor Pex5p. J Biol Chem 2007; 282: 22534–43.
43. Grou CP, Carvalho AF, Pinto MP, Wiese S, Piechura H, Meyer HE, Warscheid B, Sá-Miranda C, Azevedo JE. Members of the E2D (UbcH5) family mediate the ubiquitination of the conserved cysteine of Pex5p, the peroxisomal import receptor. J Biol Chem 2008; 283: 14190–7.
44. Dodt G, Gould SJ. Multiple PEX genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. J Cell Biol 1996; 135: 1763–74.
45. Elgersma Y, Kwast L, van den Berg M, Snyder WB, Distel B, Subramani S, Tabak HF. Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in S. cerevisiae, causes proliferation of the endoplasmic reticulum membrane. EMBO J 1997; 16: 7326–41.
46. Geisbrecht BV, Collins CS, Reuber BE, Gould SJ. Disruption of a PEX1-PEX6 interaction is the most common cause of the neurologic disorders Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease. Proc Natl Acad Sci USA 1998; 95: 8630–5.
47. Kiel JA, Hilbrands RE, van der Klei IJ, Rasmussen SW, Salomons FA, van der Heide M, Faber KN, Cregg JM, Veenhuis M. Hansenula polymorpha Pex1p and Pex6p are peroxisome associated AAA proteins that functionally and physically interact. Yeast 1999; 15: 1059–78.
48. Matsumoto N, Tamura S, Fujiki Y. The pathogenic peroxin Pex26p recruits the Pex1p-Pex6p AAA ATPase complexes to peroxisomes. Nat Cell Biol 2003; 5: 454–60.
49. Zolman BK, Bartel B. An Arabidopsis indole-3-butyric acid-response mutant defective in PEROXIN6, an apparent ATPase implicated in peroxisomal function. Proc Natl Acad Sci USA 2004; 101: 1786–91.
50. Erdmann R, Blobel G. Identification of Pex13p a peroxisomal membrane receptor for the PTS1 recognition factor. J Cell Biol 1996; 135: 111–21.
51. Gould SJ, Kalish JE, Morrell JC, Bjorkman J, Urquhart AJ, Crane DI. Pex13p is an SH3 protein of the peroxisome membrane and a docking factor for the predominantly cytoplasmic PTS1 receptor. J Cell Biol 1996; 135: 85–95.
52. Chang CC, Warren DS, Sacksteder KA, Gould SJ. PEX12 binds PEX5 and PEX10 and acts downstream of receptor docking in peroxisomal matrix protein import. J Cell Biol 1999; 147: 761–74.
53. Van Ael E, Fransen M. Targeting signals in peroxisomal membrane proteins. Biochim Biophys Acta 2006; 1763: 1629–38.
54. Sparkes IA, Hawes C, Baker A. AtPEX2 and AtPEX10 are targeted to peroxisomes independently of known endoplasmic reticulum trafficking routes. Plant Physiol 2005; 139: 690–700.
55. Tabak HF, Murk JL, Braakman I, Geuze HJ. Peroxisomes start their life in the endoplasmic reticulum. Traffic 2003; 4: 512–8.
56. McCartney AW, Greenwood JS, Fabian MR, White KA, Mullen RT. Localization of the tomato bushy stunt virus replication protein p33 reveals a peroxisome-to-endoplasmic reticulum sorting pathway. Plant Cell 2005; 17: 3513–31.
57. Rucktäschel R, Girzalsky W, Erdmann R. Protein import machineries of peroxisomes. Biochim Biophys Acta 2011; 1808: 892–900.
58. Jones JM, Morrell JC, Gould SJ. PEX19 is a predominantly cytosolic chaperone and import receptor for class 1 peroxisomal membrane proteins. J Cell Biol 2004; 164: 57–67.
59. Dyer JM, McNew JA, Goodman JM. The sorting sequence of the peroxisomal integral membrane protein PMP47 is contained within a short hydrophilic loop. J Cell Biol 1996; 133: 269–80.
60. Honsho M, Fujiki Y. Topogenesis of peroxisomal membrane protein requires a short, positively charged intervening loop sequence and Xanking hydrophobic segments. J Biol Chem 2001; 276: 9375–82.
61. Matsuzono Y, Matsuzaki T, Fujiki Y. Functional domain mapping of peroxin Pex19p: interaction with Pex3p is essential for function and translocation. J Cell Sci 2006; 119: 3539–50.

62. Fang Y, Morrell JC, Jones JM, Gould SJ. PEX3 functions as a PEX19 docking factor in the import of class I peroxisomal membrane proteins. J Cell Biol 2004; 164: 863–75.
63. Soukupova M, Sprenger C, Gorgas K, Kunau W-H, Dodt G. Identification and characterization of the human peroxin PEX3. Eur J Cell Biol 1999; 78: 357–74.
64. Halbach A, Rucktaschel R, Rottensteiner H, Erdmann R. The N-domain of Pex22p can functionally replace the Pex3p N-domain in targeting and peroxisome formation. J Biol Chem 2009; 284: 3906–16.
65. Fujiki Y, Matsuzono Y, Matsuzaki T, Fransen M. Import of peroxisomal membrane proteins: the interplay of Pex3p- and Pex19p-mediated interactions. Biochim Biophys Acta 2006; 1763: 1639–46.
66. Titorenko VI, Rachubinski RA. Mutants of the yeast Yarrowia lipolytica defective in proteinexit from the endoplasmic reticulum are also defective in peroxisome biogenesis. Mol Cell Biol 1998; 18: 2789–803.
67. Tabak HF, van der Zand A, Braakman I. Peroxisomes: minted by the ER. Curr Opin Cell Biol 2008; 20: 393–400.
68. Perry RJ, Mast FD, Rachubinski RA. Endoplasmic reticulum-associated secretory proteins Sec20p, Sec39p, and Dsl1p are involved in peroxisome biogenesis. Eukaryot Cell 2009; 8: 830–43.
69. Martín JF, Casqueiro J, Liras P. Secretion systems for secondary metabolites: how producer cells send out messages of intercellular communication. Curr Opin Microbiol 2005; 8: 282–93.
70. Antonenkov VD, Hiltunen JK. Transfer of metabolites across the peroxisomal membrane. Biochim Biophys Acta 2012; 1822: 1374–86.
71. Rees DC, Johnson E, Lewinson O. ABC transporters: the power to change. Nat Rev Mol Cell Biol 2009; 10: 218–27.
72. Theodoulou FL, Holdsworth M, Baker A. Peroxisomal ABC transporters. FEBS Lett 2006; 580: 1139–55.
73. Gottesman MM, Ambudkar SV. Overview: ABC transporters and human disease. J Bioenerg Biomembr 2001; 33: 453–8.
74. Dean M, Hamon Y, Chimini G. The human ATP-binding cassette (ABC) transporter superfamily. J Lipid Res 2001; 42: 1007–17.
75. Wanders RJ, Visser WF, van Roermund CW, Kemp S, Waterham HR. The peroxisomal ABC transporter family. Pflugers Arch 2007; 453: 719–34.
76. Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu U, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EJ, Yazaki K, Theodoulou FL. Plant ABC proteins – a unified nomenclature and updated inventory. Trends Plant Sci 2008; 13: 151–9.
77. Shani N, Valle D. Peroxisomal ABC transporters. Method Enzymol 1998; 292: 753–76.
78. Morita M, Imanaka T. Peroxisomal ABC transporters: structure, function and role in disease. Biochim Biophys Acta 2012; 1822: 1387–96.
79. Pao SS, Paulsen IT, Saier MH Jr. Major facilitator superfamily. Microbiol Mol Biol Rev 1998; 62: 1–34.
80. Reddy VS, Shlykov MA, Castillo R, Sun EI, Saier MH Jr. The major facilitator superfamily (MFS) revisited. FEBS J 2012; 279: 2022–35.
81. Davies J. Small molecules: the lexicon of biodiversity. J Biotechnol 2007; 129: 3–5.
82. Martín JF, Liras P. Organization and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. Annu Rev Microbiol 1989; 43: 173–206.
83. García-Estrada C, Ullán RV, Albillos SM, Fernández-Bodega MA, Durek P, von Döhren H, Martín JF. A single cluster of coregulated genes encodes the biosynthesis of the mycotoxins roquefortine C and meleagrin in Penicillium chrysogenum. Chem Biol 2011; 18: 1499–512.
84. Cundliffe E, Demain AL. Avoidance of suicide in antibiotic-producing microbes. J Ind Microbiol Biotechnol 2010; 37: 643–72.
85. Müller WH, van der Krift TP, Krouwer AJ, Wosten HA, van der Voort LH, Smaal EB, Verkleij AJ. Localization of the pathway of the penicillin biosynthesis in Penicillium chrysogenum. EMBO J 1991; 10: 489–95.
86. Lamas-Maceiras M, Vaca I, Rodríguez E, Casqueiro J, Martín JF. Amplification and disruption of the phenylacetyl-CoA ligase gene of Penicillium chrysogenum encoding an aryl-capping enzyme that supplies phenylacetic acid to the isopenicillin N-acyltransferase. Biochem J 2006; 395: 147–55.
87. Wang FQ, Liu J, Dai M, Ren ZH, Su CY, He JG. Molecular cloning and functional identification of a novel phenylacetyl-CoA ligase gene from Penicillium chrysogenum. Biochem Biophys Res Commun 2007; 360: 453–8.
88. Koetsier MJ, Gombert AK, Fekken S, Bovenberg RA, van den Berg MA, Kiel JA, Jekel PA, Janssen DB, Pronk JT, van der Klei IJ, Daran JM. The Penicillium chrysogenum aclA gene encodes a broad-substrate-specificity acyl-coenzyme A ligase involved in activation of adipic acid, a side-chain precursor for cephem antibiotics. Fungal Genet Biol 2010; 47: 33–42.
89. Ullán RV, Casqueiro J, Bañuelos O, Fernández FJ, Gutiérrez S, Martín JF. A novel epimerization system in fungal secondary metabolism involved in the conversion of isopenicillin N into penicillin N in Acremonium chrysogenum. J Biol Chem 2002; 277: 46216–25.
90. Imazaki A, Tanaka A, Harimoto Y, Yamamoto M, Akimitsu K, Park P, Tsuge T. Contribution of peroxisomes to secondary metabolism and pathogenicity in the fungal plant pathogen Alternaria alternata. Eukaryot Cell 2010; 9: 682–94.
91. Tanaka A, Shiotani H, Yamamoto M, Tsuge T. Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-specific AK-toxin in the Japanese pear pathotype of Alternaria alternata. Mol Plant Microb Interact 1999; 12: 691–702.
92. Tanaka A, Tsuge T. Structural and functional complexity of the genomic region controlling AK-toxin biosynthesis and pathogenicity in the Japanese pear pathotype of Alternaria alternata. Mol Plant Microb Interact 2000; 13: 975–86.
93. Chanda A, Roze LV, Pastor A, Frame MK, Linz JE. Purification of a vesicle-vacuole fraction functionally linked to aflatoxin synthesis in Aspergillus parasiticus. J Microbiol Methods 2009; 78: 28–33.
94. Chanda A, Roze LV, Kang S, Artymovich KA, Hicks GR, Raikhel NV, Calvo AM, Linz JE. A key role for vesicles in fungal secondary metabolism. Proc Natl Acad Sci USA 2009; 106: 19533–8.
95. Saikia S, Nicholson MJ, Young C, Parker EJ, Scott B. The genetic basis for indole-diterpene chemical diversity in filamentous fungi. Mycol Res 2008; 112: 184–99.
96. Saikia S, Scott B. Functional analysis and subcellular localization of two geranylgeranyl diphosphate synthases from Penicillium paxilli. Mol Genet Genomics 2009; 282: 257–71.
97. Schneider K, Kienow L, Schmelzer E, Colby T, Bartsch M, Miersch O, Wasternack C, Kombrink E, Stuible HP. A new type of peroxisomal acyl-coenzyme A synthetase from Arabidopsis thaliana has the catalytic capacity to activate biosynthetic precursors of jasmonic acid. J Biol Chem 2005; 280: 13962–72.

98. Hayashi M, Yagi M, Nito K, Kamada T, Nishimura M. Differential contribution of two peroxisomal protein receptors to the maintenance of peroxisomal functions in Arabidopsis. J Biol Chem 2005; 280: 14829–35.
99. Nyathi Y, Baker A. Plant peroxisomes as a source of signalling molecules. Biochim Biophys Acta 2006; 1763: 1478–95.
100. Li T, Chiang JY. Regulation of bile acid and cholesterol metabolism by PPARs. PPAR Res 2009; 2009: 501739.
101. Sá-Correia I, dos Santos SC, Teixeira MC, Cabrito TR, Mira NP. Drug: H+ antiporters in chemical stress response in yeast. Trends Microbiol 2009; 17: 22–31.
102. Morishita M, Engebrecht J. Sorting signals within the Saccharomyces cerevisiae sporulation-specific dityrosine transporter, Dtr1p, C terminus promote Golgi-to-prospore membrane transport. Eukaryot Cell 2008; 7: 1674–84.
103. Fernández-Aguado M, Ullán RV, Teijeira F, Rodríguez-Castro R, Martín JF. The transport of phenylacetic acid across the peroxisomal membrane is mediated by the PaaT protein in Penicillium chrysogenum. Appl Microbiol Biotechnol 2012; DOI: 10.1007/s00253-012-4425-1 [Epub ahead of print].
104. Maggio-Hall LA, Wilson RA, Keller NP. Fundamental contribution of beta-oxidation to polyketide mycotoxin production in planta. Mol Plant Microbe Interact 2005; 18: 783–93.
105. Veiga T, Gombert AK, Landes N, Verhoeven MD, Kiel JA, Krikken AM, Nijland JG, Touw H, Luttik MA, van der Toorn JC, Driessen AJ, Bovenberg RA, van den Berg MA, van der Klei IJ, Pronk JT, Daran JM. Metabolic engineering of β-oxidation in Penicillium chrysogenum for improved semi-synthetic cephalosporin biosynthesis. Metab Eng 2012; 14: 437–48.
106. Hesse SJ, Ruijter GJ, Dijkema C, Visser J. Intracellular pH homeostasis in the filamentous fungus Aspergillus niger. Eur J Biochem 2002; 269: 3485–94.
107. van der Lende TR, Breeuwer P, Abee T, Konings WN, Driessen AJ. Assessment of the microbody luminal pH in the filamentous fungus Penicillium chrysogenum. Biochim Biophys Acta 2002; 1589: 104–11.
108. Alvarez E, Cantoral JM, Barredo JL, Díez B, Martín JF. Purification to homogeneity and characterization of acyl coenzyme A:6-aminopenicillanic acid acyltransferase of Penicillium chrysogenum. Antimicrob Agents Chemother 1987; 31: 1675–82.
109. Alvarez E, Meesschaert B, Montenegro E, Gutiérrez S, Díez B, Barredo JL, Martín JF. 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 1993; 215: 323–32.
110. Müller WH, Bovenberg RA, Groothuis MH, Kattevilder F, Smaal EB, Van der Voort LH, Verkleij AJ. Involvement of microbodies in penicillin biosynthesis. Biochim Biophys Acta 1992; 1116: 210–3.
111. Evers ME, Trip H, van den Berg MA, Bovenberg RA, Driessen AJ. Compartmentalization and transport in β-lactam antibiotics biosynthesis. Adv Biochem Eng Biotechnol 2004; 88: 111–35.
112. Fernández-Aguado M, Teijeira F, Martín JF, Ullán RV. A vacuolar membrane protein affects drastically the biosynthesis of the ACV tripeptide and the beta-lactam pathway of Penicillium chrysogenum. Appl Microbiol Biotechnol 2013; 97: 795–808.
113. García-Estrada C, Vaca I, Lamas-Maceiras M, Martín JF. In vivo transport of the intermediates of the penicillin biosynthetic pathway in tailored strains of Penicillium chrysogenum. Appl Microbiol Biotechnol 2007; 76: 169–82.
114. Aharonowitz Y, Cohen G, Martin JF. Penicillin and cephalosporin biosynthetic genes: structure, organization, regulation, and evolution. Annu Rev Microbiol 1992; 46: 461–95.
115. Elander RP. Industrial production of beta-lactam antibiotics. Appl Microbiol Biotechnol 2003; 61: 385–92.
116. Martín JF, Ullán RV, García-Estrada C. Regulation and compartmentalization of β-lactam biosynthesis. Microb Biotechnol 2010; 3: 285–99.
117. Baldwin JE, Bird JW, Field RA, O'Callaghan NM, Schoffield CJ. Isolation and partial characterization of ACV synthetase from Cephalosporium acremonium and Streptomyces clavuligerus. J Antibiot 1990; 43: 1055–7.
118. Martin JF. Alpha-aminoadipyl-cysteinyl-valine synthetases in beta-lactam producing organisms. From Abraham's discoveries to novel concepts of non-ribosomal peptide synthesis. J Antibiot (Tokyo) 2000; 53: 1008–21.
119. Gutiérrez S, Díez B, Montenegro E, Martín JF. Characterization of the Cephalosporium acremonium pcbAB gene encoding α-aminoadipyl-cysteinyl-valine synthetase, a large multidomain peptide synthetase: linkage to the pcbC gene as a cluster of early cephalsoporin biosynthetic genes and evidence of multiple functional domains. J Bacteriol 1991; 173: 2354–65.
120. Samsom SM, Belagaje R, Blankenship DT, Chapman JL, Perry D, Skatrud PL, van Frank RM, Abraham EP, Baldwin JE, Queener SW, Ingolia TD. Isolation, sequence determination and expression in Escherichia coli of the isopenicillin N synthetase gene from Cephalosporium acremonium. Nature 1985; 318: 191–4.
121. Martín JF, Ullán RV, Casqueiro FJ. Novel genes involved in Cephalosporin biosynthesis: the three-component isopenicillin N epimerase system. In: Brakhage A, editor. Advances in biochemical engineering-biotechnology. Berlin: Springer-Verlag, 2004: 91–109.
122. Dotzlaf JE, Yeh WK. Copurification and characterization of deacetoxycephalosporin C synthetase/hydroxylase from Cephalosporium acremonium. J Bacteriol 1987; 169: 1611–8.
123. Scheidegger A, Kuenzi MT, Nuesch J. Partial purification and catalytical properties of a bifunctional enzyme in the biosynthetic pathway of β-lactams in Acremonium chrysogenum. J Antibiot 1984; 37: 522–31.
124. Samsom SM, Dotzlaf JF, Slisz ML, Becker GW, van Frank RM, Veal LE, Yeh WK, Miller JR, Queener SW, Ingolia TD. Cloning and expression of the fungal expandase/hydroxylase gene involved in cephalosporin biosynthesis. Nat Biotechnol 1987; 5: 1207–14.
125. Gutiérrez S, Velasco J, Fernández FJ, Martín JF. The cefG gene of Cephalosporium acremonium is linked to the cefEF gene and encodes a deacetylcephalsoporin C acetyltransferase closely related to homoserine O-acetyltransferase. J Bacteriol 1992; 174: 3056–64.
126. Velasco J, Gutierrez S, Campoy S, Martin JF. Molecular characterization of the Acremonium chrysogenum cefG gene product: the native deacetylcephalosporin C acetyltransferase is not processed into subunits. Biochem J 1999; 337: 379–85.

127. Gutiérrez S, Fierro F, Casqueiro J, Martín JF. Gene organization and plasticity of the b-lactam genes in different filamentous fungi. Antonie Van Leeuwenhoek 1999; 75: 21–31.
128. Ullán RV, Liu G, Casqueiro J, Gutiérrez S, Bañuelos O, Martín JF. The cefT gene of Acremonium chrysogenum C10 encodes a putative multidrug efflux pump protein that significantly increases cephalosporin C production. Mol Genet Genomics 2002; 267: 673–83.
129. Ullán RV, Teijeira F, Guerra SM, Vaca I, Martín JF. Characterization of a novel peroxisome membrane protein essential for conversion of isopenicillin N into cephalosporin C. Biochem J 2010; 432: 227–36.
130. Teijeira F, Ullán RV, Guerra SM, García-Estrada C, Vaca I, Martín JF. The transporter CefM involved in translocation of biosynthetic intermediates is essential for cephalosporin production. Biochem J 2009; 418: 113–24.
131. Jayatilake GS, Huddleston JA, Abraham EP. Conversion of isopenicillin N into penicillin N in cell-free extracts of Cephalosporium acremonium. Biochem J 1981; 194: 645–7.
132. Baldwin JE, Keeping JW, Singh PD, Vallejo CA. Cell-free conversion of isopenicillin N into deacetoxycephalosporin C by Cephalosporium acremonium mutant M-0198. Biochem J 1981; 194: 649–51.
133. Lübbe C, Wolfe S, Demain AL. Isopenicillin N epimerase activity in a high cephalosporin-producing strain of Cephalosporium acremonium. Appl Microbiol Biotechnol 1986; 23: 367–8.
134. Kiel JA, van den Berg MA, Fusetti F, Poolman B, Bovenberg RAL, Veenhuis M, van der Klei IJ. Matching the proteome to the genome: the microbody of penicillin-producing Penicillium chrysogenum cells. Funct Integr Genomics 2009; 9: 167–84.
135. Keller NP, Turner G, Bennett JW. Fungal secondary metabolism – from biochemistry to genomics. Nat Rev Microbiol 2005; 3: 937–47.
136. Hoffmeister D, Keller NP. Natural products of filamentous fungi: enzymes, genes, and their regulation. Nat Prod Rep 2007; 24: 393–416.
137. Rottensteiner H, Kramer A, Lorenzen S, Stein K, Landgraf C, Volkmer-Engert R, Erdmann R. Peroxisomal membrane proteins contain common Pex19p-binding sites that are an integral part of their targeting signals. Mol Biol Cell 2004; 15: 3406–17.
138. Roze LV, Chanda A, Linz JE. Compartmentalization and molecular traffic in secondary metabolism: a new understanding of established cellular processes. Fungal Genet Biol 2011; 48: 35–48.
139. van de Kamp M, Driessen AJ, Konings WN. Compartmentalization and transport in β-lactam antibiotic biosynthesis by filamentous fungi. Antonie Van Leeuwenhoek 1999; 75: 41–78.
140. Ullán RV, Teijeira F, Martín JF. Expression of the Acremonium chrysogenum cefT gene in Penicillum chrysogenum indicates that it encodes a hydrophilic beta-lactam transporter. Curr Genet 2008; 54: 153–61.
141. Nijland JG, Kovalchuk A, van den Berg MA, Bovenberg RA, Driessen AJ. Expression of the transporter encoded by the cefT gene of Acremonium chrysogenum increases cephalosporin production in Penicillium chrysogenum. Fungal Genet Biol 2008; 45: 1415–21.
142. Callahan TM, Rose MS, Meade MJ, Ehrenshaft M, Upchurch RG. CFP, the putative cercosporin transporter of Cercospora kikuchii, is required for wild type cercosporin production, resistance, and virulence on soybean. Mol Plant Microbe Interact 1999; 12: 901–10.
143. van den Berg MA, Albang R, Albermann K, Badger JH, Daran JM, Driessen AJ, Garcia-Estrada C, Fedorova ND, Harris DM, Heijne WH, Joardar V, Kiel JA, Kovalchuk A, Martín JF, Nierman WC, Nijland JG, Pronk JT, Roubos JA, van der Klei IJ, van Peij NN, Veenhuis M, von Döhren H, Wagner C, Wortman J, Bovenberg RA. Genome sequencing and analysis of the filamentous fungus Penicillium chrysogenum. Nat Biotechnol 2008; 26: 1161–8.
144. Ullán RV, Campoy S, Casqueiro J, Fernández FJ, Martín JF. Deacetylcephalosporin C production in Penicillium chrysogenum by expression of the isopenicillin N epimerization, ring expansion, and acetylation genes. Chem Biol 2007; 14: 329–39.
145. Andrade AC, Van Nistelrooy JG, Peery RB, Skatrud PL, De Waard MA. The role of ABC transporters from Aspergillus nidulans in protection against cytotoxic agents and in antibiotic production. Mol Gen Genet 2000; 263: 966–77.

Dr. Juan F. Martín is Professor of Microbiology at the University of León (Spain). He graduated in Chemistry and did his PhD thesis on fungal microbial biochemistry at the University of Salamanca (Spain) in 1971. He was a postdoctoral researcher at the Waksman Institute of Microbiology (1971–1974) Rutgers University, New Jersey (USA) and in 1974 moved to the M.I.T. (Department of Food Science) at Boston, Mass (USA) working with Prof. Arnold L. Demain. After returning to Spain with a tenure position at the Spanish Research Council (CSIC) he got a position as Professor at the University of Salamanca and in 1980 a Professor tenure at the University of León. He founded in 1991 the Institute of Biotechnology of León (INBIOTEC) and directed it until 2011. His field of expertise is centered in microbial genetics and biochemistry, particularly in fungi, *Streptomyces* and Corynebacteria. He is author of 460 scientific publications and review articles and serves as member of the editorial board of several scientific journals. In 1999 was awarded with the ARIMA prize by the IUMS.

Carlos García-Estrada received his Bachelor degree in Veterinary Sciences from the University of León (Spain, 1998) and obtained his DPhil from the same University in 2003. During this period, he completed his professional training at the National Center for Natural Products Research, University of Mississippi, MS, USA (2002). Subsequently he started his postdoctoral studies under the supervision of Professor Juan-Francisco Martín at the Institute of Biotechnology (INBIOTEC), León, Spain, where he worked with fungal secondary metabolism. He completed his postdoctoral stage at the Department of Eukaryotic Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Haren, The Netherlands (2005). Currently, he is the Head of the Biopharma and Biomedicine Area at INBIOTEC and combines this activity with a position as associate professor at the University of León (Spain). His research interests center on the biosynthesis of antibiotics and other secondary metabolites by fungi.

Ricardo Vicente Ullán received his Bachelor degree in Biology Sciences from the University of Salamanca (Spain, 1995) and obtained his PhD from the University of León in 2002. He started his postdoctoral studies under the supervision of Professor Juan-Francisco Martín at the University of León, Spain, where he worked on the biosynthesis of secondary metabolites by fungi. He completed his postdoctoral stage at the Universities of Manchester (United Kingdom) and Santiago de Compostela (Spain). Currently, he is the Head of the Environmental Area at the Institute of Biotechnology (INBIOTEC) and Associate Professor at the University of León (Spain). His research interests are focused on the Environmental Biotechnology.