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Compartmentalization and Transport in β-Lactam Antibiotics Biosynthesis

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Abstract Classical strain improvement of β-lactam producing organisms by random mutagenesis has been a powerful tool during the last century. Current insights in the biochemistry and genetics of β-lactam production, in particular in the filamentous fungus *Penicillium chrysogenum*, however, make a more directed and rational approach of metabolic pathway engineering possible. Besides the need for efficient genetic methods, a thorough understanding is needed of the metabolic fluxes in primary, intermediary and secondary metabolism. Controlling metabolic fluxes can be achieved by adjusting enzyme activities and metabolite levels in such a way that the main flow is directed towards the desired product. In addition, compartmentalization of specific parts of the β-lactam biosynthesis pathways provides a way to control this pathway by clustering enzymes with their substrates inside specific membrane bound structures sequestered from the cytosol. This compartmentalization also requires specific membrane transport steps of which the details are currently uncovered.

Keywords β-Lactam · Biochemical engineering · Compartmentalization · Penicillium chrysogenum · Transporter proteins

List of Abbreviations

AAP	Amino acid permease
ABC	ATP-binding-cassette
ACS	Acetylcoenzyme A synthetase
ACVS	δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase
ad-7-ACA	Adipoyl-7-aminocephalosporanic acid
ad-7-ADC	A3-Aminodeacetoxycephalosporanic acid
A. niger	*Aspergillus niger*
APS	Adenosine-5-phosphosulfate
A. nidulans	*Aspergillus nidulans*
C. acremonium	*Cephalosporium acremonium*
CoA	Coenzyme A
DAC	Deacetylcephalosporin C
DAOC	Deacetoxycephalosporin C
Gap	General amino acid permease
GFP	Green fluorescent protein
GST	Glutathione S-transferase
IAT	Acyl-coenzyme A:isopenicillin N acyltransperase
IPNS	Isopenicillin N synthase
MFS	Major Facilitator Superfamily
N. crassa	*Neurospora crassa*
N. lactamdurans	*Nocardia lactamdurans*
NBD	Nucleotide binding domain
OPC	6-Oxopiperidine-2-carboxylic acid
PA	Phenylacetic acid
PAP	S3-Phospho-adenosine-5-phosphosulfate
P. chrysogenum	*Penicillium chrysogenum*
PCL	Phenylacetyl-coenzyme A ligase
pmf	Proton motive force
PMP	Peroxisomal membrane protein
POA	Phenoxyacetic acid
PTS	Peroxisomal targeting signal

S. cerevisiae	*Saccharomyces cerevisiae*
St. clavuligerus	*Streptomyces clavuligerus*
Sut	Sulfate transporter

1 Introduction

Industrial penicillin and cephalosporin fermentation is performed using filamentous fungi. However, sequence analysis of one of the biosynthesis genes encoding isopenicillin N synthase (*pcbC*) of various organisms involved in the biosynthesis of these compounds, suggests that the origin of these genes stems from prokaryotic organisms. In bacteria apparently the production of β-lactams has evolved as a means of improving their ability to compete with other prokaryotes. Via horizontal gene transfer, biosynthetic genes may have been acquired by filamentous fungi some 370 million years ago [1–4]. The advantage for these fungi of possessing these genes is thought to be of ecological significance. Antibiotic production provides fungi with the possibility to protect released enzymes and released nutrients against bacteria competing for the same substrates. Other explanations are found in detoxification mechanisms, for instance, to prevent the accumulation of acids such as phenylacetic acid in the cell [5]. After the discovery of the application of penicillin as an antimicrobial agent in humans in the early 1940s, classical strain improvement has been applied to obtain higher production yields. High production and secretion of the β-lactams, however, drains intracellular pools of primary metabolites. In addition, specific metabolic engineering of industrial strains has been applied; however, this requires extensive knowledge of control of the metabolic fluxes in order to obtain predictive models and the desired results. The importance of compartmentalization and transport processes in industrial penicillin biosynthesis has become clear and the different aspects of these topics are being studied in several laboratories. The aim of this chapter is to describe recent developments that are important towards the compartmentalization and transport in β-lactam antibiotics by filamentous fungi. The localization of biosynthesis enzymes and the compartmentalization of biosynthesis, precursors, intermediates and products will be discussed in relationship to their consequences for intra- and extracellular transport.

2 Localization of Biosynthesis Enzymes of the Penicillin Biosynthesis Pathway

Figure 1 in brief depicts the major enzymatic steps involved in β-lactam biosynthesis. This section discusses the cellular localization of the key enzymes.

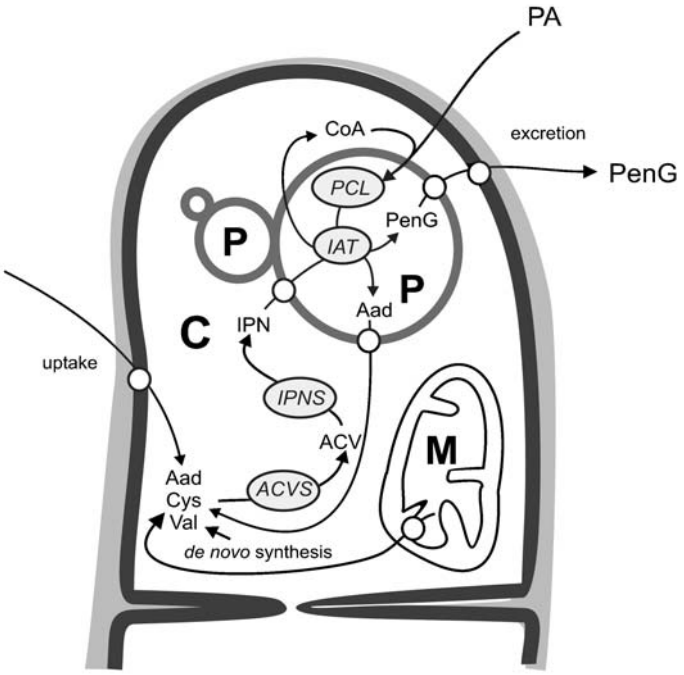

Fig. 1 Localization of the penicillin biosynthesis; δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) and isopenicillin N synthase (IPNS) are present in the cytosol (C) whereas acyl-coenzyme A:isopenicillin N acyltransferase (IAT) and possibly also phenylacetyl-coenzyme A ligase (PCL) are localized in peroxisomes (P). In the mitochondria (M), part of the synthesis of precursor amino acids takes place

2.1 δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine Synthetase (ACVS)

The first step in the biosynthesis of penicillins and cephalosporins is the condensation of three precursor amino acids, namely L-α-aminoadipate, L-cysteine and L-valine into the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV). This step is catalysed by a multi-enzyme complex of 424 kDa with non-ribosomal peptide synthetase activity termed *ACV synthetase* (ACVS) [6–10]. ACVS is encoded by the *acvA* gene that is part of a cluster which includes the other two key enzymes of the penicillin biosynthesis pathway. The localization of ACVS has been a matter of debate for some time. Initially, it was described as a membrane associated protein and found to co-sediment with vesicles of either Golgi or vacuolar origin [11–13]. However, the amino acid sequence of *P. chrysogenum* ACV synthetase contains no recognizable targeting information for the endoplasmic reticulum or the vacuole, and although the protein is hydrophobic of nature, it does not harbour any trans-membrane regions. Localization studies by traditional fractionation experiments were ob-

scured by the fact that ACVS is a highly unstable enzyme and very sensitive to proteolytic degradation. For this purpose improved protocols of cell lysis were designed and used in combination with an immuno-gold electron microscopical analysis to determine the subcellular location of this protein. On the basis of these studies, ACVS turned out to be a cytosolic enzyme [11–14]. Likewise, an ACVS fusion with green fluorescent protein in *Aspergillus nidulans* also localizes to the cytosol [15]. The cytosolic localization is more in pair with the pH optimum of this enzyme, as the acidic vacuole would not support activity. Moreover, the vacuole is highly proteolytic which seems contradictory with the protease sensitivity of the multidomain ACVS and the release of a product tripeptide. ACVS consists of three major modules, one for each amino acid. These modules are divided into domains that are specialized for partial reactions of the total condensation reaction, hereby combining adenylation activity, peptide-bond formation, epimerization and product release by thioesterase activity, in one multi-enzyme [7]. The localization of ACVS in the cytosol bears consequences for the recruitment of the three precursor amino acids. In general, acquisition of L-α-aminoadipate, L-cysteine and L-valine can either proceed through de novo synthesis or uptake from the growth medium; see below.

2.2 Isopenicillin N Synthase (IPNS)

The second step in β-lactam synthesis is the oxidative cyclisation of LLD-ACV into isopenicillin N (IPN). In this step, the bicyclic penam nucleus, consisting of the β-lactam and thiazolidine rings is generated [3, 7]. This step is mediated by *IPN synthase* (IPNS) a protein of 38 kDa that is encoded by the *ipnA* gene that is part of the penicillin biosynthesis gene cluster. From X-ray diffraction experiments using the substrate analogue δ-(L-α-aminoadipoyl)-L-cysteinyl-L-S-methyl-cysteine in the crystal it was concluded that closure of the β-lactam ring precedes the closure of the five-membered thiazolidine ring [3, 16]. Based upon the results of fractionation experiments it became evident that IPNS behaves like a soluble, cytosolic enzyme [17]. This means that LLD-ACV produced by ACVS can directly be used as the substrate for IPNS. The question if these two enzymes are organized in a metabolon or large complex is not known.

2.3 Acyl-Coenzyme A: Isopenicillin N Acyltransferase (IAT)

The third and final step in β-lactam synthesis is the exchange of the L-α-aminoadipate for phenylacetyl- or phenoxyacetyl group by *acyl-coenzyme A:isopenicillin N acyltransferase* (IAT) resulting in the formation of respectively penicillin G and penicillin V [18–21]. IAT is a hetero-dimeric enzyme consisting of a 11 kDa α-subunit and a 28 kDa β-subunit. It is synthesized as a 40 kDa pre-protein from the *aat* gene and undergoes autocatalytic processing to form the heterodimer [22, 23]. Both subunits possess a C-terminal PTS1 signal that

targets this enzyme to a microbody or peroxisome. Fractionation studies as well as immuno-gold labelling experiments indeed localized IAT to microbodies [17]. Consequently the substrate IPN has to enter the microbody before it can be converted. It is not known yet whether this occurs by diffusion over the membrane, or by facilitated or active transport.

2.4 Side Chain Activation

Before the side chain can be used in the substitution reaction catalysed by IAT mentioned above, PA and POA, the side chain precursors, have to be activated to their CoA thioesters. Theoretically this activation can be carried out by an enzyme displaying either *acetyl-coenzyme A synthetase* (ACS) activity, *phenylacetyl-coenzyme A ligase* (PCL) activity, or alternatively via a glutathione-dependent pathway involving *glutathione S-transferase* (GST) activity. This phenomenon has not well been studied, but current view considers the last option unlikely. A gene encoding a cytosolic ACS of *P. chrysogenum* has been identified and isolated. Disruption of this gene did not result in a decrease in penicillin production, meaning that ACS cannot be solely responsible for activation of precursors [17, 24]. In another study a PCL of *P. chrysogenum* containing a C-terminal peroxisomal targeting signal (PTS1; SKI) was identified [25]. This suggests a peroxisomal location of the activating enzyme which would seem advantageous as it is then in the same compartment as IAT and the peroxisomal concentration of activated precursors would be higher than in the cytosol. In addition PA and POA are more likely to easily diffuse across the peroxisomal membrane than their activated counterparts thereby providing a means of retention. However, overproduction of a presumably cytosolic located heterologous PCL from *Pseudomonas putida* U increased the penicillin production by 100% whereas overproduction of the homologous peroxisomal PCL of *P. chrysogenum* did not affect penicillin production [25, 26]. Although these results give an ambiguous view on this step, a bias towards a role of the peroxisomal PCL is provided by additional observations that will be described below.

2.5 Localization of Enzymes of Cephalosporin Biosynthesis and Other β-lactams

Cephalosporins and cephamycins are produced by the filamentous fungus *Acremonium chrysogenum* (syn. *Cephalosporium acremonium*) (cephalosporin C), and by Gram-positive actinomycetes such as *Nocardia lactamdurans* and *Streptomyces clavuligerus* (cephamycin C) [27, 28]. Cephalosporin and penicillin biosynthesis have the first two steps in common, i.e. the formation of ACV by ACV synthetase and the subsequent cyclization to isopenicillin N by IPN synthase. Here the penicillin and cephalosporin pathways diverge. Cephalosporin biosynthesis proceeds with the epimerisation of IPN into penicillin N by epimerase activity of the *cef*D1 and *cef*D2 gene products in *C. acre-*

monium [29] and the completely different *cef*D gene product in *N. lactamdurans* and *St. clavuligerus* [27, 30]. After epimerisation, penicillin N undergoes expansion of the thiazolidine ring to a dihydrothiazine ring by deacetoxycephalosporin C synthetase, yielding deacetoxycephalosporin C (DAOC). DAOC is hydroxylated by DAC synthase to give deacetylcephalosporin C (DAC). In the cephamycin producers *N. lactamdurans* and *St. clavuligerus*, DAC is carbamoylated and methoxylated to form cephamycin C [31]. In *C. acremonium*, DAC is acetylated by the *cefG*-gene encoded enzyme DAC acetyltransferase [32, 33], yielding cephalosporin C. IPN epimerase, DAOC synthetase, DAC synthase and DAC acetyltransferase behave as soluble cytosolic proteins with pH optima above 7.0, reviewed in [34]. Currently, these enzymes are believed to be localized in the cytosol, but no direct studies have addressed the compartmentalization issue. Expression of the expandase gene of *St. clavuligerus* (*cefE*) or the *C. acremonium* expandase-hydroxylase gene and the acetyl transferase gene in *P. chrysogenum* and feeding adipic acid has led to efficient production of adipoyl-7-aminodeacetoxycephalosporanic acid (ad-7-ADCA) and adipoyl-7-aminocephalosporanic acid (ad-7-ACA) respectively. Removal of the adipyl side chain gives 7-ADCA and 7-ACA, respectively, which are important intermediates in the production of semi-synthetic cephalosporins [35, 36].

3
Compartmentalization of Penicillin Biosynthesis

As can be concluded from the above the first two steps of penicillin biosynthesis, the condensation into the tripeptide and the conversion into isopenicillin N, take place in the cytosol of filamentous fungi. The final step, side chain exchange and most probably the side chain precursor activation take place in the microbody. Microbodies (also termed peroxisomes, glyoxysomes, glycosomes depending on the organism and function) are indispensable organelles that can be found in practically all eukaryotic cells. Although their morphology is relatively simple (a proteinaceous matrix surrounded by a single membrane) their physiological properties are remarkably complex. The organelles are involved in pathways of primary, intermediary and secondary metabolism. They may be regarded as organelles in which specific metabolic conversions take place mostly by non-membrane bound enzymes. Various peroxisomal metabolic pathways function in the cytosol of peroxisome-deficient mutants, although in some cases with lower final cell yield compared to wild-type cells [26, 37]. For other peroxisomal pathways, the membrane needs to function as an intact boundary, otherwise metabolic pathways may be severely affected even though all the enzymes of the pathway are synthesized and active in the cytosol [38]. A general major advantage of the presence of a peroxisomal permeability barrier is that it permits the cells to precisely adjust the levels of different intermediates of primary metabolism required for specific metabolic pathways (metabolic flux control by a physical barrier). It was in 1991 that the importance

of the microbody with respect to penicillin biosynthesis (secondary metabolism) became evident when IAT was shown to be located in this organelle [17]. When the putative targeting signal was removed the enzyme was not directed to the microbody but instead localized in the vacuole and surrounding cytosol. Under these conditions, production of penicillin was halted although the enzyme was expressed in vivo and active in vitro [39]. This might be explained by the possibility that another essential enzymatic step, the precursor activation by PCL might occur only inside microbodies and that these activated precursors are now sequestered from IAT inside the microbody. The other explanation, namely that IAT is not able to perform the catalytic reaction in the cytosol seems less likely, because in a mutant of *A. nidulans* lacking functional peroxisomes, penicillin production still occurred with the peroxisomal enzymes mislocalized to the cytosol [40]. Although this suggests that peroxisomes are not essential for penicillin production per se, a positive correlation between penicillin yield and peroxisome numbers has been implicated [17]. The exact reason for this correlation is not known, but this may relate to an increase in the amount of enzymes of the biosynthesis pathway. For detailed information on import of peroxisomal proteins and biogenesis of peroxisomes see reviews by Purdue et al. [41] and van der Klei et al. [42] and references therein.

4
Synthesis of β-lactam Precursors

4.1
L-α-Aminoadipate Synthesis

L-α-Aminoadipate is an intermediate of the L-lysine biosynthesis pathway. The intracellular level of L-α-aminoadipate can be a limiting factor in the overall penicillin synthesis rate, as shown by the observation that addition of L-α-aminoadipate to the growth medium enhances the β-lactam production [43]. Therefore, the lysine biosynthsisc pathway is extremely important for, and interconnected with, the β-lactam biosynthesis pathway. Recent insights in the lysine biosynthesis route in yeast may alter the classical view on this pathway significantly. L-Lysine biosynthesis starts with the condensation of acetyl-CoA and α-ketoglutarate into homocitrate by *homocitrate synthase*. This enzymatic reaction was until recently believed to take place in the mitochondria of *P. chrysogenum* because of insights in this route in higher eukaryotes. However, a localization study using a GFP-fusion of *homocitrate synthase* indicates that in *P. chrysogenum* this protein is located in the cytosol, although it could not be excluded that minor amounts might be present in the nucleus and mitochondria [44]. Using an *in silico* approach by performing a *Saccharomyces cerevisiae* database context sensitive motif search to identify new peroxisomal proteins, it was established that both the LYS1 and LYS4 proteins that encode a *homoaconitase* and a *saccharopine dehydrogenase*, respectively, contain a C-terminal

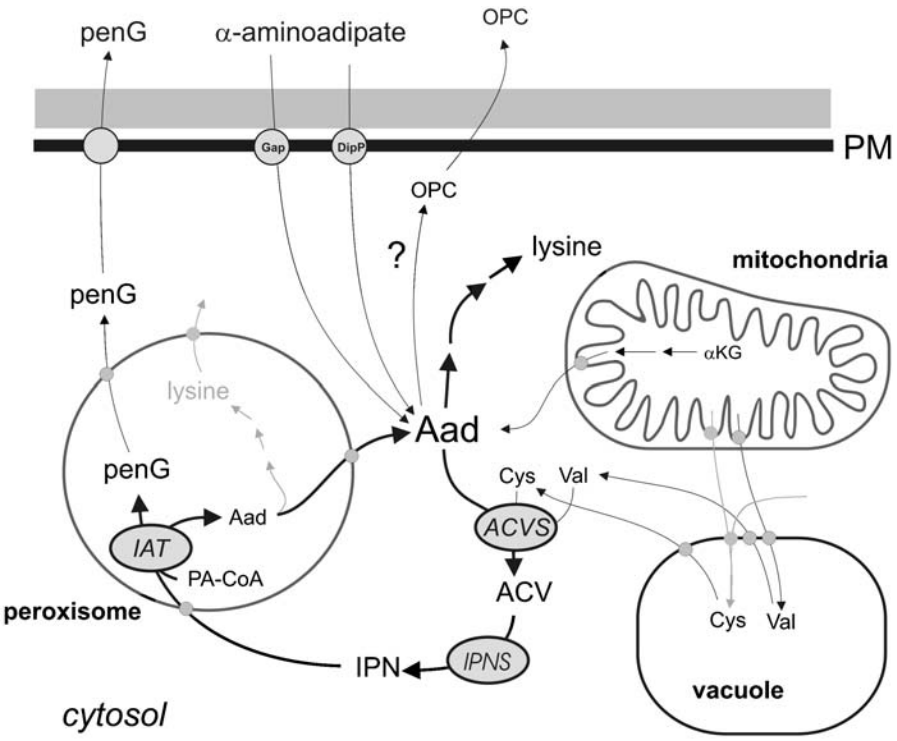

Fig. 2 Compartmentalization of lysine biosynthesis in relation to penicillin biosynthesis. α-Aminoadipate is a branch point intermediate at which the lysine and penicillin biosynthesis routes converge. In the last step of the penicillin biosynthesis, the α-aminoadipate moiety of isopenicillin N (IPN) is exchanged for phenylacetic acid and becomes available again for penicillin or lysine biosynthesis. Part of α-aminoadipate is lost by the cyclization into 6-oxopiperidine-2-carboxylic acid (OPC). OPC is excreted into the medium by an unknown mechanism. Main routes are depicted in *bold arrows*, hypothetical routes are in *light grey*. PM, plasma membrane

peroxisomal targeting signal (PTS1). GFP-fusions of these proteins localized to peroxisomes. This suggests that these proteins are peroxisomal localized [44, 45] (Fig. 2). Furthermore, examination of micro-array experiments to determine the role of peroxisomes under physiological conditions revealed that in a peroxisome-deficient mutant five genes of the lysine biosynthesis pathway are highly up-regulated, among them LYS1 and LYS4. The other three genes that are up-regulated are LYS20 (*homocitrate synthase*), LYS12 (*homoisocitrate dehydrogenase*) and LYS9 (another *saccharopine dehydrogenase*). LYS12 contains a putative PTS1 whereas LYS9 and LYS20 contain PTS2-like sequences. The observed expression pattern of the genes of a peroxisome-deficient mutant grown on rich medium surprisingly resembled a lysine starvation response even when sufficient lysine was present in the medium. The authors explain their findings by mislocalization of α-aminoadipate semialdehyde to the cytosol. When

α-aminoadipate semialdehyde is not contained inside the peroxisome the level in the cytosol will increase and stimulate the Lys14p transcriptional activator [46]. In contrast, no peroxisomal PTS could be detected in Lys5p or in the amino acid aminotransferases that are thought to be a part of the lysine biosynthetic pathway. Therefore, it is believed that it is not very likely that all the lysine biosynthesis enzymes have an exclusively peroxisomal location, and that part of the pathway may be cytosolic [46]. The question arises if these proteins are also localized in the peroxisomes of *P. chrysogenum*, which is important for the question where the L-α-aminoadipate is formed? Consequently, a peroxisomal location poses some important questions about the mechanism of release of L-α-aminoadipate, as this charged amino acid is unlikely to pass the membrane passively.

4.2 Cyclization of L-α-Aminoadipate

During β-lactam biosynthesis, part of the α-aminoadipate is lost by the irreversible formation of 6-oxopiperidine-2-carboxylic acid (OPC), the cyclized δ-lactam of α-aminoadipate. This compound is excreted into the medium. The extent of OPC formation ranges from 6 to 60% relative to the formation of penicillin (on a molar basis), depending on strain and cultivation conditions. The route leading to OPC is not understood [47] nor is it clear how this compound is excreted.

4.3 Cysteine Synthesis

The synthesis of cysteine in *P. chrysogenum* is dependent on the active uptake of sulfate from the exterior of the cell. The sulfate assimilation pathway catalyses the reduction of sulfate via sulfite to sulfide and subsequently sulfide is converted into cysteine (Fig. 3). The reduction of sulfate into sulfite is catalysed by three enzymes: *ATP sulfurase* converts inorganic sulfate into adenosine-5-phosphosulfate (APS) which is then activated into 3-phospho-adenosine-5-phosphosulfate (PAPS) by *APS-kinase* and reduced to sulfite by *PAPS reductase* [48–50]. Sulfite is reduced to sulfide by *sulfite reductase* [51]. The location of enzymes involved in the reduction of sulfate has not been described.

Sulfide is the basis for biosynthesis of L-cysteine, which occurs via two different pathways in β-lactam producing fungi: the transsulfuration and the sulfhydrylation pathway. L-Cysteine, synthesized via the transsulfuration pathway, is formed by cleavage of L-cystathionine derived from the intermediate L-homocysteine, which is formed from L-methionine or *O*-acetyl-L-homoserine [52]. Otherwise, direct acetylation of L-serine yields *O*-acetyl-L-serine that, in the presence of sulphide, is converted to L-cysteine by action of the enzyme *O-acetyl-L-serine sulfhydrylase* (OASS). Theoretically, the yield of penicillin on glucose would be substantially higher when L-cysteine is synthesized exclusively via the direct sulfhydrylation pathway [53, 54]. In *A. nidulans* and *C. acre-*

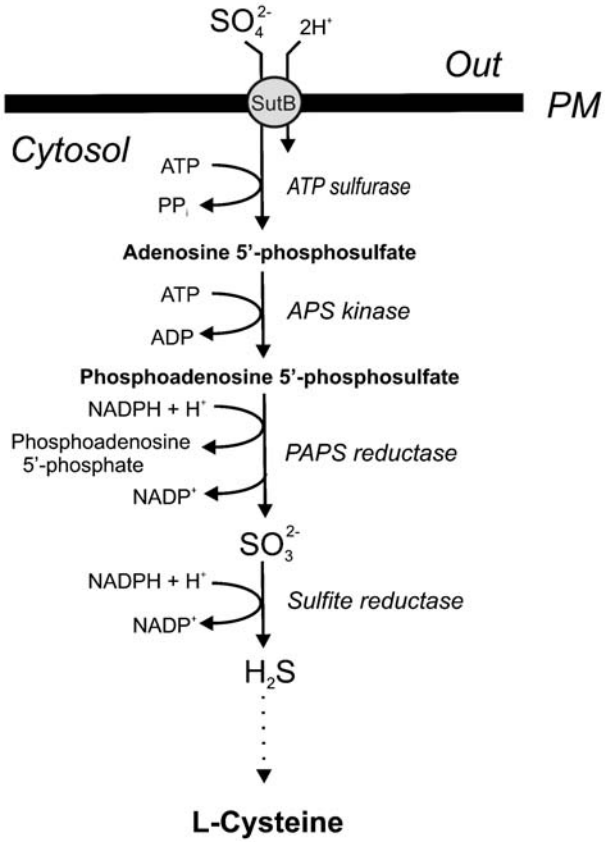

Fig. 3 Sulfate uptake and metabolism in *P. chrysogenum*. PM, plasma membrane

monium both pathways are described, although *A. nidulans* prefers the direct sulfhydrylation, while *C. acremonium* utilizes the transsulfuration pathway [55, 56]. For the industrial penicillin producer *P. chrysogenum* only the presence of the transsulfuration pathway was demonstrated as mutants, disturbed in this pathway, were unable to grow on inorganic sulfate [57], which is the main source of sulfate during industrial fermentations. Recently, Østergaard et al. [58] reported the purification of OASS from *P. chrysogenum*. This enzyme is localized in the mitochondria (van den Berg MA, Westerlaken I, Hillekens R and Bovenberg RAL, unpublished results) and the analogous enzyme of the transsulfuration pathway, *O*-acetyl-L-homoserine sulfhydrylase (OAHS), is located in the cytosol. Moreover, a cloned cDNA encoding OASS was fused to eGFP and shown to encode active OASS enzyme located in the mitochondria (van den Berg MA., Westerlaken I., Hillekens R. and Bovenberg RAL, unpublished results). Isolated UV mutants that were unable to grow on inorganic sulfate unless OAS, or a more reduced sulfate source like cysteine or methionine,

was added to the medium are likely to be disturbed in serine transacetylase. These findings suggest a distinctive role of the direct sulfhydrylation pathway for growth. This in contrast to the cytosolic transsulfuration pathway, which seems to be used for penicillin production in *P. chrysogenum*, as an increase in detectable OAHS activity correlates with the onset of penicillin G biosynthesis in shake flask experiments (van den Berg MA, Westerlaken I, Hillekens R and Bovenberg RAL, unpublished results).

4.4 Valine Synthesis

Valine synthesis starts with the condensation of pyruvate with hydroxyethyl thiamine pyrophosphate into α-acetolactate by *Acetohydroxy acid synthase*. The conversion of α-acetolactate into L-valine is catalysed by three enzymes. *Acetohydroxy acid isomeroreductase* converts α-acetolactate into dihydroxyisovalerate. *Dihydroxy acid dehydrase* converts dihydroxyisovalerate into α-ketoisovalerate which in turn is converted to L-valine by the branched chain amino acid *Glutamate transaminase*.

All four enzymes are thought to be located inside the mitochondrial matrix [59]. Consequently valine has to be translocated to the cytosol to become available for ACVS. The mechanism by which amino acids that are synthesized inside the mitochondrial matrix are transported into the cytosol has not been investigated. The inner membranes of mitochondria contain a family of transporter proteins (the mitochondrial carrier family) of related sequence and structure that are involved in the uptake and excretion of various metabolites, nucleotides and cofactors [60, 61] (and references therein). A number of these transporters have been biochemically characterized by overexpression and functional reconstitution into liposomes, most notably the ATP/ADP translocase and the phosphate transporters. The mitochondrial transporters operate by various mechanisms, which include uniport, symport, and antiport mechanisms [62] (and references therein). As to amino acid transport, a few transporters have been biochemically characterized: two human aspartate-glutamate transporters, citrin and aralar1, mediate the antiport of aspartate for glutamate [63]; a human glutamate transporter, GC, catalyses the uptake of glutamate in symport with protons [60], and an ornithine transporter from rat liver mitochondria that catalyses the uptake of ornithine in an antiport reaction for citrulline or protons [64]. In *S. cerevisiae*, an ornithine transporter, ARG11, mediates the exchange of ornithine for protons, but transports also arginine and lysine with less affinity [65]. The genomic sequence of *S. cerevisiae* suggests presence of 35 putative members of the mitochondrial transporter family and an increasing number can now be associated with a particular transport reaction [60, 66] (and references therein). It is unknown how valine, cysteine and α-aminoadipate are transported across the mitochondrial inner membrane. Interestingly, one member of the mitochondrial carrier family in *S. cerevisiae* turned out to be an adenine nucleotide transporter in peroxisomes [67].

5
Uptake of β-Lactam Precursors from the Growth Medium

5.1
Uptake of Amino Acids

Since penicillin and cephalosporin are synthesized from three amino acid precursors, amino acids might play an important role in the regulation of β-lactam synthesis. This may be either directly, e.g. precursor availability for ACV synthesis, or indirectly, e.g. by affecting expression of penicillin synthesis genes. All amino acids are synthesized by filamentous fungi, but can also be taken up from the extracellular medium and be used as nitrogen and carbon source. In industrial *P. chrysogenum* fermentations for penicillin production, corn steep liquor is often used as nitrogen source. This supplement is rich in amino acids, which are consumed in the exponential phase of a fed-batch cultivation, rather than ammonia. When the amino acids are depleted the cells start to utilize ammonia as the nitrogen source [68].

A number of effects of the addition of extracellular amino acids on β-lactam synthesis have been reported. The addition of the three amino acid precursors α-aminoadipate, cysteine and valine to *P. chrysogenum* in nitrogen-less medium leads to efficient incorporation into ACV. Only α-aminoadipate increases the rate of ACV synthesis and the overall penicillin synthesis rate [43, 68]. Based on such studies, it was suggested that the intracellular α-aminoadipate concentration may be limiting for the penicillin biosynthesis [69]. The addition of lysine to *P. chrysogenum* and *A. nidulans* cultures leads to a reduction of penicillin biosynthesis [70]. L-Lysine inhibits *homocitrate synthase*, the first enzyme in the lysine biosynthesis pathway, thereby blocking the production of α-aminoadipate, which is the branch-point metabolite between the lysine and penicillin biosynthetic pathways [71]. Lara et al. reported a stimulating effect on penicillin synthesis by addition of L-glutamate in minimal media. Interestingly, this effect was also observed with non-metabolisable analogues of L-glutamate [72].

The effect of externally added amino acids on the expression of penicillin biosynthesis genes *acvA* and *ipnA* was investigated in *A. nidulans*. The negative effect of histidine and valine is due to a reduced activation of the transcriptional factor PACC under acidic conditions. The presence of these amino acids leads to a decreased ambient pH during cultivation of the fungus. The negative effect of lysine and methionine, that also cause an acidification of the medium, does not involve PACC. The mechanism by which these amino acids act is unclear [73].

In *Acremonium chrysogenum*, the addition of DL-methionine to the medium led to increased mRNA levels of cephalosporin biosynthesis genes *pcbAB* (*acvA*), *pcbC* (*ipnA*) and *cefEF*, encoding deacetylcephalosporin C synthetase/hydroxylase and a three- to fourfold increase in the production of cephalosporin C [74, 75].

Uptake of amino acids in filamentous fungi is mediated by active amino acid permeases. In general, fungi possess a multiplicity of amino acid permeases

that are involved in the uptake of amino acids from the environment as nitrogen and/or carbon source or as building blocks for the synthesis of proteins and peptides [76]. Biochemical and genetic characterization of fungal amino acid transporters has been performed most extensively in *S. cerevisiae*. Most fungal amino acid permeases show significant sequence similarities and form a unique family referred to as the AAP family [77], a subfamily of the APC family [78]. The permeases have a common structural organization with 12 putative α-helical transmembrane segments and cytoplasmically located N- and C-terminal hydrophilic regions [79–81]. Uptake occurs as secondary transport, i.e. by proton symport, with the pmf as driving force in order to allow uptake against the concentration gradient [77, 78, 82]. An exception is an amino acid permease encoded by the *mtr* locus of *Neurospora crassa* [83, 84]. This permease is unrelated to the AAP family, but instead it belongs the amino acid/auxin permease (AAAP) family [85]. Recently, a functional and structural homologue of the *mtr* encoded permease was found in *P. chrysogenum* (unpublished results). Genome analysis of *S. cerevisiae* revealed 24 members of the AAP family, of which most have been functionally characterized [79]. Some of them are specific for one or a group of related L-amino acids, such as Dip5p (glutamate and aspartate), Put4p (proline), Can1p (arginine). Others have a broader specificity, like Agp1p, which transports most neutral amino acids [79, 86]. The general amino acid permease Gap1p, transports all L-and D-amino acids and non-proteinogenic amino acids such as citrulline and ornithine [87].

In *P. chrysogenum*, so far three amino acid permeases have been cloned and characterized [88]; (Trip et al. unpublished results), and various other activities have been classified on the basis of transport and competition assays. Nine amino acid transport systems have been reported: system I for L-methionine [89]; II for L-cysteine [90]; III for all amino acids [91, 92], analogous to Gap1p of *S. cerevisiae*; IV for acidic amino acids; V for L-proline; VI for L-lysine and L-arginine, VII for L-arginine; VIII for L-lysine and IX for L-cysteine [92]. The first two systems are expressed under sulphur starvation, while systems III-V are expressed under nitrogen and carbon starvation (NCR and CCR). Systems VI-VIII appear constitutive [76]. System VI was studied by Hillenga et al., 1996 [93], using plasma membranes fused with liposomes containing cytochrome *c*. Factors that interfere with the analysis of the plasma membrane transport processes when performed with intact mycelium, like metabolism and compartmentalisation, were circumvented this way. Inhibition studies with analogues revealed a narrow substrate specificity for arginine and lysine and quantitative analysis of arginine uptake suggest a H^+-arginine symport stoichiometry of one-to-one [93]. Uptake of α-aminoadipate, an acidic amino acid similar in structure to glutamate, might be mediated by both the acidic amino acid transport system [92], and general amino acid transport system [76]. The acidic amino acid permease gene, DipP, was cloned and biochemically characterized. This transporter is homologous to Dip5 of *S. cerevisiae* [79] and is capable of transporting α-aminoadipate, albeit with much lower affinity than the preferred substrates aspartate and glutamate (K_m of 800 and 35 µmol/l, re-

spectively) (H. Trip, unpublished results). Transport studies with penicillin producing mycelium show that α-aminoadipate uptake is strongly competed by leucine which is a substrate for the general amino acid permease, and not for DipP. This suggests that the general amino acid permease provides the main route for α-aminoadipate uptake into the cell. The expression of DipP is, like Dip5 in *S. cerevisiae*, under nitrogen catabolite repression and is strongly induced when glutamate is the only nitrogen source in the culture medium (H. Trip, unpublished results). *ARLP* encodes a permease specific for aromatic amino acids and leucine [88] and *MTRP* encodes a permease specific for neutral aliphatic and aromatic amino acids (H. Trip, unpublished results). MtrP is a structural and functional homologue of the *mtr* locus encoded protein of *N. crassa* and therefore not related to the AAP family, but a member of the AAAP family [88]. The physiological role of these permeases is unclear. It has been postulated that the ACV precursors α-aminoadipate, cysteine and valine are sequestered in the vacuole of *P. chrysogenum*. Cysteine and valine are produced in the vacuole due to proteolytic degradation of proteins. The presumed vacuolar localization of ACVS would then benefit from a direct withdrawal of these amino acids from the vacuolar pools [13]. The recent observation that ACVS is located in the cytosol [14] and the fact that in *S. cerevisiae* the acidic amino acids glutamate and aspartate are not accumulated in the vacuole, but instead, are located almost exclusively in the cytosol [94], do not support the vacuolar storage of the acidic amino acid α-aminoadipate. In *S. cerevisiae*, four vacuolar amino acid transporters have been identified, one of which, AVT6, mediates the efflux of the acidic amino acids glutamate and aspartate from the vacuole. These transporters do not show homology with amino acid permeases from the cellular membrane [95].

5.2 Uptake of Sulfate and Phosphate

The uptake of sulfate is an important step in the regulation of sulphur metabolism in *P. chrysogenum.* This uptake has been studied with mycelium and isolated plasma membrane vesicles. These experiments showed that uptake is mediated by a electroneutral sulfate/proton symport mechanism [96]. The *P. chrysogenum* membrane vesicles were fused with cytochrome-c oxidase containing liposomes to provide the system with a proton motive force. Sulfate uptake was solely dependent on the transmembrane pH gradient, and occurred with high affinity (K_m~30 µmol/l). Apart from sulfate, the transporter also showed affinity for analogous divalent oxyanions like thiosulfate, selenate and molybdate. The genes of two putative sulfate transporters (designated SutA and SutB), and *PAPS reductase* (*parA*) have been cloned and sequenced [28]. SutB is the major sulfate transporter, while the exact function of SutA remains to be elucidated. This protein has been implicated in thiosulfate uptake or is possibly involved in an intracellular sulfate uptake activity. Expression studies were performed to determine if there is a relationship between penicillin biosyn-

thesis and sulfate metabolism. Under sulphur starvation conditions the expression levels of both sulfate transporters are elevated. A positive correlation was observed between the levels of *sutB* mRNA and the penicillin biosynthesis, but such a correlation was not apparent for *sutA* and *parA* mRNAs. The *parA* mRNA levels are controlled by the sulphur content of the medium. It is generally believed that SutB is the main route for sulfate uptake during β-lactam biosynthesis [97].

Phosphate transport in *P. chrysogenum* has hardly been studied. In fungi uptake of phosphate occurs through proton and sodium phosphate symport. In fermentation media phosphate addition does not in itself inhibit penicillin production, but it strongly enhances the effect of glucose repression of transcription of the genes of the penicillin cluster [98]. In *S. cerevisiae* at least 5 transporters are involved in this process namely PHO84, 87, 89, 90 and 91. Deletion of all five genes is lethal [99]. Pho90 and Pho91 have the highest phosphate transporting capacity, whereas Pho84 and Pho87 are specific phosphate sensors. Pho89 has a very low transporting capacity and is not involved in phosphate signalling [99]. Pho84 is a phosphate proton symporter belonging to the Major Facilitator Superfamily (MFS) proteins and contains 12 membrane spanning segments.

5.3 Uptake of Nitrogen-Containing Compounds

Ammonium, nitrate, urea and amino acids are possible nitrogen sources in β-lactam synthesis. The uptake and synthesis of amino acids has been described above. Although one of the earliest reports about an active ammonium transport system concerned the uptake of methylammonium by *P. chrysogenum* [100], no major new insights have been obtained since then. At high concentration, methylammonium is toxic to cells, and this was used to screen for mutants of *S. cerevisiae* and *A. nidulans* that are impaired in methylammonium uptake [101, 102]. This screen lead to the identification of several genes that encode (methyl)ammonium transporters (MEP/AMT). In *A nidulans,* two ammonium transporters have been described. These two proteins, MeaA and MepA are also involved in the retention of ammonium as determined by cross-feeding studies [103]. Although the molecular mechanism of transport is still unclear, studies using the LeAMT1 plasma membrane ammonium transporter of tomato (*Lycopersicon esculentum*) that was functionally expressed in *Xenopus* oocytes, indicate that ammonium ions are the substrates rather than ammonia. Uptake seems to take place by means of a uniport mechanism [104].

5.4 Uptake of Side Chain Precursors

Phenylacetic acid (PA) and phenoxyacetic acid are weak acids that rapidly enter *P. chrysogenum* cells through passive diffusion and distribute across the

membrane according to the transmembrane pH gradient [105, 106]. However, various reports have implicated active transport in the acquisition of phenylacetic acid from the medium [107]. The major differences in these studies may relate to concentration of phenylacetic acid used, and eventually the type of strains (low- vs high-yielding strains). When high concentrations of PA (60–3000 µmol/l) are used, PA readily enters the cells through passive diffusion in both low- and high-yielding strains. However, at low concentrations (1.4–100 µmol/l) accumulation of PA in the low yielding strain exceeds the accumulation of PA in the high yielding by a factor 10 at the lowest concentrations, suggesting the involvement of a transporter protein [108]. However, the latter may also relate to side-chain activation. Instead of uptake, the activity of the CoA ligase may be responsible for the observed retention. However, during β-lactam biosynthesis, high concentrations (millimolar) of PA or phenoxyacetic acid are fed to the cells, which makes that passive diffusion will be the dominating route of entry into the cell.

5.5 Uptake of Sugars

The supply of sugars as the major carbon in industrial fermentation of *P. chrysogenum* is of importance, as it accounts for more than 10% of the overall costs [109]. Moreover, sugar plays an important role in the regulation of penicillin biosynthesis. Glucose and sucrose impose a strong inhibitory effect on β-lactam production by repression of penicillin biosynthesis genes (*acvA* and *ipnA* in *P. chrysogenum*, *ipnA* in *A. nidulans*) as well as by post-transcriptional (down)regulation (IAT in *A. nidulans*) [110]. Lactose does not inhibit β-lactam biosynthesis, which, for *P. chrysogenum*, was suggested to be due to the slow hydrolysis into glucose and galactose resulting from very low β-galactosidase activity [98]. Lactose has been traditionally used for penicillin biosynthesis, but during industrial fermentation, a limiting glucose-feed is now regularly used, avoiding carbon source/catabolite regulation [68].

Glucose uptake in fungi has been best studied for *S. cerevisiae*. Glucose transport occurs by facilitated diffusion [111] which involves transporters that belong to the MFS family [112, 113]. A family of 20 different hexose transporters or related proteins (Hxtp) is thought to be involved in sugar transport and regulation [111, 114]. In a *hxt1–7* disruption mutant strain, glucose uptake is abolished, whereas the expression of any one of the genes *HXT1*, *2, 3, 4, 6* or *7* can restore glucose uptake [115]. Hxt1p and Hxt3p are low affinity transporters (K_m=50–100 mmol/l), Hxt2p and Hxt4p are equipped with a moderately low affinity (10 mmol/l) and Hxt6p and Hxt7p are high affinity glucose transporters (1–2 mmol/l). A galactose permease was also shown to transport glucose with high affinity (K_m=1–2 mmol/l) [114]. In filamentous fungi, glucose uptake systems have been described for *A. nidulans* [114, 116], *A. niger* [117], and *N. crassa* [118–123]. In general, at least two systems appear to be present, a constitutive, passive, low-affinity system, and a glucose repressible, proton

motive force-driven, high-affinity system. The high-affinity system generally has a much lower K_m-value than found in *S. cerevisiae*. The K_m for the high-affinity system in *A. nidulans* is 0.04–0.06 mmol/l [81, 124]; for *P. chrysogenum* a value of 0.2 mmol/l has been reported [125]. Like in *S. cerevisiae*, more than two transporter proteins might be involved in glucose transport, but since mutants disrupted in one or more glucose transporter genes are not yet available, individual characterization is complicated.

Little information is available on lactose transport in fungi. The best characterized fungal lactose uptake system is the inducible *LAC12* gene product of *Kluyveromyces lactis* [126, 127], which transports lactose in symport with protons. Proton symport seems to be a general mechanism for disaccharide transport in fungi [126–128]. In *P. chrysogenum*, lactose is taken up by an energy-dependent system, mostly likely proton motive force-driven system. The lactose transport activity is induced when cells are growth on lactose (van de Kamp et al., unpublished).

6
Transport Across the Microbody Membrane

As mentioned previously, some of the enzymatic steps of the penicillin and lysine synthesis pathway take place inside the microbody. The exact reason why these steps are localized in this intracellular organelle is not clear, but it has been hypothesized that the microbody lumen provides an optimal environment for these enzymes for instance with respect to pH, metabolite concentration etc. The internal pH of peroxisomes in the yeast *Hansenula polymorpha* has been reported to be acidic (pH 5.8–6) [129]. However, the pH optimum of IAT is in the alkaline range, and the enzyme is inactive at pH values lower than 6 [130, 131]. The same has been reported for PCL which is likely localized in the microbody. The pH of *P. chrysogenum* microbodies has also been investigated with the enhanced yellow fluorescent protein (eYFP) that was targeted to the microbody by means of a C-terminal PTS1 signal SKL. Based on the fluorescence characteristics, it was concluded that the microbody is not acidic, but slightly alkaline (pH 7.0–7.5) [132]. This is more in accordance with the pH optimum of the abovementioned biosynthesis enzymes. Studies on microbodies in human fibroblasts even suggest that the luminal pH may be as alkaline as pH 8.2 [133].

Other possible advantages for compartmentalization of key enzymatic steps may relate to the higher concentrations of both enzymes and substrates, the prevention of draining catalytic intermediates into unwanted side reaction pathways, and/or regulation of the biosynthesis pathway. The subcellular distribution of the various enzymatic steps over different organelles poses, however, important problems towards the transport of the metabolites. For a long time it was believed that peroxisomes are permeable to small compounds. For instance, it was not possible to obtain peroxisomes while maintaining the permeability barrier of the membrane. Also, a porin-like protein has been found

to be associated with the peroxisomal membrane [134–137]. However, the in vivo studies on the luminal pH and identification of various transporters now suggest that the peroxisomal membrane represents a permeability barrier. NAD(H), NADP(H), acetyl-CoA, ATP and protons cannot freely pass peroxisomal membranes of different organisms [67, 133, 138, 139]. The necessity for peroxisomal membrane proteins (PMPs) with a transport function is therefore obvious. Biochemical studies, however, have suffered from the fact that the organelles are very fragile, while PMPs appear of low abundance [140]. Of the known peroxisomal transporters only one has been studied in detail with respect to substrate specificity, namely the peroxisomal adenine nucleotide transporter Ant1p of *S. cerevisiae*. This system is very homologous to the mitochondrial transporter family. Ant1p has been overproduced, purified from the peroxisomal membrane fractions and reconstituted into liposomes [67]. The system has been suggested to function as an ATP/AMP antiporter, supply the microbody lumen with cytosolic ATP. So far, experimental evidence is lacking for the involvement of transporters in the uptake of IPN and PA or the extrusion of α-aminoadipate and penicillins.

7
Excretion of β-lactams into the Medium

The mechanism of excretion of β-lactams into the medium has been a subject of speculation for a long time. Various options need to be considered, i.e., passive diffusion, vesicular transport and the involvement of transport proteins. Passive diffusion phenomena are strongly dependent on the physicochemical characteristics of the membrane, like fluidity, degree of saturation and the acyl chain length of the lipid fatty acids but also on the intrinsic properties of the compound, like charge, size and hydrophobicity. Penicillins V and G are amphiphatic, moderately hydrophobic molecules and negatively charged at the cytosolic pH. The diffusion of these molecules has been studied in model membranes, and it was suggested that they can permeate a membrane composed of phospholipids [106]. The permeability characteristics of the membrane were, however, greatly reduced when sterols were present in the membrane. Since plasma membranes of *P. chrysogenum* contain 30% ergosterol, a concentration that suffices to block most of the passive permeation, passive diffusion seems very unlikely [106].

During recent years it has become increasingly evident that all living cells are equipped with multidrug transporters that are capable of expelling unrelated, mostly hydrophobic compounds across the membrane. These transporters convey multidrug resistance to cells. Due to the physiochemical characteristics of penicillins, MDR transporters are likely candidates for β-lactam secretion. MDR transporters can be subdivided into six families (for a review see [141, 142]). Two of the transporter families have already been implicated in β-lactam extrusion and will be briefly discussed here; namely the ATP-binding-

cassettes (ABC) transporter superfamily and the MFS of proteins. The ABC-transporters form a very large family of proteins with a very broad spectrum of substrate specificity, they translocate both small and large molecules across membranes. They are characterized by the presence of two cytosolic nucleotide binding domains (NBD's) each containing the highly conserved Walker A and Walker B motifs that specify the nucleotide binding site, and two transmembrane domains consisting of six transmembrane spanning segments [141]. The MFS is also referred to as the uniporter-symporter-antiporter family. These proteins are secondary transporters that transport small molecules in a proton motive force-dependent manner. They can be classified into 17 families. This includes the drug:H^+ antiporter families that specify membrane proteins with either 12 or 14 membrane spanning segments [142].

Recently, the first experimental evidence has been obtained that secretion of β-lactam in filamentous fungi may indeed involve active transport. This concerned a study on the involvement of ABC-transporters of *A. nidulans* in drug resistance. After identification of a number of ABC-transporter genes, a disruption mutant for the *atrD* gene displayed increased sensitivity towards the chemically unrelated compounds valinomycin, nigericin and cycloheximide. Moreover, in a halo size assay, used as a measure of the amount of penicillin produced, a reduced penicillin production was detected for an *atrD* deletion strain [143]. This suggests a role of the ATRDp in penicillin secretion, although this needs to be verified by direct transport assays. In another study, the region downstream of the *acvS* gene of *Acremonium chrysogenum* was examined which identified a gene encoding a membrane protein (CefT) belonging to the MFS. The deduced protein sequence revealed that this protein belongs to the family of drug: H^+ antiporters with 12 transmembrane segments. Disruption of the gene showed that it was not required for cephalosporin synthesis and that growth of *A. chrysogenum* was not affected. However, amplification of the full length gene (2 to 4 copies) resulted in a twofold increase in the cephalosporin C production [29]. Both studies, however, await direct proof that the identified transporters (AtrD and CefT) mediate antibiotic transport. Also in *P. chrysogenum*, a series of ABC transporters have been identified that are expressed under penicillin producing conditions [144]. Some of these MDR-like ABC transporters are induced when cells are challenged with β-lactam, suggesting a role in β-lactam excretion. In various antibiotic producing organisms, genes have been identified that confer resistance to the produced antibiotic presumably by transporting the drug out of the cells. In the gene cluster for antibiotic biosynthesis of *Streptomyces argillaceus* the genes *mtrA* and *mtrB* are present that encode a putative ABC-transporter and render the organism resistant to mithramycin [145]. In β-lactam producing actinomycetes like *Nocardia lactamdurans* genes are found that code for transporter proteins either belonging to the ABC-transporter superfamily or the MFS [27].

8 Concluding Remarks

In recent years, major insights have been obtained in the compartmentalization of the β-lactam biosynthesis pathway in filamentous fungi. The exact molecular reasons for the localization of the last step of biosynthesis steps in a microbody are not known, although the specific pH in this organelle seems favourable for the catalytic activity of the key enzymes. Control of the cellular distribution of α-aminoadipate to direct it either into the lysine or penicillin biosynthesis pathway may be crucial now it appears that critical enzymatic steps take place in the microbody. However, many of the molecular details still need to be resolved (see chapter Brakhage et al.). Other questions concern if β-lactam synthesis is limited by transport process, as for instance, cellular secretion? Intrinsic to the approach of removing bottlenecks from a metabolic production process, new limiting factors are found one of which may related to transport, a process often ignored in metabolic pathway engineering programs.

The antibiotic resistance of bacteria necessitates the discovery and production of new antibiotics. Genetic engineering enables us to intervene in metabolic and biosynthetic pathways thereby providing new opportunities of product formation. Such challenging metabolic reprogramming efforts also require insights in critical transport steps and possible limitation by exciting substrate specificities. Metabolic flux analyses of genetically altered strain, genome sequencing and transcriptome profiling, and directed evolution promise to be interesting tools for the near future.

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