# Amino acid transport in *Penicillium chrysogenum* in relation to precursor supply for β-lactam production

Cover: sporulating *Penicillium chrysogenum* (obtained from Enius AG (Germany), website http://schimmel-schimmelpilze.de)

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**RIJKSUNIVERSITEIT GRONINGEN**

# Amino acid transport in *Penicillium chrysogenum* in relation to precursor supply for β-lactam production

**Proefschrift**

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**Wiskunde en Natuurwetenschappen**
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**Hein Trip**

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# Voorwoord

Dat was het dan. Meer dan vier jaar aio geweest, resulterend in dit proefschrift. Rond de tijd dat ik afstudeerde, wist ik niet of ik wel aio zou willen worden. Ik heb het toch gedaan op advies van onder andere mijn begeleider van mijn eerste afstudeeronderwerp, Ted van der Lende. En ik heb er geen spijt van gekregen. Het was een mooie tijd waarin ik veel geleerd heb en mooie en nieuwe ervaringen heb gehad zoals bijvoorbeeld het publiceren van het eerste artikel. Dat het een geslaagde tijd was heeft voor een groot deel gelegen aan de omgeving waarin ik heb kunnen werken. Daarom wil ik een aantal mensen bedanken.

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Hein

# Contents

# Chapter 1

## 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.

# 1. Introduction

Industrial penicillin and cephalosporin fermentation is performed using filamentous fungi. 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, the production of β-lactams may have evolved as a means of improving their ability to compete with other prokaryotes. Filamentous fungi acquired the biosynthetic genes possibly by horizontal gene transfer some 370 million years ago (25, 32, 34, 88). 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 (43). After the discovery of the application of penicillin as an antimicrobial agent in humans in the early 1940's, 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, which 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.

This chapter describes recent developments that are important towards the compartmentalization and transport of β-lactam antibiotics in 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 biosynthetic 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.

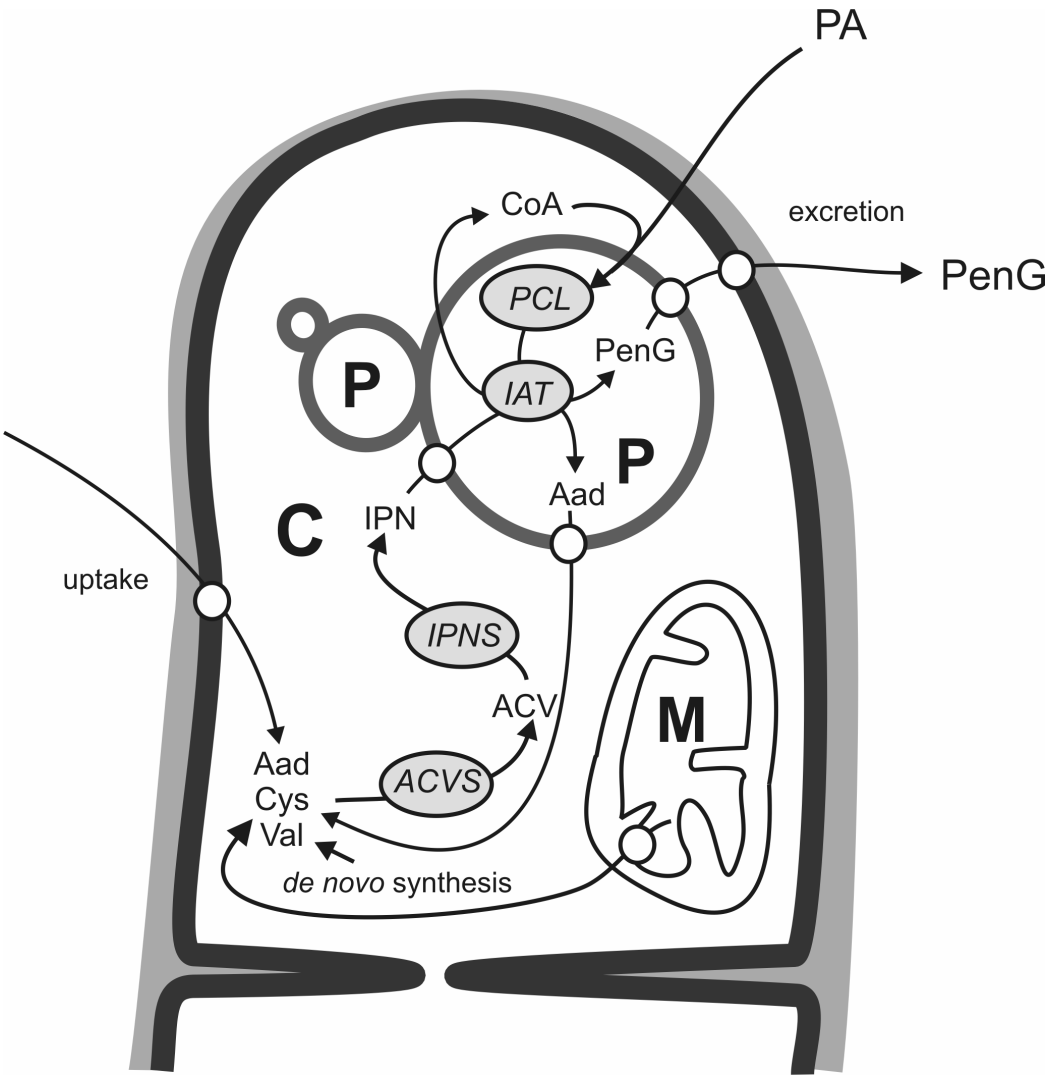


**Figure 1.** Localization of the penicillin biosynthetic; δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) and Isopenicillin N synthase (IPNS) are present in the cytosol (C) whereas Acyl-coenzyme A:isopenicillin N acyltransferase (IAT) and possibly also phenylacetyl-coenzyme A ligase (PCL) 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 catalyzed by a multi-enzyme complex of 424 kDa with non-ribosomal peptide synthetase activity termed *ACV synthetase* (ACVS) (1, 100-102, 234). 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 (52, 106, 111). 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 harbor any trans-membrane regions. Localization studies by traditional fractionation experiments were obscured 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 (52, 106, 111, 215). Likewise, an ACVS fusion with green fluorescent protein in *Aspergillus nidulans* also localizes to the cytosol (226). 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 (102). 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 cyclization of LLD-ACV into isopenicillin N (IPN). In this step, the bicyclic penam nucleus, consisting of the β-lactam and thiazolidine rings is generated (34, 102). 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-aminoadipyl)-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 (22, 34). Based upon the results of fractionation experiments it became evident that IPNS behaves like a soluble, cytosolic enzyme (136). 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 moiety for a phenylacetyl- or phenoxyacetyl group by *acyl-coenzyme A:isopenicillin N acyltransferase* (IAT) resulting in the formation of respectively penicillin G and penicillin V (11, 117, 205, 227). IAT is a hetero-dimeric enzyme consisting of an 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 (7, 8). Both subunits possess a C-terminal PTS1 signal that targets this enzyme to a microbody or peroxisome. Fractionation studies as well as immuno-gold labeling experiments indeed localized IAT to microbodies (136). 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 catalyzed 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 (66, 136). In another study a PCL of *P. chrysogenum* containing a C-terminal peroxisomal targeting signal (PTS1; SKI) was identified (65). 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 (65, 131). 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) (113, 212). 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 epimerization of IPN into penicillin N by epimerase activity of the *cef*D1 and *cef*D2 gene products in *C. acremonium* (210) and the completely different *cef*D gene product in *N. lactamdurans* and *St. clavuligerus* (113, 125). After epimerization, 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 (127). In *C. acremonium*,

DAC is acetylated by the *cefG*-gene encoded enzyme DAC acetyltransferase (129, 130), 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 (211). 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, 171).

## 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 (131, 196). 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 (219). 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 (136). 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* (135). 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 (39). 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 (136). 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. (156) and van der Klei et al. (220) 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 (115). Therefore, the lysine biosynthesis 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

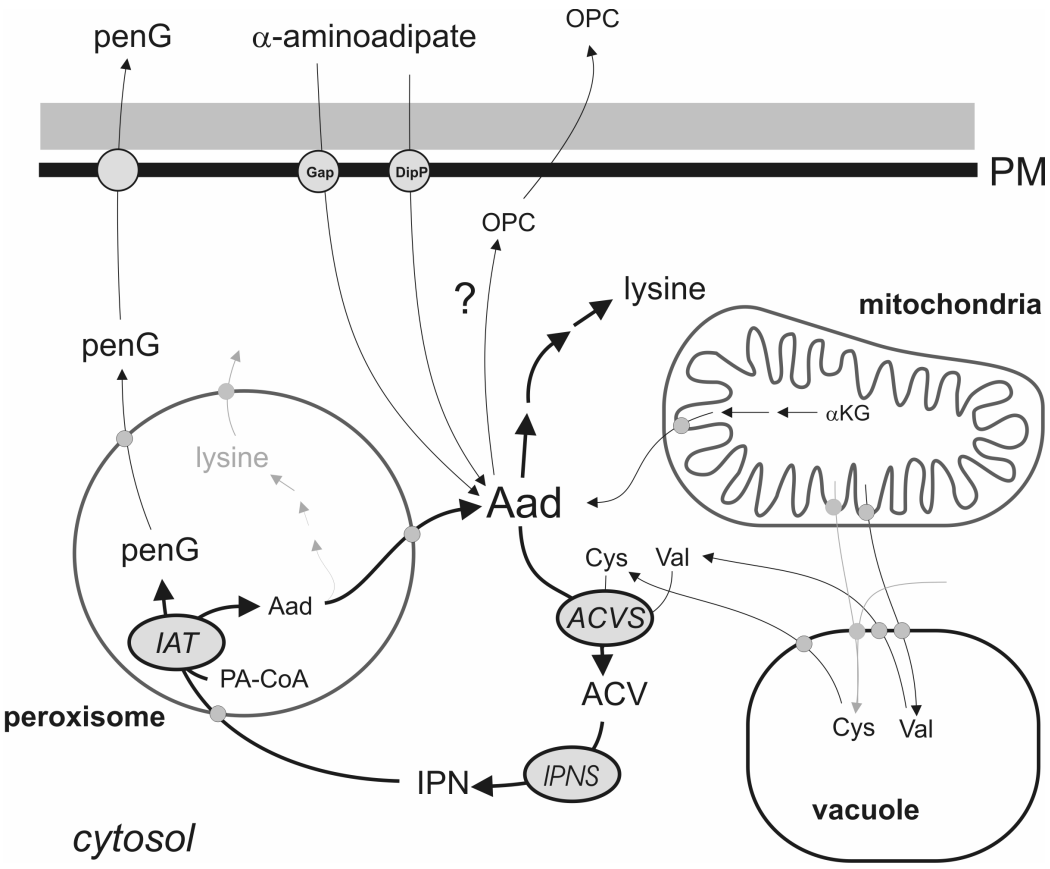


**Figure 2.** Compartmentalization of lysine biosynthesis in relation to penicillin biosynthesis. α-Aminoadipate (Aad) is a branch point intermediate at which the lysine and penicillin biosynthesis routes converge. In the last step of the penicillin biosynthesis, the α-aminoadipate moiety of isopenicillin 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.

*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 (10). 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 peroxisomal targeting signal (PTS1). GFP-fusions of these proteins localized to peroxisomes. This suggests that these proteins are peroxisomal localized (10, 61) (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 aminoadipate semialdehyde to the cytosol. When aminoadipate semialdehyde is not contained inside the peroxisome the level in the cytosol will increase and stimulate the Lys14p transcriptional activator (20). 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 (20). 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 (74) 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 catalyzes the reduction of sulfate via sulfite to sulfide and subsequently sulfide is converted into cysteine (Fig. 3). The reduction of sulfate into sulfite is catalyzed by three enzymes: *ATP sulfurase* converts inorganic sulfate into adenosine-5-phosphosulfate (APS)

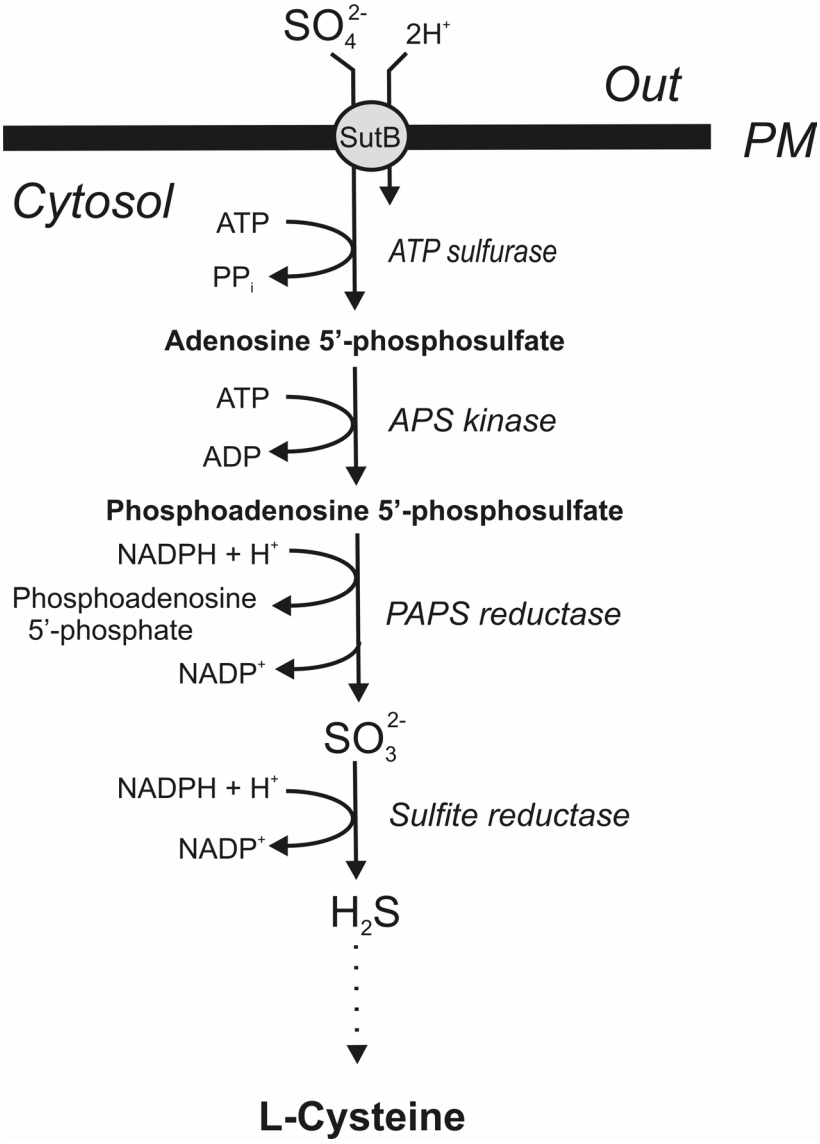


**Figure 3.** Sulphate uptake and metabolism in *P. chrysogenum*. PM, plasma membrane.

which is then activated into 3-phospho-adenosine-5-phosphosulfate (PAPS) by *APS-kinase* and reduced to sulfite by *PAPS reductase* (57, 164, 165). Sulfite is reduced to sulfide by *sulfite reductase* (128). 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 (145). 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 (75, 97). In *A. nidulans* and *C. acremonium* both pathways are described, although *A. nidulans* prefers the direct sulfhydrylation, while *C. acremonium* utilizes the transsulfuration pathway (155, 207). 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 (47), which is the main source of sulphate during industrial fermentations. Recently, Østergaard et al. (147) 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 sulphate unless OAS, or a more reduced sulphate 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 catalyzed 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 (105). 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 (55, 148 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 (152 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 (150); a human glutamate transporter, GC, catalyzes the uptake of glutamate in symport with protons (55), and an ornithine transporter from rat liver mitochondria that catalyzes the uptake of ornithine in an antiport reaction for citrulline or protons (87). In *S. cerevisiae*, an ornithine transporter, ARG11, mediates the exchange of ornithine for protons, but transports also arginine and lysine with less affinity (149). The genomic sequence of *S. cerevisiae* suggests the presence of 35 putative members of the mitochondrial transporter family and an increasing number can now be associated with a particular transport reaction (55, 124 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 (151).

# 5. Uptake of β-lactam precursors from the growth medium

## 5.1. Uptake of amino acids

### 5.1.1. The role of amino acids in penicillin production

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. Filamentous fungi are able to synthesize all amino acids, but they can also take them up from the extracellular medium and use them as nitrogen and carbon source or as building blocks for protein or peptide synthesis. 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 (143).

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 (115, 143). Based on such studies, it was suggested that the intracellular α-aminoadipate concentration may be limiting for the penicillin biosynthesis (81). The addition of lysine to *P. chrysogenum* and *A. nidulans* cultures leads to a reduction of penicillin biosynthesis (41). 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 (118). 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-metabolizable analogues of L-glutamate (107).

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 (204).

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 (48, 224).

### 5.1.2. Uptake of amino acids in fungi

Uptake of amino acids in filamentous fungi is mediated by 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 (82). 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 (6) or YAT family, a subfamily of the large and ubiquitous Amino Acid-Polyamine-Organocation (APC) Superfamily (91). The permeases have a common structural organization with 12 putative α-helical transmembrane segments and cytoplasmically located N- and C-terminal hydrophilic regions (63, 186). 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 (6, 91, 181).

In *S. cerevisiae*, 18 members of the AAP family can be distinguished of which most have been functionally characterized (157). 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 (89, 157).The general amino acid permease Gap1p, transports all L-and D-amino acids and non-proteinogenic amino acids such as citrulline and ornithine (93). Two methionine permeases, Mup1 and Mup3, are more distantly related to the AAP family. It has been suggested that they form a separate family, mainly based on a different topology of 11 transmembrane segments as predicted by a prediction program used at that time (90). However, more up to date programs clearly predict 12 TMSs. The amino acid sequence of Mup1 and Mup3 still

differs significantly from AAP family members, classifying them outside this family, but within the APC family.

Two amino acid permeases of the pathogenic yeast *Candida albicans*, one specific for L-arginine, L-lysine and L-histidine (CaCan1) and one specific for L-proline have been isolated from the plasma membrane and functionally reconstituted into proteoliposomes, allowing a study of AAPs in an isolated system. Arginine and respectively proline transport characteristics were similar to those observed in intact yeast cells (96, 134).

From filamentous fungi, several amino acid permeases have been cloned and biochemically characterized. These include the well studied proline permeases prnB of *Aspergillus nidulans* (187, 202), three permeases (AAT1p, AAT2p and UfAAT3p) of the rust fungus *Uromyces fabae* (71, 193, 194), a general amino acid permeases (HcGap1) of *Hebeloma cylindrosporum* (230), a broad specificity amino acid permease (AmAAP1) of *Amanita muscaria* (139), a general amino acid permease of *Neurospora crassa*, Naap1 (122) and Mtr, an aromatic and neutral aliphatic amino acid permease of *N. crassa*, which is not related to the AAP family (46, 190). All other described transport systems have been identified on basis of transport and competition assays (82 and references therein).

From *P. chrysogenum*, so far four amino acid permeases have been cloned and characterized: the general amino acid permease PcGap1, the acidic amino acid permease PcDip5 (208), an aromatic amino acid and leucine permease ArlP (209) and PcMtr, a structural and functional homolog of Mtr of *N. crassa* (Trip et al., unpublished results). On the basis of transport and competition assays, nine amino acid transport systems have been reported: system I for L-methionine (13); II for L-cysteine (183); III for all amino acids (14, 85) (corresponding to PcGap1); IV for acidic amino acids (corresponding to PcDip5); V for L-proline; VI for L-lysine and L-arginine, VII for L-arginine; VIII for L-lysine and IX for L-cysteine (85). 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 (82). System VI was studied by Hillenga *et al*., 1996 (78), 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 compartmentalization, 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 (98).

As mentioned above, Mtr of *N. crassa* and PcMtr of *P. chrysogenum* are structurally different from the AAP family permeases. Mtr and PcMtr are specific for aromatic and neutral aliphatic amino acids (46, 190) (Trip et al., unpublished results), and belong to the eukaryotic-specific amino acid/auxin permease (AAAP) family. This family is distantly related to the large APC family and contains permeases specific for amino acids and their derivatives, in plants and animals as well as vacuolar amino acid permeases in *S. cerevisiae* (172, 231). Members of the AAAP family show relatively low sequence similarity but share a common topology of 11 (or in some occasions 10) transmembrane segments. As with AAPs, amino acid transport by Mtr and PcMtr, appears to occur by proton symport (231).

### 5.1.3. Uptake of α-aminoadipate in *Penicillium chrysogenum*

Uptake of the β-lactam precursor α-aminoadipate, an acidic amino acid similar in structure to glutamate, is mediated by both the acidic amino acid permease PcDip5, and general amino acid permease PcGap1 (82, 85, 208). Their genes, *PcDIP5* and *PcGAP1* were cloned and functionally expressed in *S. cerevisiae* strain M4276, which is disrupted in the *DIP5* and *GAP1* genes and thereby deficient in acidic amino acid uptake (159). PcDip5 is capable of transporting α-aminoadipate, albeit with much lower affinity than the preferred substrates aspartate and glutamate ($K_m$ of 800 and 35 μM, respectively). PcGap1 transports all amino acids including non-protein amino acids and transports α-aminoadipate with intermediate affinity ($K_m$ = 230 μM). Studies on the uptake of α-aminoadipate and competition with leucine, a substrate for the PcGap1, and not for PcDip5, with penicillin producing mycelium of *P. chrysogenum*, show that both PcDip5 and PcGap1 contribute equally in α-aminoadipate uptake, when present at low concentration (25 μM) (208). At higher concentrations, PcDip5 may become the major uptake route as it appears to have a higher capacity for α-aminoadipate transport.

### 5.1.4. AAP family and Mtr type permeases

With an increasing number of sequenced fungal genomes becoming accessible, the distribution of AAP family members as well as Mtr homologs in fungi

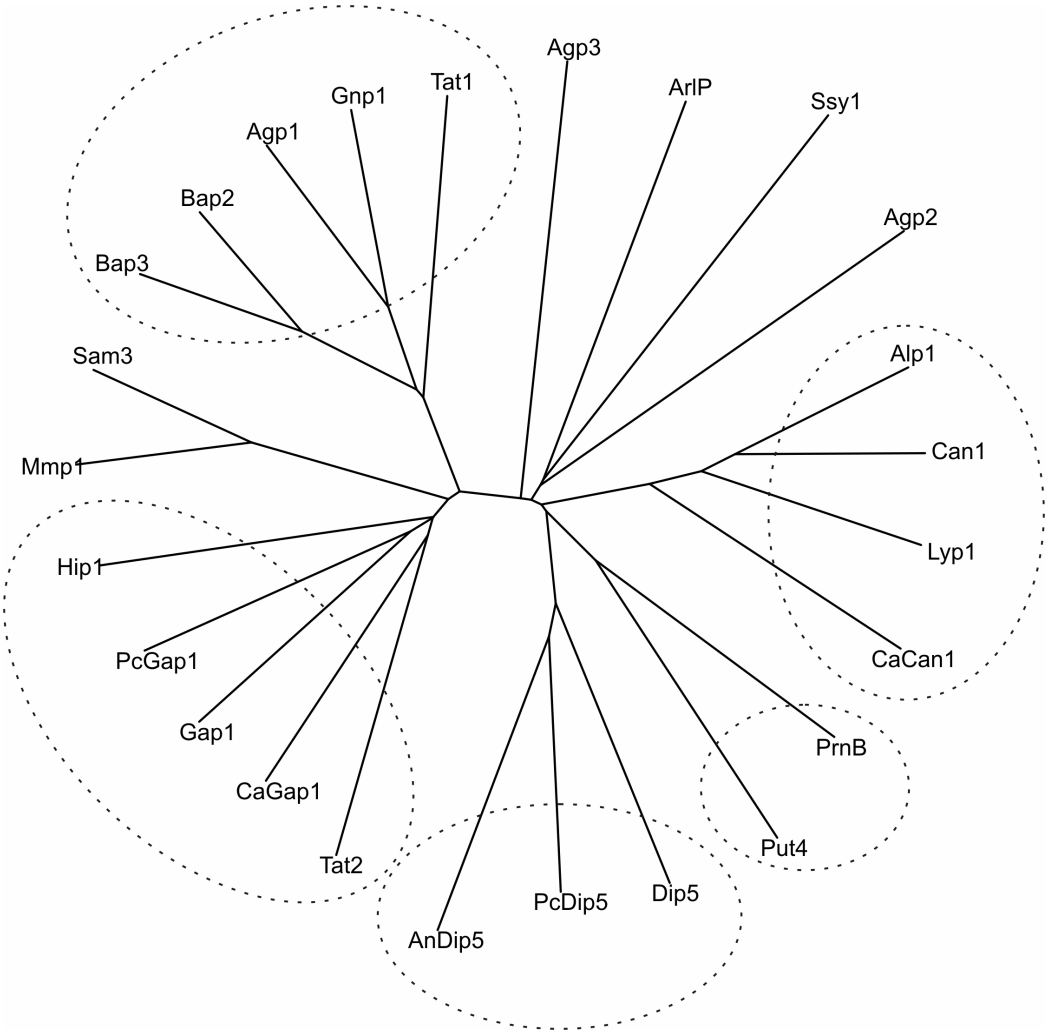


**Figure 4.** Evolutionary tree of AAP family permeases with known substrate specificity. Permeases with similar substrate specificity group in clusters.

was investigated (Trip et al., unpublished results). In general, a well distinguishable group of AAPs is found, varying in number from 8 in *Ustilago maydis*, to approx. 20 in *A. nidulans*. Being closely related to *A. nidulans*, *P. chrysogenum* may be expected to have a similar number of AAPs. Strikingly, in most filamentous fungal genomes, one Mtr homolog (> 50 % amino acid identity) is found, with the exception of *F. graminearum*, which has 5 Mtr homologs. In the yeasts *S. cerevisiae*, *Ashbya gossypii*, *Candida albicans* and the fission yeast *Schizophyllum pombe*, no Mtr homolog is present (Trip et al., unpublished results).An alignment of AAPs and a derived evolutionary tree show clustering of permeases with similar substrate

specificity (Fig. 4), (157). The acidic amino acid permeases (Dip5 and homologs), the basic amino acid permeases (Can1 etc.) and neutral amino acid permeases (Agp1, Bap2 etc.) are present in separate groups. A cluster containing the general amino acid permeases (Gap1, PcGap1 etc.) seems a more mixed group in which also the histidine permease Hip1 is present. Non-characterized AAPs that group with characterized permeases in this tree might therefore be expected to have similar substrate specificity.

### 5.1.5. Structure-function relationships in AAPs

So far, little is known about the structure-function relationships in AAPs. They all share a 12 transmembrane segment topology with cytoplasmic N- and C-termini (63, 186). Alignment of AAP family members shows conserved regions in predicted transmembrane segments as well as in cytoplasmic and extracellular loops. Also, positions are conserved within clusters of amino acid permeases with similar substrate specificity (Fig. 5). A lysine residue in transmembrane segment 3 of 3 acidic amino acid permeases (Dip5 (159), position 173; AnDip5 of *A. nidulans* (C. Scazzocchio, personal communication) and PcDip5 (208), is conserved, whereas in other AAPs this position is occupied by uncharged amino acids. In the same transmembrane segment, a threonine residue is conserved in three basic amino acid permeases (Can1 (position 180), Lys1, Alp1) of *S. cerevisiae* and one of *C. albicans* (CaCan1 (198)). When a random mutagenesis of arginine transporter Can1 was performed, resulting in the isolation of different clones that had acquired low capacity citrulline transport ability, mutations were found in transmembrane segments 3 and 10 as well as in cytoplasmic and extracellular loops. Most of the affected positions are conserved among most AAPs, but none, apart from a serine in the cytoplasmic loop between TMS 2 and 3, are specifically conserved in cationic amino acid permeases. All mutations seem to have an indirect effect on substrate recognition, rather than being directly involved in substrate binding or selectivity (160). In PrnB of *A. nidulans*, two mutations located in transmembrane segment 6, one at an absolutely AAP family-conserved phenylalanine (F248L) and one conserved specifically in proline permeases (K245L), clearly affect proline uptake kinetics. From this it was suggested that this region may play a role in substrate binding and/or transport. However, considering that the more drastic K245E mutation had a smaller effect on proline transport than the K245L mutation, indicates that this residue is not directly involved in substrate binding or recognition (201).

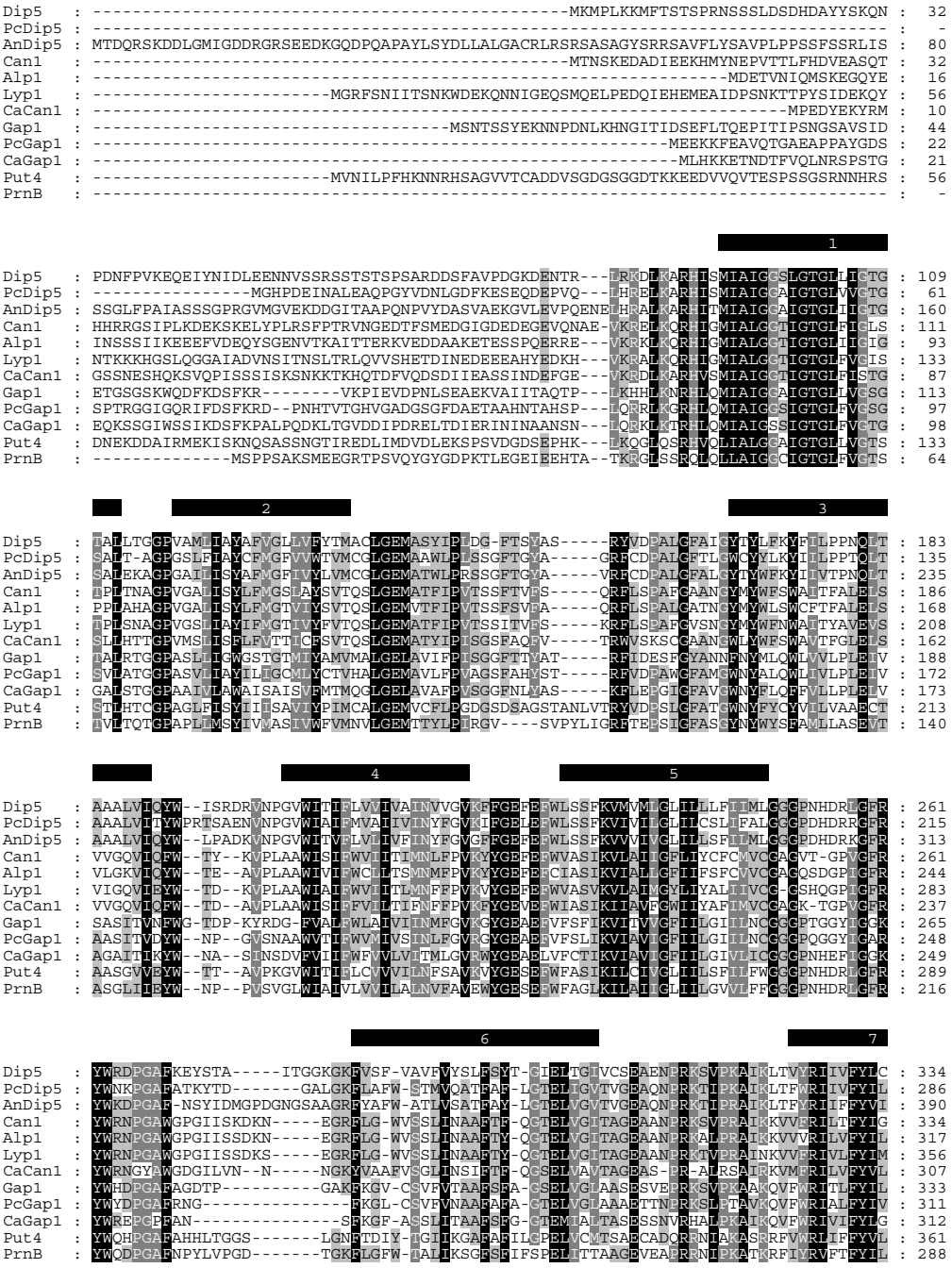


**Figure 5.** Alignment of characterized AAP family members from 4 clusters: acidic amino acid permeases (Dip5 etc.), basic amino acid permeases (Lyp1 etc.), proline permeases (Put4 and PrnB) and general amino acid permeases (Gap1 etc.). The approximate positions of the 12 transmembrane segments are indicated by the black bars above the sequences.

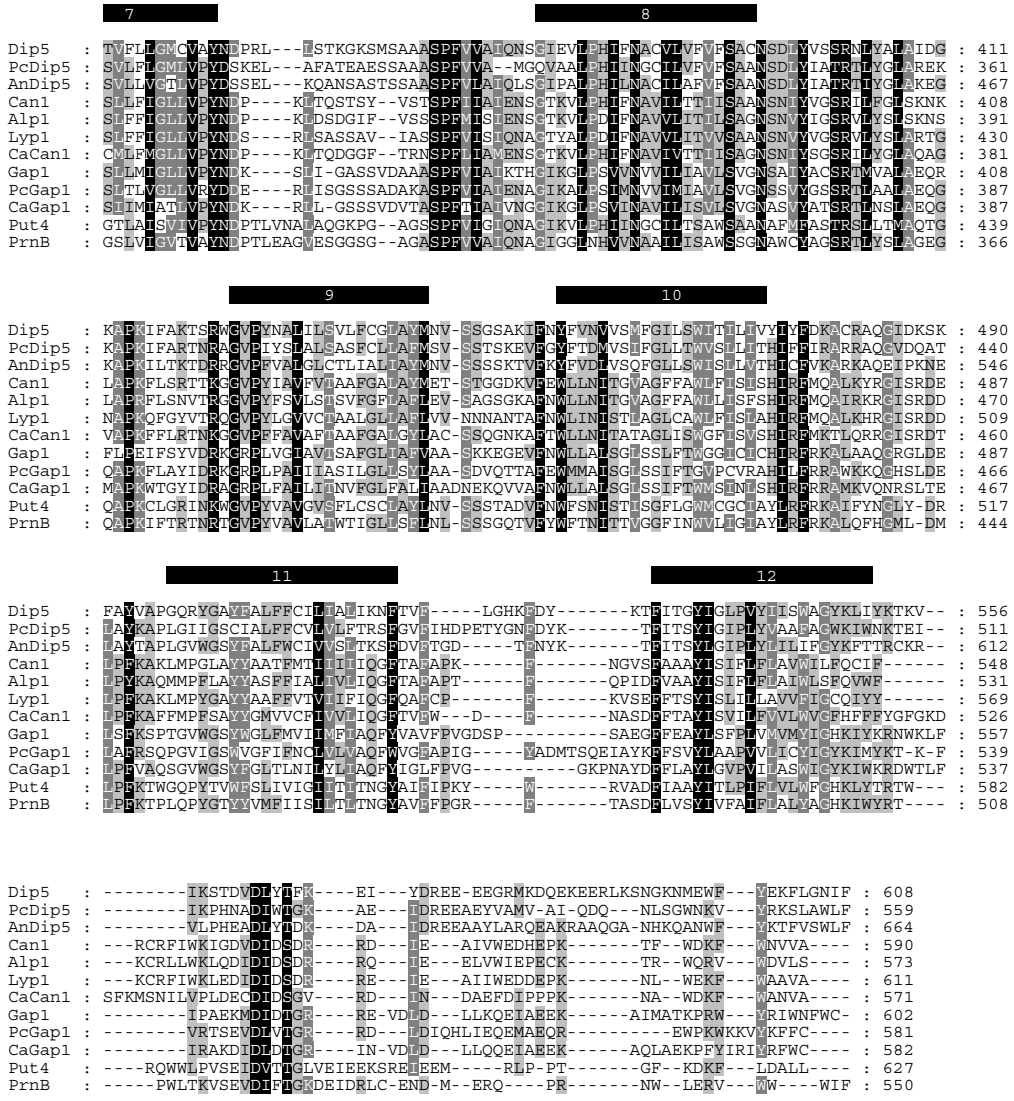


Figure 5 (continued)

### 5.1.6. Regulation of amino acid transport on the transcriptional level

The expression of amino acid permeases in fungi is regulated at the transcriptional and post-translational level (6, 82, 186). In general, transcriptional regulation provides a mechanism to tune the expression of genes to the environmental conditions, thereby preventing the synthesis of proteins required for utilization of substrates when other, preferred substrates are present, whereas post-translational regulation can more quickly adapt cells to a changing environment by inactivating or reactivating enzymes or processes within minutes (68). This prevents the energy consuming uptake and accumulation of substrates when other, preferred substrates are available. Post-translational regulation can negate transcriptional regulation, i.e. genes may be transcribed and translated but their proteins can be inactivated and targeted to the vacuole for breakdown (191).

The best known transcriptional regulation mechanism involved in utilization of nitrogen containing compounds is nitrogen catabolite repression (NCR), which decreases the level of enzymes required for utilization of unfavorable nitrogen sources when a preferred nitrogen source like ammonia, asparagine or glutamine is present (228). The general amino acid permease (Gap) activity in *S. cerevisiae*, *P. chrysogenum*, *C. albicans* and *N. crassa* is repressed when $NH_4^+$ or glutamine is present. In *S. cerevisiae* (strain Σ1278b and derived strains) the transcript level of *GAP1* is low when glutamine, asparagine or ammonia is the nitrogen source (93, 185). In *S. cerevisiae* strain S228c, also a commonly used lab strain, *GAP1* expression is not repressed by ammonia, possibly due to the lack of two genes, *MPR1* and *MPR2*, in this strain (120, 199). Still, strain S228c shows low Gap1 activity due to post-translational deactivation by ammonia (191) (see below). Two independent systems involving transcription factors Gln3 and Nil1, regulate *GAP1* transcription (191, 192). In *P. chrysogenum*, expression of *PcGAP1* is repressed when growing with ammonia as nitrogen source and derepressed when glutamate or urea is the nitrogen source (208). Likewise, in *C. albicans*, *CaGAP1* expression is repressed in the presence of ammonia (17). In *N. crassa*, the general amino acid permease activity is repressed by rich nitrogen sources (40), although the transcription level of the *NAAP1* gene, encoding the general amino acid permease, in response to nitrogen availability has not been tested.

The major proline permease PrnB of *A. nidulans* is regulated by NCR and carbon catabolite repression (CCR), i.e. transcription is only repressed when ammonia

is present together with glucose as a carbon source (36, 188, 202). In addition, *prnB* transcription is strongly induced when proline is added to a culture growing in minimal medium (203), a phenomenon called substrate induction. Substrate induction is also observed for PcDip5, the acidic amino acid permease of *P. chrysogenum*. When glutamate is the nitrogen source, *PcDIP5* is highly expressed, whereas the transcription is at an intermediate level when a poor nitrogen source like urea is the sole nitrogen source and completely abolished when ammonia is present (208).

The expression of a number of amino acid permease genes (*BAP2*, *BAP3*, *TAT1*, *TAT2*, *AGP1*, *GNP1, DIP5*) in *S. cerevisiae* is induced by the presence of a low concentration of extracellular amino acids, such as leucine and phenylalanine. These are sensed by Ssy1, an AAP family member that does not transport amino acids, but has in comparison with other AAPs an extended N-terminus (approx. 200 amino acids) which is involved in a signal transduction process. Presumably, amino acid binding causes an alteration in Ssy1, producing a signal that is transmitted to various target promoters (15, 45, 56, 60, 89, 99). An Ssy1 homolog, also having a long N-terminal domain, was found in the protein database of *A. gossypii*, although homology between the N-terminal domains of both is low. In other fungal protein databases, no AAP family members containing a long N-terminal domain was found (data not shown).

Some amino acid transporter genes of parasitic or biotrophic fungi are expressed at the interaction sites with their hosts. The expression *AAT1*, *UfAAT2* (a.k.a. *PIG2*) and *UfAAT3*, broad specificity permeases of the obligatory biotrophic rust fungus *Uromyces fabae*, is upregulated in infection structures (haustoria) in host cells (71, 194). *AmAAP1*, encoding a broad range amino acid transporter of the ectomycorrhizal fungus *Amanita muscaria* was isolated from an ectomycorrhizal cDNA library and is under nitrogen regulation (139). *Inda1*, an amino acid permease gene of the mycoparasite *Trichoderma harzianum* is upregulated during growth on host cell walls (223).

### 5.1.7. Regulation at the posttranslational level

Regulation at the posttranslational level has been studied for Gap1, Bap2 (67) and Tat2 (178) of *S. cerevisiae* and PrnB of *A. nidulans*. The addition of a preferential nitrogen source (ammonia, glutamine) to proline- or urea-grown cells triggers complete loss of Gap1 activity (68). Deactivation starts with dephosphorylation of

Gap1, followed by ubiquitination, endocytosis and degradation in the vacuole (189, 191). In cells growing with glutamate as nitrogen source, *GAP1* is translated, but newly synthesized Gap1 is sorted directly from the Golgi to the vacuole, instead of the plasma membrane (170, 191). Down-regulation of Tat2 and Bap2 is, inversely to Gap1, induced by nutrient starvation. Tat2 in the plasma membrane is transported to and degraded in the vacuole, while an internal pool of Tat2 is routed directly from the Golgi to the vacuole. Both processes require ubiquitination and are controlled by the TOR signaling pathway (12). The degradation of Bap2 appears to be largely dependent on cellular ubiquitination and endocytosis, without indication for direct targeting from the Golgi to the vacuole (146).

### 5.1.8. Amino acid transport across organellar membranes

In addition to transport of amino acids across the cellular membrane, transport of penicillin precursor amino acids across organellar membranes may play an important role in penicillin production (77, 211). 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 (111). The recent observation that ACVS is located in the cytosol (215) 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 (98), 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 but have been subdivided in the AAAP family (172).

## 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 (79). 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 μM). Apart from sulphate, 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 (212). SutB is the major sulfate transporter, while the exact function of SutA remains to be elucidated. This protein has been implicated in thiosulphate uptake or is possibly involved in an intracellular sulphate uptake activity. Expression studies were performed to determine if there is a relationship between penicillin biosynthesis 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 sulphate uptake during β-lactam biosynthesis (213).

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 (126). In *S. cerevisiae* at least 5 transporters are involved in this process namely PHO84, 87, 89, 90 and 91. Deletion of all 5 genes is lethal (64). 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 signaling (64). 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* (70), 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 (9, 49). 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 (133). 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 (116).

**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 (50, 77). However, various reports have implicated active transport in the acquisition of phenylacetic acid from the medium (54). The major differences in these studies may relate to concentration of phenylacetic acid used, and possibly the type of strains (low- versus high- yielding strains). When high concentrations of PA (60-3000 μM) are used, PA readily enters the cells through passive diffusion in both low- and high-yielding strains. However, at low concentrations (1.4-100 μM) 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 (51). 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 (33). 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*) (114). 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 (126). 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 (143).

Glucose uptake in fungi has been best studied for *S. cerevisiae*. Glucose transport occurs by facilitated diffusion (104) which involves transporters that belong to the MFS family (83, 121). A family of 20 different hexose transporters or related proteins (Hxtp) is thought to be involved in sugar transport and regulation (104, 162). 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 (163). Hxt1p and Hxt3p are low affinity transporters ($K_m$ = 50-100 mM), Hxt2p and Hxt4p are equipped with a moderately low affinity (10 mM) and Hxt6p and Hxt7p are high affinity glucose transporters (1-2 mM). A galactose permease was also shown to transport glucose with high affinity ($K_m$ = 1-2 mM) (162). In filamentous fungi, glucose uptake systems have been described for *A. nidulans* (123, 162), *A. niger* (206), and *N. crassa* (119, 175, 176, 179, 180, 184). 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 mM (21, 123); for *P. chrysogenum* a value of 0.2 mM has been reported (30). 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* (27, 44), which transports lactose in symport with protons. Proton symport seems to be a general mechanism for disaccharide transport in fungi (27, 44, 216). 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) (141). However, the pH optimum of IAT is in the alkaline range, and the enzyme is inactive at pH values lower than 6 (3, 4). 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) (214). 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 (37).

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 (110, 166-168). 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 (37, 149, 151, 218, 225). 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 (177).

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 (151). 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 (77). 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 (77).

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 153, 173). 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 (173). 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 (153).

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 (5). 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. Nonetheless, amplification of the full length gene (2 to 4 copies) resulted in a twofold increase in the cephalosporin C production (210). Both studies 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 (18). 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 (53). 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 (113).

## 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 favorable 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. Still, many of the molecular details still need to be resolved. 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 biosynthesis 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.

## Scope of this thesis

Like all organisms, fungi are capable of accumulating solutes from the environment for energy supply and as precursors for macromolecule synthesis. Some hydrophobic compounds may enter the cell by passive diffusion, but most solutes require a specific transport protein to mediate their uptake or secretion. Amino acids can be used as nitrogen and carbon source as well as building blocks for proteins and secondary metabolites. The uptake of these amino acids is achieved by amino acid permeases that belong to the group of secondary transporters, i.e. transporters that directly use the proton motive force as driving force for accumulation of the solute. Fungi possess a number of these permeases that differ in specificity. Some are specific for a single amino acids or a range of related amino acids while others seem to mediate the uptake of nearly all amino acids. Most of these permeases belong to one family

*Penicillium chrysogenum* synthesizes the antibiotic penicillin starting with the condensation of the three amino acid precursors α-aminoadipate, cysteine and valine. The intracellular concentration of α-aminoadipate, and not of cysteine and valine, is limiting for the penicillin synthesis rate. The addition of α-aminoadipate to the growth medium leads to an increased intracellular concentration and thereby an increase in penicillin production. In the work leading to this thesis, we set out to characterize the mechanism of transport of α-aminoadipate from the medium into penicillin producing cells of *Penicillium chrysogenum*. In this study, four different amino acid permeases of *Penicillium chrysogenum* were cloned and characterized. In chapter 2, the cloning of an amino acid permease gene is described that was obtained by a relatively unspecific degenerated primer strategy. Upon the functional expression of the gene in an acidic amino acid defective *Saccharomyces cerevisiae* mutant, the cloned transporter appeared not to be involved iṇ α-aminoadipate transport, but instead was shown to be specific for aromatic amino acids and leucine. In chapter 3, two permeases are described: PcDip5, specific for acidic amino acids, including α-aminoadipate, and PcGap1, the general amino acid permease that transports all amino acids. The relative contribution of these systems in the uptake of α-aminoadipate in penicillin producing mycelium was studied by transport and competition assays using radioactively labeled α-aminoadipate. In addition, the expression of both permease

genes in response to nitrogen availability and in different media was studied. Chapter 4 describes the cloning and characterization of a permease specific for aromatic and neutral aliphatic amino acids, called PcMtr. This system is homologous to the *Neurospora crassa* Mtr that was previously shown to be structurally different from the common amino acid permeases that belong to the AAP family. To examine the distribution of amino acid permeases in fungi, protein databases of completely sequenced genomes of fungi were analyzed for presence of AAP type permeases and Mtr homologs. Chapter 5 describes the random mutagenesis of the *PcDIP5* gene and a positive selection for improved α-aminoadipate transport by PcDip5 using a newly constructed *S. cerevisiae* mutant, capable of using α-aminoadipate as a nitrogen source, but deficient in α-aminoadipate uptake. The results of this work are summarized and discussed in Chapter 6.

# Chapter 2

## Cloning and characterization of an aromatic amino acid and leucine permease of *Penicillium chrysogenum*

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## Abstract

The gene encoding the amino acid permease ArlP (*Ar*omatic and *l*eucine *P*ermease) was isolated from the filamentous fungus *Penicillium chrysogenum* after PCR using degenerated oligonucleotides based on conserved regions of fungal amino acid permeases. The cDNA clone was used for expression of the permease in *Saccharomyces cerevisiae* M4054, which is defective in the general amino acid permease Gap1. Upon overexpression, an increase in the uptake of L-tyrosine, L-phenylalanine, L-tryptophan and L-leucine was observed. Further competition experiments indicate that ArlP recognizes neutral and aromatic amino acids with an unbranched β-carbon atom.

## Introduction

Fungi can use amino acids either as nitrogen and/or carbon source or as building blocks for protein synthesis. The uptake of amino acids is mediated by a multiplicity of carriers with different, overlapping degree of specificity. In addition, cells harbor one or more broad-specificity, large capacity, general amino acid permeases (82), (186). Most of the sequenced and characterized fungal amino acid permeases from fungi belong to one related group, referred to as the AAP family (6). This family also includes some bacterial amino acid permeases. These transport systems have a common structural organization with 12 α-helical putative transmembrane segments and cytoplasmically located N- and C-terminal hydrophilic regions (186). Transport occurs by proton symport with the pmf as driving force (78, 181).

In *Neurospora crassa,* an amino acid permease specific for neutral aliphatic and aromatic amino acids was identified, which is not related to the AAP family (46, 103, 195). It is a member of the amino acid/auxin permease (AAAP) family which includes auxin and amino acid permeases from plants and animals (231).

In the yeast *Saccharomyces cerevisiae*, 24 members of the AAP family have been found (6, 140, 154) of which most have been functionally characterized (157). Based on their regulation at the transcriptional level, the permeases in *S. cerevisiae* are divided in two classes. The general amino acid permease Gap1p, which transports all D- and L-amino acids with high capacity (93), the proline permease Put4p (94) and the acidic amino acid permease Dip5p (159) are subject to nitrogen catabolite repression. The transcription of their genes is repressed when a rich nitrogen source like ammonia or arginine is available (222). The expression of most low-capacity, high specificity and affinity permeases, requires induction by sensing of an extracellular amino acid (38). Examples are the branched-chain amino acid permeases, Bap2p (67) and Bap3p, the tyrosine and tryptophan permease Tat1p (178) and the histidine permease, Hip1p (200),

In filamentous fungi, amino acid uptake has been studied mainly by transport assays and characterization of mutants defective in uptake of one or more amino acids. In *Neurospora crassa,* five distinct transport systems (system I – V) have been identified. System III corresponds to the general amino acid permease. The other systems are specific for a group of related amino acids. A similar situation seems to

hold for *Aspergillus nidulans* (82, and references therein). The L-proline specific transport system, encoded by *prnB,* was the first amino acid permease from a filamentous fungus which was sequenced (21) and characterized with respect to kinetics and regulation (202). For the penicillin producing filamentous fungus *Penicillium chrysogenum*, nine different transport systems have been reported: system I for L-methionine (13); II for L-cysteine (183); III for neutral and basic amino acids (14); 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 (85). 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 (82). Here we describe the cloning of a gene that specifies a novel amino acid permease from *P. chrysogenum* with an unusual specificity.

## Materials and methods

**Strains and media.** The *P. chrysogenum* strain used in this study is Wisconsin 54-1255, a low-level penicillin-producing strain (kindly provided by DSM). Cultures were started on YPG medium (1% yeast extract, 2% peptone and 2 % glucose) and incubated for 16 h and subsequently diluted into minimal medium supplemented with 4 % glucose and 0.4 % ammonia acetate as described by (107). *Saccharomyces cerevisiae* M4054 strain (*MAT*α**,** *ura3, Δgap1*) (67) was used for the functional expression and transport assays. Cells were grown in a minimal buffered medium containing 1 % succinate, 0.6 % NaOH, 0.16 % Yeast Nitrogen Base without ammonium sulphate and without amino acids (Difco), 2 % glucose and 0.1 % proline as a nitrogen source (MP), or on YPG (1 % yeast extract, 2 % peptone, and 2 % glucose). Minimal medium containing 0.5% $(NH_4)_2SO_4$ instead of proline was used for selection of transformants containing the plasmid yCA and yCN (see below). *E. coli* DH5α was employed for all cloning steps, carried out as described in (174).

**Amplification of amino acid permease gene fragment by PCR.** Degenerated oligonucleotides (5′-ATGATSKCCMTSGGYGSYISYMTIGGIACIGG-3′ and 5′-GAAYTCGRIYTCRSCRWAIGGICG-3′) were designed based on conserved regions of fungal amino acid permease genes. PCR was performed on

chromosomal DNA of *P. chrysogenum* Wisconsin 54-1255 in a Perkin-Elmer thermocycler for 30 cycles, with each cycle consisting of 94ºC for 45 s, 40ºC for 1 min and 72ºC for 1 min. At the end of 30 cycles, samples were incubated for 5 min at 72ºC. Gel purified products, ranging from approx. 350 bps to 500 bps were subcloned into the pGEM-T Easy vector (Promega) for DNA sequence determination. Of the 25 clones sequenced, two identical clones showed homology with known amino acid transporter-encoding genes.

**Isolation of permease gene from genomic library.** A genomic library of *P. chrysogenum* DNA in phage Lambda ZAP II (Stratagene), was screened by plaque hybridization as described by (174). Approximately 30,000 plaques were transferred to Zeta-Probe blotting membranes (BioRad), and probed with the radioactively labeled, above-mentioned PCR product. One positive plaque was isolated, phages were released and pBluescript was rescued from the Lambda ZAP II vector by *in vivo* excision, using M13. The 8-kb insert carried the complete permease gene.

**Expression in S. cerevisiae M4054.** For expression, a construct was created based on the yeast-*E. coli* shuttle vector yEP352 (76). The copper inducible promoter *pCUP1* of *S. cerevisiae* was inserted into yEP352. The cDNA of the amino acid permease gene was amplified by PCR from a cDNA library and cloned behind *pCUP1*. The terminator of *AatA*, encoding acyl coenzyme A:isopenicillin N acyltransferase of *P. chrysogenum*, was cloned behind the permease gene resulting in the plasmid yCA. For control experiments, the same construct without the permease gene was used, denoted yCN. Plasmids were transformed into *S. cerevisiae* strain M4054. To test transcript levels, total RNA was isolated from cells disrupted by vortexing with glassbeads and extracted with Trizol reagent (Gibco BRL). Specific *arlP* mRNA levels were measured by quantitative RT-PCR.

**Transport studies.** Overnight cultures grown in minimal medium containing proline as the sole nitrogen source, were diluted with fresh medium, supplemented with 0.2 mM $CuSO_4$, and growth was continued at 30ºC until an $OD_{600}$ of 0.4 - 0.8. Cells from 50 ml of culture were harvested, washed in minimal medium without any nitrogen source and resuspended to $OD_{600}$ of 10 in the same medium. After 10 min of preincubation at 30ºC, 100 µl of cells were added to 150 µl of the same medium

containing 25 μM $^{14}$C-labeled L-amino acid (0.05 μCi). For competition studies, the uptake of 250 μM [$^{14}$C-] L-tyrosine was analyzed in the presence of a 10-fold excess of an unlabeled L-amino acid. For the determination of the $K_m$ value, the concentration of L-tyrosine was varied between 25 μM and 2.5 mM, while for $K_i$ determinations, the kinetics was analyzed in the presence of 500 μM of the indicated unlabeled amino acid. The uptake of amino acid was stopped by the addition of 2 ml of ice-cold 0.1 M LiCl, immediately followed by filtration through a 0.45-μm-pore-size nitrocellulose filter. The filters were washed with 2 ml of ice-cold 0.1 M LiCl, submerged in scintillation fluid and the retained radioactivity was measured in a scintillation counter.

**Expression of *arlP*.** For expression studies, main cultures were incubated for 48 h (early stationary phase) in a rotary shaker at 200 rpm and 25ºC, then transferred to fresh minimal medium containing either urea (0.4 %, w/v), ammonia acetate (0.4 %, w/v) or ammonia acetate plus 10 mM tyrosine, phenylalanine or leucine as nitrogen source. Incubation was continued for 5 h, and mycelial samples were taken at various times, washed and grounded with liquid nitrogen. RNA was extracted using Trizol reagent (Gibco BRL) and specific *arlP* mRNA levels were measured by quantitative RT-PCR.

## Results

**Cloning of amino acid permeases of *Penicillium chrysogenum*.** To clone genes encoding the *P. chrysogenum* amino acid permeases, PCR was performed using degenerated primers based on conserved regions of fungal amino acid permeases (Fig. 1). Products that varied in length from about 350 to 550 bps were cloned, sequenced, and analyzed by means of BLAST searches (2). Of the 20 different PCR products, only one fragment of 406 bp showed significant homology (up to 37 % amino acid identity) with genes encoding amino acid permeases of *S. cerevisiae* and other fungi. To clone the complete gene, a genomic library of *P. chrysogenum* in lambda ZAP II (Stratagene) was screened by plaque hybridization. One positive plaque was isolated and by *in vivo* excision from the lambda ZAP II phage vector, pBluescript, containing

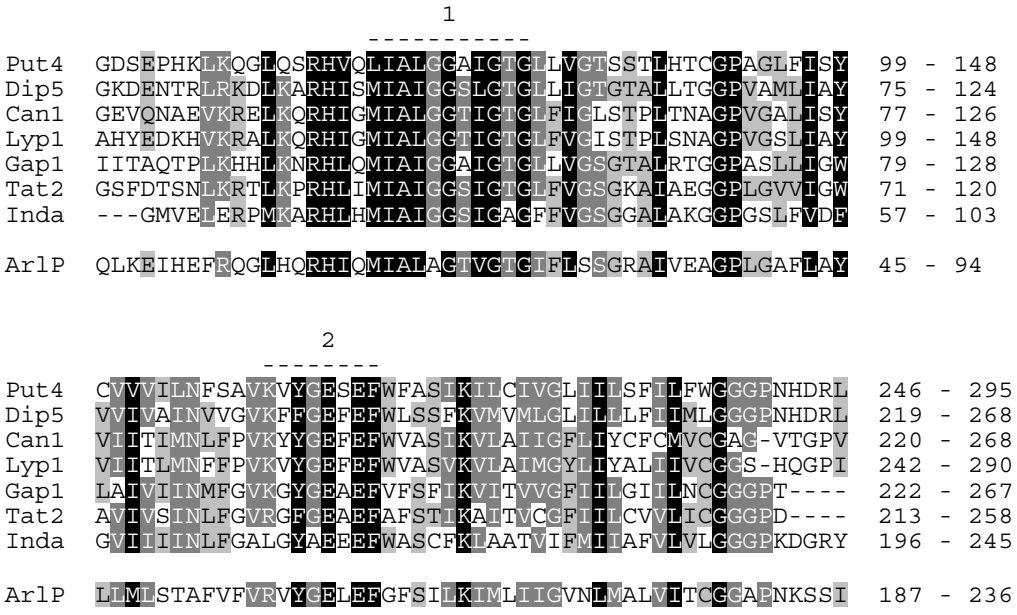


**Figure 1.** Amino acid sequence alignment of conserved regions of fungal amino acid permeases. Sequences were searched from PubMed database at NCBI (www.ncbi.nlm.nih.gov/PubMed) using the program BLAST (2), except for the ArlP sequence (this work). Alignments were made with ClustalX (95). Degenerated oligonucleotides were based on the indicated sequences (dashed lines). Put4 (221), Dip5 (159), Can1 (80), Lyp1 (197), Gap1 (93) and Tat2 (178) are *S. cerevisiae* permeases for proline, glutamate and aspartate, arginine, all amino acids and aromatic amino acids respectively; Inda is a general amino acid permease from *T. harzianum* (223).

an insert of 8 kb, was obtained. The insert was sequenced and found to contain an ORF that specifies a permease gene. The full-length sequence of *arlP* is shown in Fig. 2. The ORF of 1680 bp is interrupted by one intron of 59 bp. The encoded transport protein shows the highest homology with putative amino acid transporters of *Uromyces fabae* (41% amino acid identity), *Amanita muscaria* (39%), *Schizosaccharomyces pombe* (39%) and with characterized amino acid transporters of *Saccharomyces cerevisiae* (Dip5, 37%; Put4, 36%) (BLAST search (2)).

```
-320  GAGACGCTAG ATGCCCTATT TAAACCCCGC TGAAAAGTTT CTTTTTTTCT GATTTCTTTG

-240  TTTTACGCGA AAGCCGCGCT GTAAAGGTGG CCCAGTTTAT CAGCCAACAA TACAATGTGA

-180  ATTGCAAATT GGCCCTTTCC AAGTAGCTGT GATTGTTCAA CTATGCCTGG GGCCACGATC

-120  TGCGCAATAG GGGGGAGAAT TAGTTACGTG AAAAAAAAAG TACGAGCAAT AATGACTTGT

- 60  TATTCCTTAG CTTAAGTCTT TTCCTTCCCA AAGATTTTGT GTCACGCCAC GGGGTTCTCA

+  1  ATGTCACCTT TTATAACGAC CCATGTTTGG AAGGACGATG GCAGTACTAC CCCCATCGTG
         M  S  P  F  I  T  T  H  V  W  K  D  D  G  S  T  T  P  I  V

  61  TCCAACCCAA ATGTCCCAAT CTACGGAGAC GGCGAAATGA GCGACAATAG CGAAATAGAG
         S  N  P  N  V  P  I  Y  G  D  G  E  M  S  D  N  S  E  I  E

 121  AATGCGGCCG GTCAGCTCAA GGAGATTCAC GAGTTCAGAC AGGGCCTGCA TCAGCGACAC
         N  A  A  G  Q  L  K  E  I  H  E  F  R  Q  G  L  H  Q  R  H

 181  ATTCAGATGA TTGCTCTCGC CGGTACGGTG GGGACGGGTA TTTTCCTCAG TTCGGGCCGT
         I  Q  M  I  A  L  A  G  T  V  G  T  G  I  F  L  S  S  G  R

 241  GCCATTGTGG AAGCTGGACC ATTGGGCGCG TTCTTGGCAT ATACAATCAT TGGTGCTACT
         A  I  V  E  A  G  P  L  G  A  F  L  A  Y  T  I  I  G  A  T

 301  GTTGCGAGTG TCGTCTACGG AGTTGGTGAG ATGGGAGCCC TGGTACCTCT CAATGGTGGT
         V  A  S  V  V  Y  G  V  G  E  M  G  A  L  V  P  L  N  G  G

 361  GTTATTCGCT ACGCAGAGAT CTTCTGTGAC CCCGCGCTAG CGTTCGCCAA TGGGTGGAAC
         V  I  R  Y  A  E  I  F  C  D  P  A  L  A  F  A  N  G  W  N

 421  CAGATATACT CCTACTGTGT GTCGATTCCG TCGGAGATTG TCGCTGCGGC TGTTATCATT
         Q  I  Y  S  Y  C  V  S  I  P  S  E  I  V  A  A  A  V  I  I

 481  GAATTCTGGA TCACGGTCAA CAACGCAATA TGGATCACTG TGCTTGGTCT TCTTATGCTT
         E  F  W  I  T  V  N  N  A  I  W  I  T  V  L  G  L  L  M  L

 541  TCCACCGCCT TTGTCTTTGT TCGCGTGTAT GGAGAGCTCG AGTTCGGCTT TTCTATTCTC
         S  T  A  F  V  F  V  R  V  Y  G  E  L  E  F  G  F  S  I  L

 601  AAAATTATGC TCATTATCGG CGTAAACCTG ATGGCTCTGG TGATTTCACC CTCTAGGTCG
         K  I  M  L  I  I  G  V  N  L  M  A  L

 661  GGAACATGTC TACTTTGCGT GCTAATCCTT TTTGATAGG TAATTACCTG CGGTGGAGCC
                                                  V  I  T  C  G  G  A

 720  CCGAATAAGT CGTCCATTGG ATTTGCTTAC TGGAAAGCAC CCTACGGCCC CTTCGTCCAG
         P  N  K  S  S  I  G  F  A  Y  W  K  A  P  Y  G  P  F  V  Q

 780  TACTTGGGGG TGGGCGGGCC CCTTGGCCGG TTTTTGGGCT TCTGGAAAAC CTTCGACAAC
         Y  L  G  V  G  G  P  L  G  R  F  L  G  F  W  K  T  F  D  N

 840  GCGCTCTTCG CTTACTCTGG TATCGAGAAC TTTACTCTCG CTGCGGCTGA GACTCGAAAC
         A  L  F  A  Y  S  G  I  E  N  F  T  L  A  A  A  E  T  R  N

 900  CCTCGACACT CTATCCCCAT GGCCGCTAGA CGTATCTTCG TCCGCATTCT GCTCTTTTAC
         P  R  H  S  I  P  M  A  A  R  R  I  F  V  R  I  L  L  F  Y
```

**Figure 2.** Nucleotide sequence and deduced amino acid sequence of the genomic clone of *P. chrysogenum* ArlP. Sequences with gray background indicate the position of the conserved regions of AAP family permeases, on which degenerated primers were based. The 59 bp intron is boxed in black. The 12 predicted transmembrane segments are underlined.

```
960   GTGATCACCA TCTTTATGAT CGGCCTGATT GTTTCGTCTG CCGATAAAAG ACTCCTGGGC
        V  I  T   I  F  M   I  G  L  I   V  S  S   A  D  K   R  L  L  G

1020  TCGTCGGGGA CAGCCTCGCA GTCCCCTTTC GTTATCGCAG CGCGCCATGC TGGCATCAAG
        S  S  G   T  A  S   Q  S  P  F   V  I  A   A  R  H   A  G  I  K

1080  GTGGTTCCTT CGATCATCAA CGCAGTTGTG CTCACATCAG CATGGTCCTC CGGGAACAGC
        V  V  P   S  I  I   N  A  V  V   L  T  S   A  W  S   S  G  N  S

1140  AATATCTTGG GTGGCTCCAG AATCCTATAC GGTATGGCAA CCCAGGGTCA TGCCCCCGCC
        N  I  L   G  G  S   R  I  L  Y   G  M  A   T  Q  G   H  A  P  A

1200  GTCTTCACAC GTATCAACCG CTTCGGTATC CCTTGGGTTG CTGTCGCGCT CTATGGCGTC
        V  F  T   R  I  N   R  F  G  I   P  W  V   A  V  A   L  Y  G  V

1260  TTCATGTCAC TTGGATACAT GAGTCTTTCC AGCTCGGCCA GCACGGTATT CACATGGCTA
        F  M  S   L  G  Y   M  S  L  S   S  S  A   S  T  V   F  T  W  L

1320  CAGAACCTTG TCTCTATCTC GACTCTAGTG AACCTGATGT GCATCTGTAT TGTCTACCTT
        Q  N  L   V  S  I   S  T  L  V   N  L  M   C  I  C   I  V  Y  L

1380  AGATTCTACT ACGGATGCAA GAAGCAGGGA ATTGATCGCT TCAAGGAGCT CCCGTGGGCA
        R  F  Y   Y  G  C   K  K  Q  G   I  D  R   F  K  E   L  P  W  A

1440  GCACCTTTCC AGCCATACAT AACTTGGATC TCGTTGTTCA TTTATGTTGT CCTTTTCTTC
        A  P  F   Q  P  Y   I  T  W  I   S  L  F   I  Y  V   V  L  F  F

1500  ACCGGAGGGT TTACGACATT TATGCGCGGT CATTGGAGCA CTGCAACCTT CGTTTCGACT
        T  G  G   F  T  T   F  M  R  G   H  W  S   T  A  T   F  V  S  T

1560  TATTTCAACC TGCCATTCAT CGTCATTGTG TATTTTGCCT ACAAGTTCTG GGCAAAAACG
        Y  F  N   L  P  F   I  V  I  V   Y  F  A   Y  K  F   W  A  K  T

1620  AAGATCATCC CGCTGGCAGA AATTCCCATT CGGCCTTTCA TCGAAAGCTG GCACAAAAAT
        K  I  I   P  L  A   E  I  P  I   R  P  F   I  E  S   W  H  K  N

1680  CCCGAGCCAG AGCCCAAGCC GAAGCGAGGC CTCAGCAAAC TTAATATTTT GTGGTCATAA
        P  E  P   E  P  K   P  K  R  G   L  S  K   L  N  I   L  W  S  -

1740  TTTGTACCGT GTACAGTTTC ACCATGAACC TGACGCTGGA CCACCGGACA CCGGCCTGTC

1800  CGGTTTTAGG GCTTAGTGGC CGGGTGATGC CGGGCCCGCT TTCATGCATC GAAAACCGTG

1860  ACATTGTGCT TGCTGACACT AAATAAAAAG TTTCTTTGCT TCATTATATT TGAGAATACT
```

Figure 2 (continued)

**Expression in *S. cerevisiae* M4054.** The gene was expressed in *S. cerevisiae* strain M4054 (*Δgap1*) to analyze the substrate specificity of the permease. This strain lacks the general amino acid permease, Gap1. Gap1 is a non-specific, high capacity permease that transports most L- and D-amino acids. Due to the deletion, strain M4054 exhibits a low endogenous amino acid uptake activity (93), (157). The permease gene was amplified from a cDNA library, thereby obtaining the intron-less ORF, and inserted into the yEP352-based vector yCN, under control of the copper inducible *CUP1* promoter, yielding yCA. This construct was introduced into strain

M4054. For control experiments, the "empty vector" yCN was transformed into M4054. To determine if the gene was expressed upon addition of $CuSO_4$, RNA levels were analyzed by RT-PCR. The presence of $CuSO_4$ resulted in a high transcription of the permease gene, whereas without $CuSO_4$ only a low level of transcript was found (see also Fig. 5).

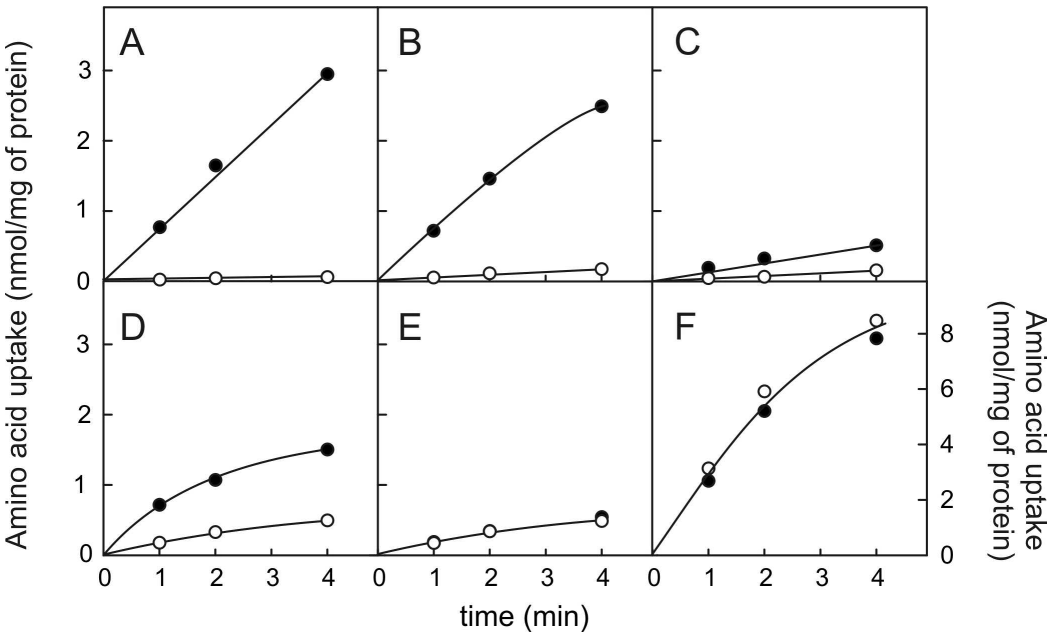


**Figure 3.** Time dependent uptake of L-tyrosine (A), L-phenylalanine (B), L-tryptophan (C), L-leucine (D), L-isoleucine (E) and L-alanine (F) in *S. cerevisiae* M4054 expressing ArlP (solid symbols) or with empty vector (open symbols). Uptake experiments were performed with cells grown to exponential phase in minimal proline medium. Expression of ArlP was induced with 2 mM $CuSO_4$ 5 h before harvesting.

**Transport characteristics.** To determine the substrate specificity of the permease gene expressed in *S. cerevisiae* M4054, uptake was analyzed of 15 different [$^{14}$C]-radiolabeled L-amino acids that represent all groups of amino acids. Cells were grown to mid-exponential phase in minimal medium with proline as sole nitrogen source to prevent nitrogen catabolite inhibition known to elicit the degradation of some amino-acid permeases in *S. cerevisiae* (73). The uptake of L-[$^{14}$C]tyrosine in M4054 expressing the permease was at least 10 times higher than the background

uptake level (Fig. 3). A significant increase in the uptake rate was also observed for L-[$^{14}$C]phenylalanine, L-[$^{14}$C]leucine and L-[$^{14}$C]tryptophan. For the other tested amino acids (L-[$^{14}$C]alanine, L-[$^{14}$C]isoleucine, L-[$^{14}$C]lysine, L-[$^{14}$C]glutamine, L-[$^{14}$C]asparagine, L-[$^{14}$C]cysteine, L-[$^{14}$C]glutamate, L-[$^{14}$C]histidine, L-[$^{14}$C]threonine, L-[$^{14}$C]serine and L-[$^{14}$C]proline), the uptake rates were indistinguishable for the control and permease-containing cells.

The apparent $K_m$ of tyrosine uptake was about 360 μM (Table 1). The substrate specificity of the permease was further tested by determining the inhibition of uptake of L-[$^{14}$C]tyrosine by a ten-fold excess of unlabeled amino acids (Fig. 4). Interestingly, some of the amino acids that inhibited tyrosine uptake by more than 60%, such as alanine, arginine and methionine, appeared not to be transported by the permease in direct transport assays. Leucine, tryptophan and phenylalanine were strong competitive inhibitors of L-[$^{14}$C]tyrosine with $K_i$ values ranging from 150 to 750 μM (Table 1). None of the amino acids that contained a branched β-carbon (i.e., isoleucine, valine and threonine) inhibited tyrosine uptake. The acidic amino acids exerted a low inhibition, which might be related to their negative charge. Based on these data the transporter which is now termed ArlP, appears to be involved in the uptake of aromatic amino acids and leucine.

**Table 1.** Kinetic parameters for tyrosine uptake and competitive inhibition of tyrosine uptake by phenylalanine, tryptophan and leucine

| Amino acid | $K_m$ (μM) | $K_i$ (μM) |
|---|---|---|
| Tyrosine | 361 | |
| Phenylalanine | | 355 |
| Tryptophan | | 743 |
| Leucine | | 169 |

The values were obtained from linear regression analysis of data in Lineweaver-Burk plots for competitive inhibition.

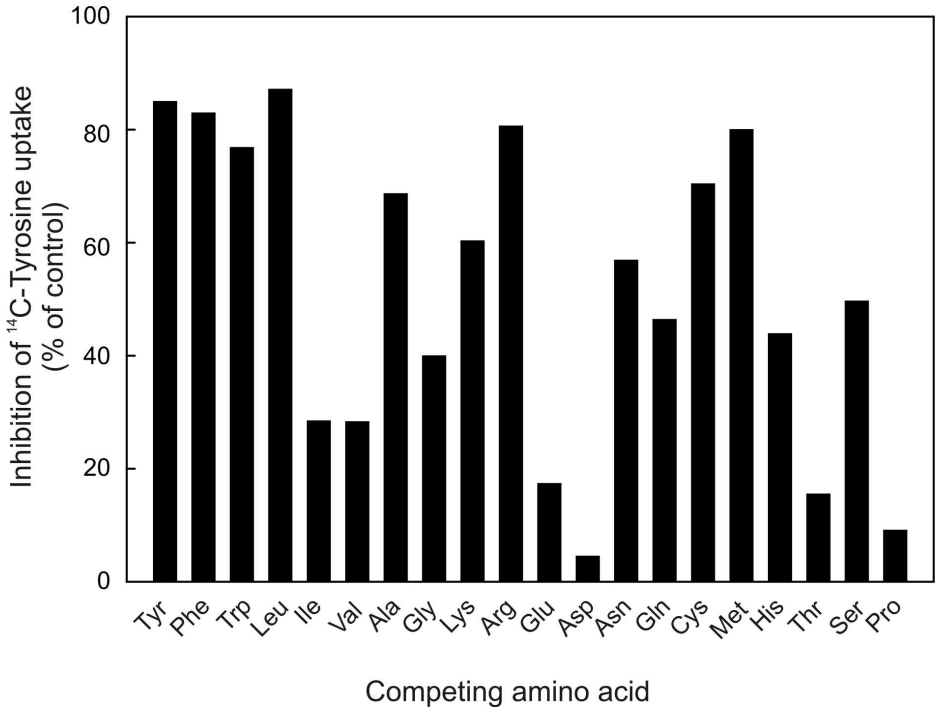


**Figure 4.** Competition of nonlabeled amino acids for uptake of [$^{14}$C]tyrosine for uptake by *S. cerevisiae* strain M4054 expressing ArlP. [$^{14}$C]tyrosine was present at a final concentration of 25 μM, and the indicated nonlabeled amino acids were added in a 10-fold excess. The rate of uptake was determined after 3 min and results shown are the means of three independent experiments.

The activity of the permease upon $Cu^{2+}$-induced overexpression of *arlP* in *S. cerevisiae* was tested not only after growing cells in minimal medium with proline as sole nitrogen source but also with $NH_4^+$. Uptake of tyrosine in cells grown in the presence of $(NH_4)_2SO_4$ was much lower than in cells grown with proline as sole N-source. Since the transcript levels of the *arlP* gene were not drastically different with both nitrogen sources, this suggests a negative regulation of the amino acid permease at a post-transcriptional level in response to ammonia (Fig 5). Post-transcriptional regulation by rapid ammonia inactivation has been shown for the proline permease PrnB in *A. nidulans* (202), and for Gap1 in *S. cerevisiae* (72).

**Expression of *arlP* in *Penicillium chrysogenum*.** The two most common mechanisms involved in regulation at the transcriptional level of AAP family transporters are substrate induction (45) and nitrogen catabolite repression (6). To test

if the expression of *arlP* is regulated by either one of these mechanisms, *P. chrysogenum* was grown in minimal media with proline (poor nitrogen source) or ammoniaoxaloacetate (rich nitrogen source) as the sole nitrogen source, as well as in presence of millimolar concentrations of tyrosine, phenylalanine or leucine. Messenger RNA levels of the permease gene were measured by RT-PCR. At none of the tested conditions a significant level of expression could be detected during logarithmic growth, while low expression levels were found at late stationary phase, irrespective of carbon or nitrogen source.

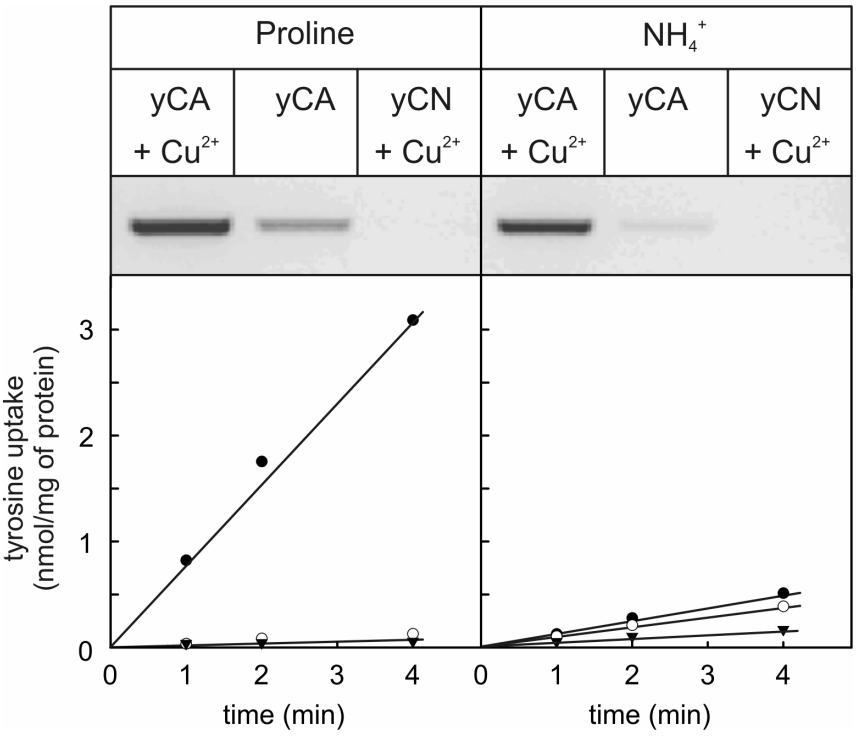


**Figure 5.** Expression (upper panel) and tyrosine uptake activity (lower panel) of ArlP in *S. cerevisiae* strain M4054 transformed with yCA (containing *arlP*) or yCN (empty vector) grown in the presence of $Cu^{2+}$ or without $Cu^{2+}$, in minimal proline medium (MP, left) or minimal ammonia medium (MA, right). *ArlP* mRNA levels were measured by a quantitative RT-PCR. Tyrosine uptake was measured at an extracellular concentration of 25 μM in minimal medium without nitrogen source (MM). ArlP mediated uptake of tyrosine was measured in cells grown in MA (with $Cu^{2+}$; closed triangles; without $Cu^{2+}$; open circles) and MP ($Cu^{2+}$; closed circles)

## Discussion

In order to amplify gene fragments of amino acid permeases of *P. chrysogenum,* PCR was performed using a set of degenerated primers that were based on conserved regions of fungal amino acid permeases, forming the AAP family (6, 186). The PCR fragment was used to clone the entire gene from a genomic library. The cloned permease gene showed 30-39 % amino acid sequence identity with members of this family. When the cDNA clone was expressed in *Saccharomyces cerevisiae* strain M4054 (Δ*gap1*), which has a decreased amino acid uptake (67), a significantly increased rate of tyrosine, phenylalanine, leucine and tryptophan uptake was observed. For other amino acids the increase in uptake was only marginal. Based on this observation, the permease was identified as an aromatic amino acid and leucine transport system, termed ArlP.

An interesting feature of ArlP is the selectivity towards the configuration of the β-carbon of amino acids. When the side chain is branched at this position, the amino acid does not compete with tyrosine for uptake. This suggests that the active site has an unusual specificity that has not been observed before for aromatic amino acid permeases. However, many of the competing amino acids are not transported by ArlP as the uptake of these amino acids is not elevated upon expression of *arlP* in *S. cerevisiae* M4054.

The physiological function of ArlP is not understood. The expression of the *arlP* gene was tested under conditions of nitrogen limitation/starvation and in the presence of millimolar concentrations of its substrates. No significant level of the *arlP* mRNA could be detected under these conditions. Some expression was observed at late stationary phase, irrespective of carbon or nitrogen source, but this was not further analyzed. Some fungal amino acid permeases have been shown to be expressed in differentiating cells. For instance, the expression of an amino acid permease of the rust fungus *Uromyces fabae*, which is homologous to the amino acid permeases of the AAP family, is induced only in haustoria, i.e., specialized hyphae that are involved in plant infection (71). The expression of *arlP* in *P. chrysogenum* may also be restricted to a morphologically or developmentally differentiated state.

We also noted that ArlP activity after overexpression was strongly decreased in *S. cerevisiae* when grown with ammonia as nitrogen-source. Post-transcriptional regulation by rapid ammonia inactivation has been shown for the proline permease

PrnB in *A. nidulans* (202), and in more detail, for Gap1 in *S. cerevisiae,* where $NH_4^+$ induces ubiquitination, endocytosis and vacuolar degradation (73, 189).

In summary, we have described the cloning and characterization of a permease of *Penicillium chrysogenum*, specific for aromatic amino acids and leucine. *Saccharomyces cerevisiae* proved to be a suitable host for overexpression and characterization.

## Acknowledgements

*S. cerevisiae* strain M4054 was kindly provided by M.C. Kielland-Brandt, (Department of Physiology, Carlsberg Laboratory, Copenhagen, Denmark). We thank Dr. M. van der Kamp and Dr. J. Kiel for valuable discussions. This work was supported by the European Community (grant no QLK3-CT-1999-00729).

# Chapter 3

## Uptake of the β-lactam precursor α-aminoadipic acid in *Penicillium chrysogenum* is mediated by the acidic and the general amino acid permease

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## Abstract

External addition of the ß-lactam precursor α-aminoadipic acid to the filamentous fungus *Penicillium chrysogenum* leads to an increased intracellular concentration of α-aminoadipic acid and an elevation of the penicillin production. The exact route for α-aminoadipic acid uptake is unknown, although the general amino acid and acidic amino acid permeases have been implicated in this process. Their corresponding genes, *PcGAP1* and *PcDIP5* of *P. chrysogenum* were cloned and functionally expressed in a mutant of *Saccharomyces cerevisiae* (M4276) in which the acidic amino acid (*DIP5*) and the general amino acid permease (*GAP1*) genes are disrupted. Transport assays show that both PcGap1 and PcDip5 mediated the uptake of α-aminoadipic acid, albeit PcGap1 showed a higher affinity for α-aminoadipic acid than PcDip5 ($K_m$ values of 230 and 800 μM, respectively). Leucine strongly inhibits α-aminoadipic acid transport via PcGap1, but not via PcDip5. This difference was exploited to estimate the relative contribution of both transport systems to the α-aminoadipic acid flux in β-lactam producing *P. chrysogenum*. The transport measurements demonstrate that both PcGap1 and PcDip5 contribute to the α-aminoadipic acid flux.

## Introduction

The production of penicillin, still the most widely used antibiotic together with its derivatives, is mainly based on an industrial fermentation utilizing the filamentous fungus *Penicillium chrysogenum*. Although the biochemistry of the penicillin biosynthesis has been researched extensively, much less is known about the transport processes involved. This for instance relates to the transport of penicillin or its precursors across membranes of the intracellular compartments and the transport across the plasma membrane (211). The penicillin biosynthesis pathway starts in the cytosol with the condensation of three amino acids, L-α-aminoadipic acid (α-AAA), L-cysteine and L-valine, to form the tripeptide L-α-amino-adipyl-L-cysteinyl-D-valine (ACV) (215). ACV is converted into isopenicillin N (IPN) by IPN synthase. In this step the characteristic β-lactam ring structure is formed by ring closure. IPN is subsequently transported into microbodies or peroxisomes, where the α-aminoadipic acid moiety is replaced by a phenylacetic acid group involving a CoA derivative of this aromatic acid. This process yields penicillin G that subsequently must be transported across the microbody membrane and the plasma membrane in order to accumulate in the extracellular medium (1, 19, 125, 211). α-Aminoadipic acid is released in the microbody and can be recycled either for lysine or penicillin biosynthesis. However, a portion of the α-aminoadipic acid is lost by the irreversible formation of 6-oxopiperidine-2-carboxylic acid (OPC), a cyclized form of α-aminoadipic acid. 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. A major fraction of the OPC seems to be secreted from the cell, but the mechanism of secretion is unknown (74).

The intracellular concentration of α-aminoadipic acid, and not of cysteine and valine, appears limiting for the synthesis of the tripeptide ACV which affects the overall penicillin biosynthesis rate in low producing strains (59, 81). This has become apparent from experiments in which α-aminoadipic acid is supplemented to the mycelium whereupon the intracellular α-AAA concentration is elevated concomitantly with an enhanced level of ACV and penicillin production (59, 81). In addition, higher penicillin-producing strains have increased levels of intracellular α-AAA (92). In *P. chrysogenum*, α-AAA is a branching intermediate of the lysine and

penicillin biosynthesis pathway that share common enzymatic steps starting from α-ketoglutarate. The routes diverge at the point where the α-AAA can be converted into α-AAA-semialdehyde by α-aminoadipate reductase (16, 58, 92). Disruption of the gene encoding this enzyme, i.e., *LYS2*, results in a higher intracellular α-AAA concentration and concomitantly higher levels of penicillin production (26). In *Aspergillus nidulans*, also producing penicillin but not used for industrial production, the relative amount of α-AAA entering either the penicillin synthesis or lysine synthesis pathway is tightly regulated (23). Another important aspect of the α-AAA biosynthesis route is that in yeast, some of the enzymes of the lysine biosynthesis pathway are localized in the microbodies instead of the mitochondria (20). It is unclear how this pathway is organized in filamentous fungi.

The observation that externally added α-AAA enhances penicillin biosynthesis strongly suggests that there is a mechanism of α-AAA uptake. Fungi have different amino acid permeases that vary in specificity. Some systems are specific only for a group of related amino acids, while the general amino acid permease can transport all amino acids (82, 157, 186). These systems all belong to one family, referred to as the amino acid permease (AAP) family (6). Exceptions are the *mtr* locus encoded permease from *Neurospora crassa* and its homologue of *P. chrysogenum*, which are specific for neutral aliphatic and aromatic amino acids (103, 195) Trip et al., unpublished results). In general, amino acid transport occurs by proton symport and thus is driven by the proton motive force (78, 181). Although α-AAA is not one of the natural amino acid but an intermediate of amino acid metabolism, it shares the characteristics with glutamate and aspartate as a dicarboxylic amino acid. In *S. cerevisiae*, these amino acids can enter the cell via the general amino acid permease, Gap1, and the acidic amino acid permease, Dip5 (159). In *P. chrysogenum*, transport systems have mostly been described only on the basis of functional studies, and these systems have not been genetically described. The general amino acid permease is a non-specific system that is involved in uptake of all amino acids (14), while the acidic amino acid permease mediates the uptake of glutamate and aspartate. Previous studies have shown that glutamate transport by the acidic amino acid permease can be inhibited by an excess of α-AAA, which suggest that this compound is a substrate for this transporter (85).

Here we have analyzed the uptake of α-AAA by *P. chrysogenum* in more detail, and show that there are two major uptake routes, i.e., 1) via the acidic amino acid permease, and 2) via the general amino acid permease. The genes encoding these transporters were cloned in a *S. cerevisiae* mutant that is defective in acidic amino acid uptake, allowing the functional characterization of the *P. chrysogenum* Gap1 and Dip5 homologues. The results indicate that both systems contribute to the uptake of α-AAA by *P. chrysogenum* under conditions that β-lactams are formed.

## Materials and methods

**Strains and culture conditions.** *Penicillium chrysogenum* strain Wisconsin 54-1255 (kindly provided by DSM-Anti-Infectives, Delft, The Netherlands) was grown in a minimal medium based on a penicillin production medium as described (107). The medium contained 4 % (w/v) glucose or lactose as carbon source, and 0.4 % (w/v) ammonium acetate, 0.4 % (w/v) urea, 10 mM L-glutamate, 10 mM L-serine or 10 mM L-lysine as nitrogen source. Cultures were started on YPG medium (1% yeast extract, 2% peptone and 2% glucose), incubated for 16-20 h in a rotary shaker at 200 rpm and 25ºC, then diluted into minimal medium and incubated for 24 or 48 h.

*Saccharomyces cerevisiae* strain M4276 (*MATα*, *ura3*, *Δgap1*, *Δdip5*) (159) was grown in minimal medium (1 % (w/v) succinic acid, 0.6 % (w/v) NaOH, 0.16 % (w/v) Yeast Nitrogen Base [Difco] without ammonium sulphate and amino acids, and 2 % (w/v) D-glucose (67)), containing 0.5 % (w/v) ammoniumsulphate (MA medium), 0.1% (w/v) L-proline (MP) or 0.5 g/L L-glutamate (MG) as sole nitrogen source. When indicated, these media were solidified with 1.5% agar.

*E. coli* strain DH5α was used as plasmid host and DNA manipulations were carried out essentially as described (174). *E. coli* XL10-Gold Ultracompetent cells (Stratagene) were used for amplification of the partial cDNA library.

**Expression of a partial cDNA library of *P. chrysogenum* in *S. cerevisiae* M4276.** A cDNA library of *P. chrysogenum* (kindly provided by DSM-Anti-Infectives, Delft, The Netherlands) was transferred from pCMVSPORT 4.0 (Stratagene, Amsterdam, Netherlands) to a suitable yeast expression vector. For this

purpose, the ampicillin resistance marker of the yeast/*E. coli* shuttle vector yEP352 (76) was replaced by a kanamycin resistance marker; and the copper inducible promoter of *CUP1*, *pCUP1* (182) was introduced together with the terminator of *CYC1* (232), yielding the plasmid designated yEX-C. The SbfI and NotI sites between *pCUP1* and *tCYC1* were used for insertion of the cDNA library. The cDNA library was isolated from pCMVSPORT4.0 by digestion with SbfI and NotI and subjected to gelelectrophoresis. Since the size of known fungal amino acid permease genes all range within 1.4 and 2 kb, fragments ranging from 1.0 to 2.5 kb were isolated, purified and ligated into yEX-C. To amplify the library, the ligation mixture was transformed to *E. coli* XL10-Gold Ultracompetent cells (Stratagene, Amsterdam, Netherlands) for high transformation efficiency, and spread on Luria Broth (LB) plates with kanamycin. Plasmid DNA was isolated by resuspending the colonies in water, followed by a standard plasmid isolation procedure. About 5 μg of plasmid DNA was transformed to *S. cerevisiae* M4276, deficient in glutamate and aspartate uptake, as described (62). Cells were spread on MG plates, containing 0.05 g/L L-glutamate as sole nitrogen source and 0.2 mM $CuSO_4$ for induction of the copper inducible promoter *pCUP1*. At the same time, transformation efficiency was checked by spreading transformed cells on MA plates, containing $NH_4^+$ as nitrogen source, rendering an efficiency of approx. 35000 transformants per μg of DNA. After 6 days of incubation at 30ºC, eight colonies appeared that were transferred to liquid minimal medium, containing 0.05 g/L L-glutamate as sole nitrogen source. Only one of the colonies was able to grow on glutamate, and the plasmid DNA was isolated following a standard plasmid rescue protocol. For propagation, plasmid DNA was transformed to *E. coli* DH5α. The plasmid was analyzed by restriction, revealing a 1.9 kb insert in the yEX-C vector of which the DNA sequence was determined.

**Cloning of *PcGAP1*.** The following degenerated primers were designed on basis of conserved regions of known fungal (putative) general amino acid permease genes, primer Gap F: 5'-CCCGGCGCCATCAARCARGTNTTYTGG-3' and primer Gap R1: 3'-CCGGAGATGGCCAGNAGCCARTCRAA-5'. PCR was performed with genomic DNA of *P. chrysogenum* as a template. PCR products were analyzed by gelelectrophoresis and a clear, distinct fragment of 0.4 kb was isolated, purified and ligated into pGEM-T Easy (Promega, Leiden, Netherlands). Of the 4 clones analyzed by sequencing, 3 contained a fragment showing high homology (50% amino acid identity) with Gap1 of *S. cerevisiae*, and one contained a fragment showing highest

homology (45-48% amino acid identity) with yeast basic amino acid permeases. The Gap1 homologous fragment was used to make a radioactively labeled probe for the screening of a genomic library.

**Isolation of genomic DNA of permease genes.** A genomic library of *P. chrysogenum* Wisconsin 1255-54 in phage λZAP II (Stratagene, Amsterdam, Netherlands) was screened by plaque hybridization, using a radioactively labeled, 400 bp internal fragment of the *PcDIP5*, amplified by PCR, or the *PcGAP1* gene fragment described above. Positive plaques were isolated, phages were released and pBluescript, containing the genome fragments (average size of 8 kb) was rescued from the phage by incubation with helper phage M13. DNA sequencing of the *PcDIP5* positive clone, revealed a complete, 1677 bp open reading frame, interrupted by 5 introns of approximately 60 bp in length. The *PcGAP1* positive clone contained a 1743 bp open reading frame interrupted by 3 introns of 63, 55 and 35 bp. Introns were identified on basis of consensus sequences of introns of *P. chrysogenum*, and by alignment of the cDNA and genomic DNA in the case of *PcDIP5*.

**Cloning of the cDNA of *PcGAP1* in the yeast expression vector yEX-C.** A forward primer with an EcoRI restriction site (5'-ATTCACATAGAATTCATG-GAGGAGAAGAAGTTTGAGGC-3') and a reverse primer with a NotI restriction site (3'-ACAGCGGCCGCTTTCCAGGTAAGATGC-5') were used in a PCR to amplify the cDNA of *PcGAP*1 with the cDNA library as a template. The obtained 1.8 kb product was digested with EcoRI and NotI and ligated into EcoRI and NotI digested vector yEX-C. The resulting plasmid yEX-PcGAP1 was transformed to *S. cerevisiae* M4276.

**α-Aminoadipate transport assays with mycelium of *P. chrysogenum*.** *P. chrysogenum* was precultured overnight as described above and germinating spores were added to penicillin production media. After 48 h of growth, mycelium was harvested by suction filtration, washed and resuspended in ice-cold 50 mM $KH_2PO_4/K_2HPO_4$, pH 6.0 to a density of approximately 15 mg dry weight/ml. This suspension was preincubated for 5 min at 25ºC with aeration, and D/L-$^3$H-αAAA was added to final concentrations of 10 μM to 2.5 mM. For inhibition studies, D/L-$^3$H-

αAAA was present at 25 μM, while the competitor L-leucine was used in a concentration range of 10 μM to 2.5 mM. After 3 min, the uptake was stopped by addition of 2 ml of ice-cold 0.1 M LiCl and subsequent suction filtration through a 0.45-μM pore size nitrocellulose filter. The filter was washed with 2 ml 0.1 M LiCl and radioactivity was measured in a scintillation counter. Background values were determined by measuring the uptake in mycelium, which was pretreated with the protonophore carbamoyl-cyanide-*m*-chloro-phenylhydrazone (CCCP). Uptake measurements with *P. chrysogenum* were made in four independent experiments of which average values and standard deviations are shown (Fig. 1A).

**Amino acid transport assays in *S. cerevisiae*.** *S. cerevisiae* strain M4276 yEX-PcDIP5, M4276 yEX-PcGAP1 and strain M4276 yEX-C (empty vector) were grown overnight in MP medium at 30ºC. Cultures were then diluted 10-fold in fresh MP medium, containing 0.2 mM $CuSO_4$ for induction of transcription of *PcDIP5* or *PcGAP1*, and grown to an $OD_{600}$ of 0.4-0.8. Cells were harvested by centrifugation, washed with minimal medium without nitrogen source (MM) and resuspended to an $OD_{600}$ of 10 in the same medium. Before starting the transport assay, cells were incubated for 10 min at 30ºC with stirring. Subsequently, 100 μl of the cell suspension was added to 150 μl of MM with 25 μM of $^{14}$C-labeled L-amino acid (0.05 μCi) and incubation was continued at 30ºC while stirring. Uptake reactions were stopped immediately after addition of substrate, or after 30, 60 or 120 sec by the addition of 2 ml of ice cold 0.1 M LiCl and subsequent suction filtration through a 0.45-μM pore size nitrocellulose filter. Radioactivity retained on the filters was measured in a scintillation counter. For $K_m$ determinations, uptake of glutamate and aspartate at concentrations varying from 10 to 2500 μM, was measured after 30 sec to ensure linearity with time. For the $K_m$ determinations of α-aminoadipate transport, uptake was accessed after 3 min. $K_i$ values of leucine inhibition were determined at α-aminoadipate concentrations of 10 to 2500 μM, in the presence and absence of 1 and 2.5 mM of L-leucine using *PcDIP5* expressing cells or 10 μM in *PcGAP1* expressing cells. All uptake measurements with *S. cerevisiae* M4276 were made in at least two independent experiments and average values are given.

The $K_m$ values for α-aminoadipate transport via PcDip5 and PcGap1 and the respective $K_i$ values for the competitive inhibition by leucine were used to access the

relative contributions of the two transporters in the uptake of α-aminoadipate by *P. chrysogenum* in the presence of an increasing concentration of leucine. The data were analyzed by regression according to Michaelis-Menten kinetics by using the following equation:

$$v = v_{\text{PcDip5}} + v_{\text{PcGap1}} = \frac{V_{\text{max - PcDip5}} * [\alpha - AAA]}{[\alpha - AAA] + K_{m-\text{PcDip5}} * (1 + \frac{[Leu]}{K_{i-\text{PcDip5}}})} + \frac{V_{\text{max - PcGap1}} * [\alpha - AAA]}{[\alpha - AAA] + K_{m\text{-PcGap1}} * (1 + \frac{[Leu]}{K_{i-\text{PcGap1}}})}$$

wherein $v$ is the observed rate of α-aminoadipate uptake, and $v_{\text{PcDip5}}$ and $v_{\text{PcGap1}}$ the rates of uptake via PcDip5 and PcGap1, respectively. $K_{m\text{-PcDip5}}$ and $K_{m\text{-PcGap1}}$ and $V_{max\text{-PcDip5}}$ and $V_{max\text{-PcGap1}}$, are the Michaelis constant and maximal velocity for α-aminoadipate uptake via PcDip5 and PcGap1, respectively. $K_{i\text{-PcDip5}}$ and $K_{i\text{-PcGap1}}$ are the inhibition constant by leucine of α-aminoadipate uptake via PcDip5 and PcGap1, respectively, while [α-AAA] and [Leu] are the concentration of α-aminoadipate and leucine, respectively.

**Semi-quantitative RT-PCR.** Total RNA extracts were prepared by a Trizol based method (Invitrogen, Breda, Netherlands). Mycelium was frozen in liquid nitrogen and ground. Approximately 5 volumes of Trizol were added, and samples were mixed thoroughly. After the addition of 1/5 volume of chloroform and mixing, samples were spun for 15 min at 10.000 rpm in a microcentrifuge. The RNA containing water phase was collected and further purified by phenol extraction. RNA was precipitated with ethanol and dissolved in $H_2O$. The RNA concentration was determined spectroscopically at 260 nm. Reverse transcriptase (RT)-PCR was performed using RT-PCR beads (Amersham-Pharmacia, Freiburg, Germany) and a Perkin-Elmer thermocycler. Primers were used generating internal fragments of *PcDIP5* (0.35 kb), *PcGAP1* (0.5 kb) or the internal standard *actA* (0.8 kb) that served as a control to monitor the total amount of RNA used as template. Samples were incubated at 42ºC for 30 min for reverse transcription, followed by a standard PCR with the following conditions: 5 min at 95ºC, then 22 cycles of 30 sec at 95ºC, 30 sec at 55ºC and 40 sec at 72ºC. PCR products were analyzed by gelelectrophoresis.

**Materials.** Uniformly labeled D/L-α-amino[$^3$H]adipic acid was custom synthesized by Amersham Pharmacia biotech (Freiburg, Germany), with a specific activity of 38 Ci/mmol.

**Nucleotide sequence accession numbers.** The 2,580 and 1,997 kb sequences containing the *P. chrysogenum PcDIP5* and *PcGAP1* have been deposited in GenBank under accession number AY456273 and AY456274, respectively.

## Results

**Uptake of α-aminoadipate by *P. chrysogenum* Wisconsin 54-1255.** Since the general amino acid permease of *P. chrysogenum* transports most amino acids including glutamate and aspartate, and because α-aminoadipate (α-AAA) inhibits glutamate uptake mediated by the acidic amino acid permease (85), both of these permeases have been implicated in the uptake of α-AAA. However, this assumption has not been directly tested in experiments wherein the uptake of α-AAA is measured. Nor have the individual contributions of both permeases and possible other systems been determined in α-AAA uptake in cells of *P. chrysogenum* grown at penicillin producing conditions. For that purpose, transport studies were performed with D/L-$^3$H-labeled-α-AAA. The amino acid was used at a concentration of 25 μM. L-Leucine is a good substrate for the general amino acid permease and is not transported by the acidic amino acid permease (14, 85, 86). Therefore, L-leucine was used for competition studies to discriminate between the two transporters. α-AAA was readily accumulated by the cells. The inhibition profile of α-AAA uptake by *P. chrysogenum* in the presence of increasing concentrations of L-leucine, showed two distinguishable inhibition effects (Fig. 1A). At low L-leucine concentration, up to 100 μM, the uptake of α-AAA is readily reduced by 45-50%, whereupon higher concentrations of L-leucine up to 2.5 mM only marginally inhibited the remaining α-AAA uptake. These data suggest that a major fraction of the α-AAA uptake is mediated by the general amino acid permease, but also show that there is a residual uptake which is hardly inhibited by higher concentrations of L-leucine.

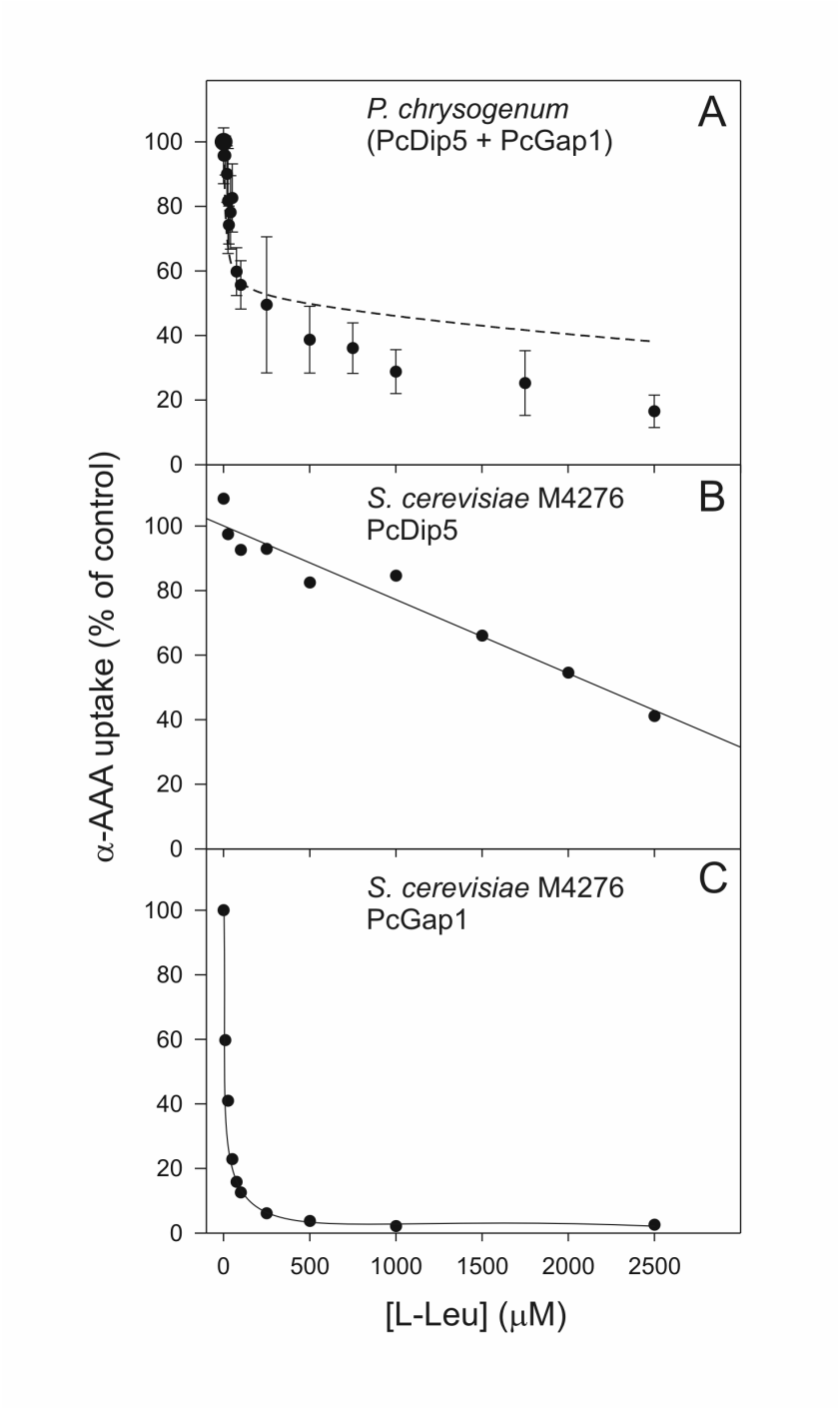


**Figure 1.** Uptake of α-aminoadipic acid and inhibition by unlabeled L-leucine. (A) The uptake of $^{3}$H-α-AAA (25 μM) by mycelium of *P. chrysogenum*, grown at penicillin producing conditions, was assayed in the presence of increasing concentrations of unlabeled L-leucine. The dashed line represents the best fitting curve after computer aided regression and curve fitting of equation 1 (see text) to the measured data. (B) and (C) are as (A), except that uptake of $^{3}$H-α-AAA was assayed with *S. cerevisiae* M4276 cells that express *PcDIP5* and *PcGAP1*, respectively.

**Cloning of the acidic amino acid permease gene *PcDIP5*.** To define the exact identity of the transporters involved in α-AAA, it is necessary to clone the genes and analyze their activity separately. Since the residual α-AAA uptake in the presence of excess L-leucine could be inhibited by unlabeled glutamate (data not shown), we decided to clone in addition to the general amino acid permease, the acidic amino acid permease. In *S. cerevisiae*, acidic amino acids (glutamate and aspartate) can be taken up by the general amino acid permease Gap1, as well as by the acidic amino acid permease Dip5 (159). A mutant strain M4276 has been described that lacks both the Gap1 and Dip5 genes. This mutant is deficient in acidic amino acid transport and therefore cannot grow with glutamate or aspartate as sole nitrogen source (159). In contrast to the parental strain, *S. cerevisiae* M4276 is also unable to accumulate α-AAA, which indicates that there are no other routes for uptake of this molecule in *S. cerevisiae* (data not shown). Strain M4276 was used for a functional complementation cloning strategy to identify transporters that mediate dicarboxylic amino acid uptake. Herein, a cDNA library of *P. chrysogenum* was expressed in *S. cerevisiae* M4276 and cells were selected on plates and liquid media containing L-glutamate as sole nitrogen source. Cells that grow on these media must have acquired a transport system that is capable of glutamate uptake. This yielded one clone that could grow with glutamate as sole nitrogen source. The cDNA of the clone contained a 1,677 bp open reading frame which encodes a 559 amino acid protein that is 47% identical to Dip5 (Fig. 2), and 30-34% identical to Gap1 and other amino acid permeases of *S. cerevisiae*. The gene was designated *PcDIP5*.

**Cloning of the general amino acid permease gene, *PcGAP1*.** Despite several attempts, selection for complementation of glutamate uptake in *S. cerevisiae* with the partial cDNA library did not yield a general amino acid permease. Therefore, an alternative PCR approach was used making use of degenerated primers that were based on the alignment of 5 known general amino acid permeases. With these primers, a 424 bp PCR fragment was obtained that, upon translation into amino acids, showed a high homology with Gap1 of *S. cerevisiae*. The fragment was used to probe a genomic library from which the full-length gene was isolated. The entire gene consists of a 1,751 bp open reading frame, interrupted by 3 introns of 63, 55 and 35 bp, and encodes a protein of 581 amino acids. After a BLAST search (2), the highest

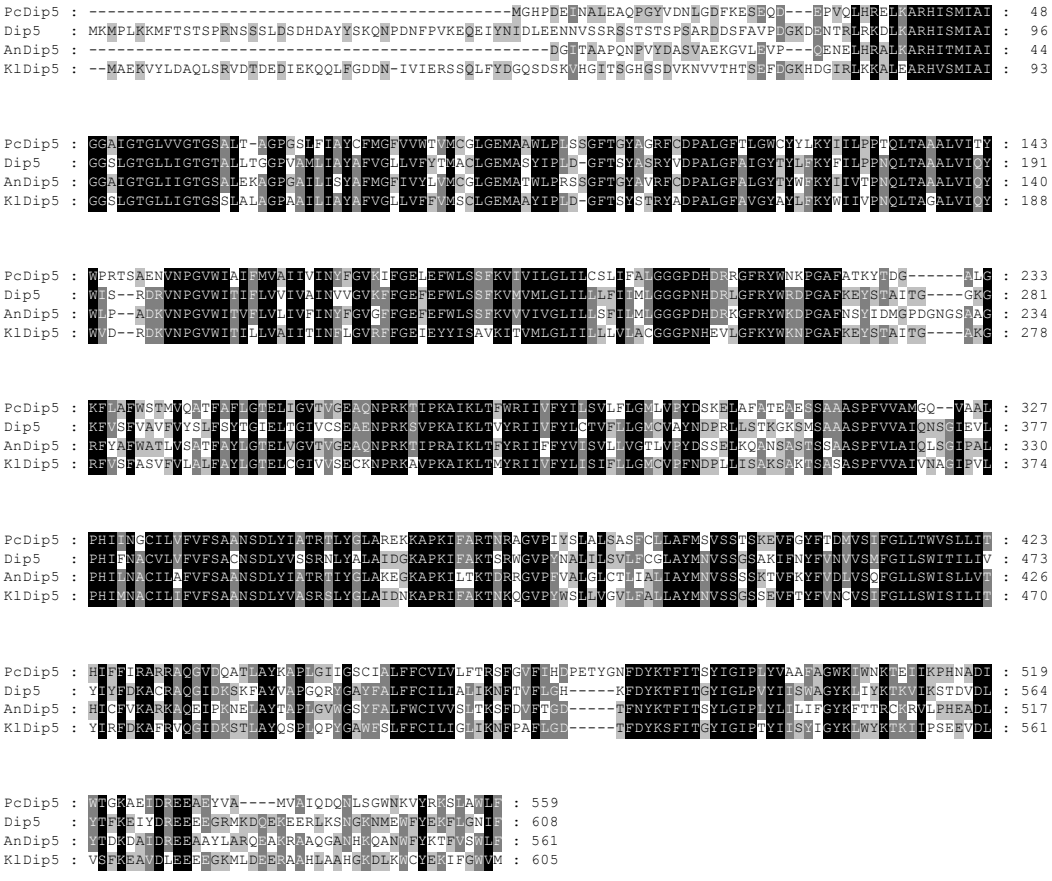

**Figure 2.** Amino acid sequence alignment of the acidic amino acid permeases of *P. chrysogenum* (PcDip5), *S. cerevisiae* (Dip5) (159) and putative acidic amino acid permeases of *Aspergillus nidulans* (AnDip5) and *Kluyveromyces lactis* (KlDip5). The *A. nidulans* sequence, 57% identical to PcDip5 and 50% identical to Dip5 was derived from the *A. nidulans* database of the Whitehead Institute after a BLAST search (2). This sequence is incomplete at the N-terminus. The *K. lactis* sequence was obtained after a BLAST search at NCBI and shares 61% amino acid identity with Dip5 and 50% identity with PcDip5. Conserved and similar amino acids are indicated in black and grey, respectively.

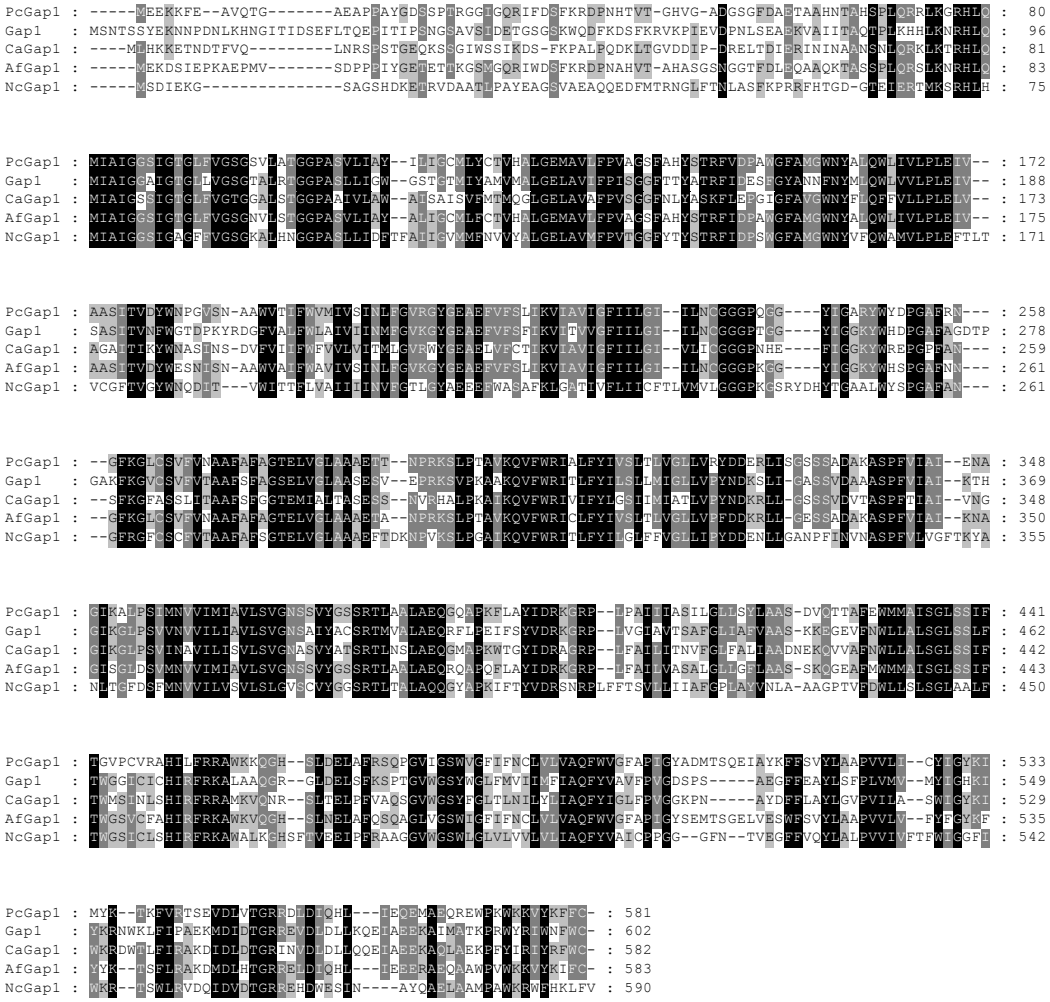

**Figure 3.** Alignment of the general amino acid permeases of *P. chrysogenum* (PcGap1), *S. cerevisiae* (Gap1) (93), *Candida albicans* (CaGap1) (17), *Aspergillus fumigatus* (AfGap1) and *Neurospora crassa* (NcGap1, encoded by the *pmg* locus) (122). AfGap1 is a putative general amino acid permease, based on its homology with PcGap1 (78% amino acid identity) and Gap1 (55% identity), and was obtained from the *A. fumigatus* Genome Database at TIGR (The Institute for Genomic Research) after a BLAST search (2). NcGap1 was originally deposited with GenBank as NAAP1. Conserved and similar amino acids are indicated in black and grey, respectively.

homology was found with known general amino permeases of *S. cerevisiae* (Gap1, 50% identical), *Candida albicans* (Gap1, 47%) and *Neurospora crassa* (Naap1, 48%) (Fig. 3). The gene was therefore designated *PcGAP1*.

**Overexpression of *PcDIP5* and *PcGAP1* in *S. cerevisiae* M4276 and transport characteristics.** The plasmids isolated from the cDNA library, yEX-PcDIP5, and yEX-PcGAP1 were used for functional characterization of the transporters in *S. cerevisiae* M4276. As a negative control, M4276 was transformed with the empty vector yEX-C. Cells were incubated with $CuSO_4$ for induction of the expression, and analyzed for the uptake of a range of amino acids (209). Cells expressing *PcDIP5* readily accumulated L-glutamate and L-aspartate, while L-α-aminoadipate was taken up at a lower rate (Fig. 4A). L-asparagine was accumulated slowly, whereas for L-glutamine and L-serine no significant difference was observed between the PcDip5 and control cells (data not shown). The affinity ($K_m$) of PcDip5 for the uptake of L-glutamate and L-aspartate was about 35 μM, while the $K_m$ value for L-α-aminoadipate uptake appeared rather high, i.e., 800 μM. Since the $^3$H-labeled α-aminoadipate represents a racemic mixture of the D- and L-configuration, further competition experiments were performed with the unlabeled stereoisomers. A 100-fold excess of unlabeled D-α-aminoadipate did not inhibit uptake of labeled D/L-α-aminoadipate, whereas L-α-aminoadipate decreased uptake by more than 90%. This shows that PcDip5 is specific for the L-configuration (data not shown). Taken together, these results demonstrate that the cloned permease, PcDip5, corresponds to the acidic amino acid permease of *P. chrysogenum*.

For cells expressing *PcGAP1*, the uptake of 19 amino acids including the non-protein amino acids, L-citrulline, D/L-α-aminoadipate, L-α-aminoisobutyric acid and L-ornithine, was measured (Fig. 5). For all amino acids except L-proline, a significant increase in the uptake by at least two-fold was observed upon the expression of *PcGAP1*. These data either suggest that L-proline is not a substrate for PcGap1, or that in cells overexpressing *PcGAP1* the background uptake via endogenous proline permease(s) is reduced due to the expression of *PcGAP1*. The acidic amino acids glutamate and aspartate were more slowly accumulated by *PcGAP1* expressing cells as compared to *PcDIP5* expressing cells (Fig. 4B). However, since the total amount of PcGap1 or PcDip5 protein in these cells is unknown, the exact $V_{max}$ values can not be

compared. Strikingly, L-α-aminoadipate was readily accumulated, with a $K_m$ value of 230 μM. This affinity is more than 3 times higher than observed for PcDip5. The general amino acid permease of *S. cerevisiae* and *N. crassa* have been reported also to transport some D-amino acids (82), but like PcDip5, PcGap1 was found to transport α-aminoadipate only in the L-configuration (data not shown). These data demonstrate that the cloned gene, *PcGAP1*, encodes the general amino acid permease of *P. chrysogenum*.

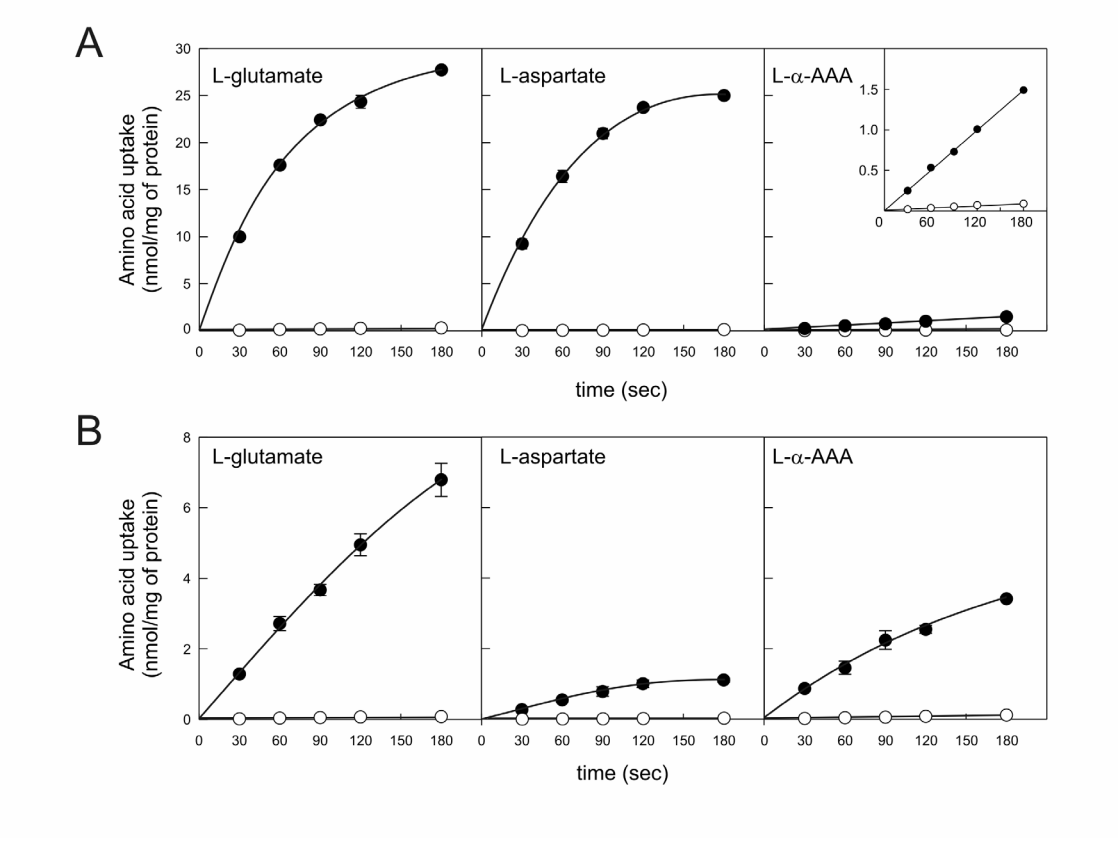


**Figure 4.** Time-dependent uptake of acidic amino acids by *S. cerevisiae* M4276, expressing *PcDIP5* (A) and *PcGAP1* (B). Cells containing the empty vector were used as control (open symbols). The inset in (A) shows the uptake of α-aminoadipate with an adjusted uptake scale. Uptake assays were performed with cells grown to exponential phase in minimal proline medium and expression of *PcDIP5* was induced with 0.2 mM $CuSO_4$, 5 h before harvesting.

**Inhibition of PcDip5 or PcGap1 mediated α-aminoadipate uptake by leucine.** The biphasic inhibition of α-aminoadipate uptake by L-leucine by *P. chrysogenum* mycelium (Fig. 1A) was explained by the presence of at least two different uptake routes for α-aminoadipate, i.e., uptake via the general amino acid permease that is strongly inhibited by L-leucine, and uptake via a specific amino acid permease that is nearly insensitive to L-leucine. To validate this hypothesis, we re-investigated the inhibition of α-aminoadipate uptake by leucine in *S. cerevisiae* M4276 that expresses *PcDIP5* and *PcGAP1* separately (Fig. 1B and C). Indeed, PcGap1-mediated α-aminoadipate was strongly inhibited by L-leucine (Fig. 1C) exactly with a concentration range that was inhibitory in *P. chrysogenum* cells. In contrast, PcDip5-mediated uptake was inhibited by L-leucine only at high concentrations (Fig. 1B). The $K_i$ value for leucine inhibition of PcGap1 mediated

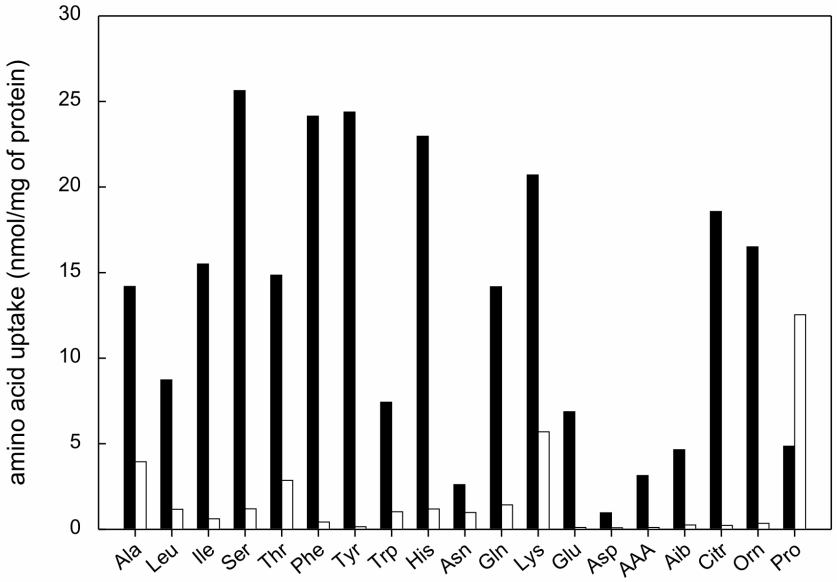


**Figure 5.** Uptake of amino acids by *S. cerevisiae* M4276, expressing *PcGAP1*. Cells were incubated for 3 min with the indicated, radioactively labeled amino acids at 25 μM final concentration. Closed and open bars represent the uptake levels by cells expressing *PcGAP1* (yEX-PcGAP1) or cells with the empty vector (yEX-C), respectively.

uptake was 10 μM, and for PcDip5 mediated uptake this value was around 6.3 mM. This high $K_i$ value is not in perfect agreement with the inhibition pattern shown in Fig. 1B, which should have a flatter slope. Therefore, a non-specific effect by leucine seems to inhibit α-aminoadipate uptake via PcDip5.

The various $K_m$ and $K_i$ values were used for computer modeling of α-aminoadipate uptake using equation 1 that describes the relative contributions of the two transporters according to Michaelis-Menten kinetics. The data was fitted by regression to the data as shown in Fig. 1A. The best fitting curve (dashed line, Fig. 1A) showed an imperfect fit with the data measured at higher leucine concentrations, indicating that the uptake process is more complex than was described by the equation. From the regression analysis the relative contributions (maximal velocities) of PcDip5 and PcGap1 for α-aminoadipate uptake the absence of leucine could be calculated corresponding to 51 % and 49 %, respectively. This is in good agreement with a more phenomenological analysis in which it is considered that nearly 95 % of the α-aminoadipate uptake activity of PcGap1 is already inhibited at 250 μM L-leucine, while the inhibition of PcDip5 at this concentration is at the most 5 % (Fig. 1C and B). This would also yield an estimated 50 % contribution of both permeases (Fig. 1A). Thus, PcDip5 and PcGap1 seem to have an equal contribution in the uptake of α-aminoadipate when present at 25 μM. Taking the difference in $K_m$ value into account, a greater fraction of the α-aminoadipate flux will be mediated by PcDip5 with increasing α-aminoadipate concentration. Taken together, these data strongly suggests that the uptake of α-aminoadipate by *P. chrysogenum* involves two transporters, i.e., PcGap1 and PcDip5.

**Expression of *PcDIP5* and *PcGAP1* in response to nitrogen source and carbon source.** In fungi, the activity of amino acid permeases is regulated both at the transcriptional and the posttranslational level (82, 186, 191, 202, 203). To analyze the expression of *PcDIP5* and *PcGAP1* in *P. chrysogenum* an RT-PCR method was used. Mycelium was grown in liquid culture with different nitrogen and carbon sources and in penicillin production medium (lactose as carbon source, urea and glutamate as nitrogen source). After preculturing *P. chrysogenum* Wisconsin 54-1255 on YPG medium, germinating conidia were transferred to minimal media containing $NH_4^+$ (a rich nitrogen source), urea (a poor nitrogen source), glutamate (substrate of PcDip5

and PcGap1), serine or lysine (substrates of PcGap1) as sole nitrogen source and with glucose as carbon source. After 24 h of incubation, mRNA was isolated and used as a template for semi-quantitative RT-PCR (Fig. 6A). The expression of *PcDIP5* and *PcGAP1* was induced when mycelium was grown with glutamate, whereas in the presence of $NH_4^+$, expression was very low. With urea, *PcDIP5* was not expressed, while *PcGAP1* showed only a low expression. With serine, both genes were expressed at a moderate level. In contrast to *PcDIP5*, lysine was as effective as glutamate in inducing the expression of *PcGAP1*. However, lysine appears not to be a very good

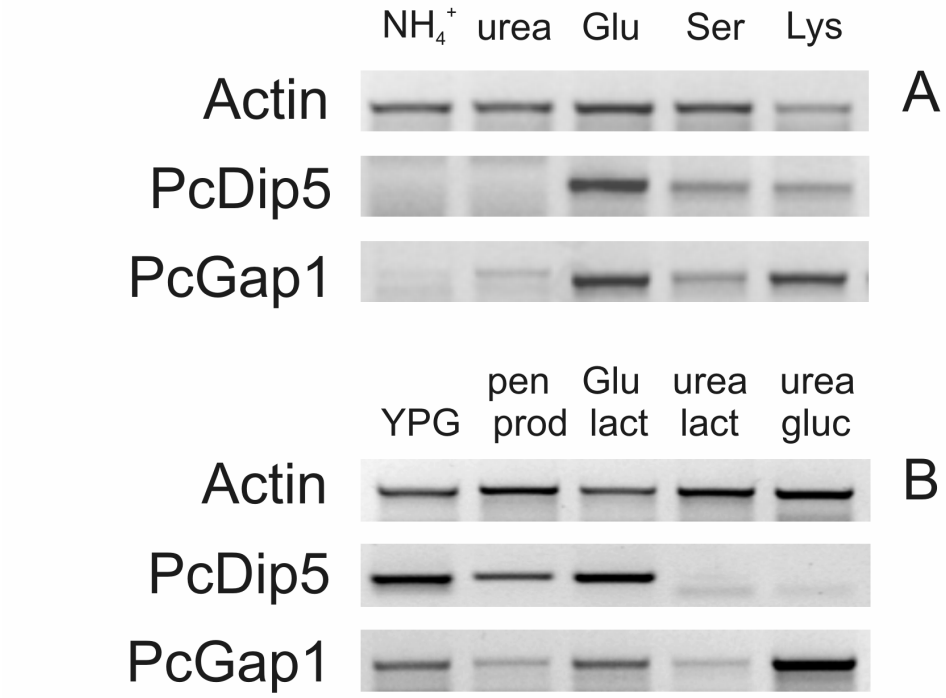


**Figure 6.** RT-PCR expression analysis of *PcDIP5* and *PcGAP1* in *P. chrysogenum* grown under different conditions. Expression of *PcDIP5* and *PcGAP1* after 24 h of growth on (A) minimal media containing glucose as carbon source and different nitrogen sources, and (B) in various media as indicated. Penicillin production medium contains urea and glutamate as nitrogen and carbon source. The other three media are minimal media with lactose or glucose as sole carbon source and glutamate or urea as sole nitrogen source. Isolated mRNA was used as a template for semi-quantitative RT-PCR. The expression analysis provides relative information for one gene only, and should not be compared among genes.

nitrogen source as the cultures grew slowly as mycelial pellets. This morphological behavior is indicative for stress.

To test whether the *PcDIP5* and *PcGAP1* are expressed under conditions of penicillin production and/or are subject to carbon catabolite repression, *P. chrysogenum* Wisconsin 54-1255 was grown for 48h on YPG, penicillin production medium containing lactose as carbon source, and on minimal medium with glutamate or urea as sole nitrogen source and lactose or glucose as carbon source (Fig. 4B). At penicillin producing conditions, *PcDIP5* and *PcGAP1* were moderately expressed in comparison with the other tested growth conditions. Nevertheless, substantial rates of α-aminoadipate uptake were observed via PcGap1 and PcDip5 under these conditions (See Fig. 1A). The pattern of expression did not significantly change after 72 hr of growth (data not shown). Carbon catabolite repression was not observed in any of the tested media. The expression of *PcGAP1* when growing on lactose and urea was even much lower than with glucose and urea (Fig. 4B, lanes 4 and 5). In addition, growth on YPG with glucose as carbon source, did not repress *PcGAP1* or *PcDIP5* expression. Although nitrogen catabolite repression was observed in the presence of $NH_4^+$, *PcDIP5* and *PcGAP1* seem not to be under control of carbon catabolite repression. Both transporters are expressed when cells are grown with glutamate and serine as sole nitrogen source, whereas *PcGAP1* is also expressed when urea is used as sole nitrogen source.

## Discussion

When α-aminoadipic acid is fed to penicillin producing mycelium of *Penicillium chrysogenum*, a higher intracellular α-aminoadipic acid concentration and penicillin synthesis rate can be observed (59, 81). The exact mechanism of uptake of α-aminoadipic acid has not been studied before. Here, we demonstrate that both the acidic and general amino acid permease (PcDip5 and PcGap1) contribute to α-aminoadipate uptake in *P. chrysogenum* when cells are grown under conditions that result in penicillin production. Although the studies with the cloned genes in *S. cerevisiae* indicate that PcGap1 has a higher affinity for α-aminoadipate, it appears that both systems equally contribute to the α-aminoadipate uptake by *P. chrysogenum*

when present at 25 μM. Taking the kinetic parameters into account, one can predict that the contribution of PcDip5, will increase with the α-aminoadipate concentration and will maximize to about 70-77% of the total uptake. The transport assays were performed at pH 6.0, but since PcGap1 has a preference for the uncharged form of glutamate (85), the contribution of PcGap1 in α-aminoadipate uptake may increase at lower pH. Although we can not exclude that in addition to PcGap1 and PcDip5, other permeases might be involved in α-aminoadipate uptake, the studies with the *S. cerevisiae* mutant indicate that at least in yeast Gap1 and Dip5 are the major permeases for uptake of this molecule. Also in other fungi no other transporters have been identified that could participate in the uptake of negatively charged amino acids.

Feeding of penicillin fermentations with external α-aminoadipate is not a cost-effective process. Therefore, at first sight it appears that the identified transporters would be of little benefit to the fermentation. However, transporters not only function in the uptake of compounds, but they also prevent that critical nutrients are lost by the cells. In this respect, the identified permeases may be important for the retention of intracellular α-AAA. In *A. nidulans*, two ammonium transporters have been identified, i.e., MeaA and MepA, that are required for the retention of intracellular ammonium (132). Surprisingly, MeaA that exhibits a lower affinity for ammonium ($K_m$ = 3 mM), appears more important for retention than the high-affinity ammonium permease, MepA ($K_m$ = 44 μM). It is unknown to what extent losses of intracellular α-aminoadipate in *P. chrysogenum* into the medium occur during industrial fermentation. Processes such as fragmentation of the hyphae by agitation or leakage across the membrane could potentially lead to significant losses. PcGap1 and PcDip5 might be involved in re-uptake. Our results suggest that *PcDIP5* and *PcGAP1* are both moderately expressed in shaking flask cultures when penicillin producing conditions prevail, i.e., when lactose is used as carbon source and urea and glutamate are nitrogen sources. These are conditions that are not identical to those in industrial fermentation (97, 142) when glucose is fed to the cells in a fed-batch fermentation. Carbon catabolite repression, however, was not observed for *PcGAP1* and *PcDIP5*. On the contrary, the mRNA level of *PcGAP1* was much higher when cells were grown in minimal medium with urea as sole nitrogen source and glucose as carbon source as compared to a medium with lactose as sole carbon source. Also in YPG medium with glucose, a significant level of *PcGAP1* and *PcDIP5* expression was

observed. It should be stressed that the final activity of these permeases also depends on posttranslational regulatory phenomena. For instance, for Gap1 of *S. cerevisiae* growing with glutamate as sole nitrogen source, a high mRNA level is observed but the transport activity is low. This has been attributed to the sorting of Gap1 to the vacuole instead of the plasma membrane (29, 191).

In conclusion, externally added α-aminoadipate can be taken up by *P. chrysogenum* via the acidic amino acid permease PcDip5 and the general amino acid permease PcGap1. Even though transport occurs with a relatively low affinity, a significant flux of α-aminoadipate uptake can be detected in mycelia grown under penicillin producing conditions. These transporters may contribute to the maintenance of a high intracellular α-aminoadipate concentration within the cell to allow a high β-lactam production capacity.

## Acknowledgements

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# Chapter 4

## PcMtr, an aromatic and neutral aliphatic amino acid permease of *Penicillium chrysogenum*

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## Abstract

The gene encoding an aromatic and neutral aliphatic amino acid permease of *Penicillium chrysogenum* was cloned, functionally expressed and characterized in *Saccharomyces cerevisiae* M4276. The permease, designated PcMtr is structurally and functionally homologous to Mtr of *Neurospora crassa*, and unrelated to the amino acid permease (AAP) family which includes most amino acid permeases in fungi. Database searches of completed fungal genome sequences reveal that Mtr type permeases are not widely distributed among fungi suggesting a specialized function.

## Introduction

Fungi posses a number of permeases for the uptake of amino acids, which can serve as nitrogen and/or carbon source or as building blocks for protein and peptide synthesis. These permeases have different properties with respect to substrate specificity, affinity, capacity and regulation (82, 157). Most fungal amino acid permeases belong to a unique transporter family, referred to as the AAP (Amino Acid Permease) family (6) or the YAT (yeast amino acid transporter) family (91), which is a subfamily of the ubiquitous APC (amino acid/polyamine/organocation) superfamily (231) which also includes transporters from bacteria, archaea and higher eukaryotes. These systems share a common membrane topology with 12 transmembrane segments and cytoplasmically located N- and C-terminal hydrophilic regions (186). Transport occurs by proton symport (78, 181). Some permeases are highly specific for one or a few related L-amino acids, whereas others have a broader specificity (89, 157) . The general amino acid permease, Gap1p of *Saccharomyces cerevisiae*, transports all L- and some D-amino acids and non-protein amino acids such as citrulline and ornithine (69, 93). Ssy1 of *S. cerevisiae* does not function as a transporter but as an amino acid sensor, involved in transcriptional regulation of the expression other amino acid permease genes (45).

Amino acid transport has been studied most extensively in *S. cerevisiae*, in which 18 (or 24, if the more distantly related Mup1 and Mup3 (methionine permeases), Uga4 (GABA permease), Hnm1 (choline permease), Bio5 (7-keto 8-aminopelargonic acid (KAPA) permease) and Ykl174C (unknown function) are included (157, and references therein) members of the AAP family were identified and characterized (91, 154, 157). In filamentous fungi, our current knowledge mainly relates to functional studies of amino acid transport specificity. Few of these systems have been characterized genetically and biochemically. For the β-lactam producing filamentous fungus *Penicillium chrysogenum*, nine different amino acid transport systems have been reported on basis of their substrate specificity (13, 85). This may reflect only a minimal number as some systems may not be distinguished on the basis of classical transport assays only. For instance due to overlapping substrate specificity or different expression patterns such systems might not easily be detected. Recent cloning and biochemical characterization of amino acid permeases of *P. chrysogenum* have led to the identification of the general amino acid permease PcGap1, the acidic

amino acid permease PcDip5 and a permease specific for aromatic amino acids and leucine, ArlP (208, 209). In *Neurospora crassa*, five distinct transport activities (system I-V) have been described (82, and references therein), whereas only two genes encoding amino acid permeases have so far been characterized. Naap1 encodes a general amino acid permease (122) belonging to the AAP family, whereas Mtr functions in the uptake of neutral aliphatic and aromatic amino acids. Interestingly, the *mtr* gene was found after selection of mutants resistant to 4-methyltryptophan, a toxic tryptophan analogue. These mutants are defective in the uptake of neutral aliphatic and aromatic amino acids (46, 103, 112, 190, 195). Mtr does not belong to the AAP family nor does it show significant homology with any characterized permease (less than 22 % amino acid sequence identity with first hit after Blast search). However, based on its hydropathy profile it was classified as a member of the Amino Acid/Auxin Permease (AAAP) family, which includes auxin and amino acid permeases from plants and animals and vacuolar amino acid transporters in *S. cerevisiae*. The AAAP family exhibits minimal sequence conservation, but they contain 11 transmembrane segments which discriminates them from the typical 12 transmembrane segment configuration of the AAP family members (172, 231). The AAAP family might represent a subfamily of the APC superfamily (91).

Here, we describe the cloning and characterization of PcMtr, an amino acid permease of *Penicillium chrysogenum*, which is structurally and functionally homologous to Mtr of *Neurospora crassa*.

## Material and Methods

**Strains and media.** The *P. chrysogenum* strain used in this study is Wisconsin 54-1255, a low-level penicillin-producing strain (kindly provided by DSM-Anti-Infectives, Delft, The Netherlands). Cultures were started on YPG medium (1% yeast extract, 2% peptone and 2% glucose), incubated for 16 h and subsequently diluted into minimal medium supplemented with 4% glucose and 0.4% ammonia acetate as described (107). *Saccharomyces cerevisiae* M4276 strain (*MAT*α**,** *ura3, Δgap1, Δdip5*) (159) was used for the functional expression and transport assays. Cells were grown in a minimal buffered medium containing 1% succinate, 0.6 % NaOH, 0.16% Yeast Nitrogen Base without ammonium sulphate and without amino

acids (Difco), 2 % glucose and 0.1% L-proline (MP), 0.5% $(NH_4)_2SO_4$ (MA) or 0.04% L-citrulline (MC) as a nitrogen source, or on YPG. MC medium was prepared by adding filter sterilized L-citrulline to the medium after autoclaving. Media were solidified with 1.5% agar, when needed. *E. coli* DH5α was employed for all cloning steps, carried out as described (174).

**Yeast complementation.** A partial cDNA library of *P. chrysogenum*, was cloned in the yeast/*E. coli* expression vector yEX-C as described (208). *S. cerevisiae* M4276 was transformed with the plasmid DNA and spread on MC plates, containing L-citrulline as sole nitrogen source (see above). Plates were incubated at 30°C for 5 days and colonies that appeared were transferred to fresh MC plates. Single colonies were used to inoculate liquid MA medium and after overnight growth, plasmid DNA was isolated using a standard plasmid rescue protocol and finally transformed to *E. coli* DH5α for propagation. The plasmid was analyzed by restriction, revealing a 1.5 kb insert of which the DNA sequence was determined. It contained an entire open reading frame of 1386 bp, which encodes a protein that shows 51% amino acid identity with the *mtr* locus encoded amino acid permease of *Neurospora crassa* (46). The new plasmid was designated yEX-PcMTR.

**Transport assays.** *S. cerevisiae* M4276, containing the plasmid yEX-PcMTR or the empty vector yEX-C, was used for assaying the transport characteristics of PcMtr. Transport assays were performed as described (208). Uptake of 14 radioactively labeled amino acids (Amersham) and of $^{14}$C-L-citrulline (NEN) was measured in cells expressing *PcMTR* or containing the empty vector. Unless indicated otherwise, amino acids were present at a concentration of 25 μM. Apparent $K_m$ values for uptake were determined by measuring initial uptake rates (uptake after 30 seconds of incubation with substrate) with increasing substrate concentration within a range of 25 μM and 2.5 mM. Apparent $K_i$ values were determined in a similar manner, except that unlabeled L-tryptophan (100 μM), L-serine (100 μM), L-alanine (100 μM) or L-citrulline (1 mM) was added as competitor. In competition experiments, L-phenylalanine uptake was measured at 25 μM in the presence of a ten-fold excess of the unlabeled amino acid. All uptake measurements were made in at least two independent experiments and average values are given. The standard deviation was

less than 10% at uptake levels higher than 0.5 nmol/mg protein. At lower uptake levels, variations did not exceed 30%.

**Genomic search for Mtr homologs and AAP family members.** Complete genome sequences of *Aspergillus nidulans*, *Neurospora crassa*, *Ustilago maydis*, *Magnaporthe grisea, Fusarium graminearum*, *S. cerevisiae, Candida albicans*, *Eremothecium gossypii*, *Schizosaccharomyces pombe*, *A. fumigatus*, *Podospora anserina*, *Cryptococcus neoformans* and *Coprinus cinereus* were searched for PcMtr homologs using the BlastP and BlastX search tool (2). Proteins with more than 50% amino acid identity with Mtr were classified as Mtr-homologs. AAP family members were searched only in protein databases of completed fungal genomes and identified on basis of homology (186). Proteins scoring a lower E-value than $10^{-25}$ in a Blast search (2) of the Gap1 sequence against protein databases were classified as AAP family members.

**Nucleotide sequence accession number.** The 1550 kb sequence containing *PcMTR* has been deposited in GenBank under accession number AY628328.

## Results

**Isolation of *PcMTR*.** In an attempt to clone the general amino acid permease from *P. chrysogenum* (14), a cDNA library of *P. chrysogenum* was expressed in *S. cerevisiae* M4276, deficient in citrulline uptake, followed by selection on plates containing citrulline as sole nitrogen source (MC plates). In *S. cerevisiae*, citrulline can only enter the cell via Gap1, the general amino acid permease (69, 158). All colonies that appeared on MC plates after 5 to 6 days contained the same cDNA clone. Sequences revealed the presence of a gene which is 54% identical to the *mtr* locus encoded amino acid permease Mtr of *Neurospora crassa* (46, 103, 195), while no significant homology was observed with AAP family permeases (6, 186). Mtr of *N. crassa* is specific for aromatic and neutral aliphatic amino acids, but has not been reported to mediate the uptake of citrulline. The cloned permease gene was designated *PcMTR* and the encoded protein PcMtr.

Mtr is a member of the eukaryotic-specific amino acid/auxin permease (AAAP) family of secondary transporters. The AAAP family includes amino acid and auxin permeases of plants and animals as well as vacuolar amino acid permeases of *S. cerevisiae*. Members of this sequence diverse group of transporters commonly contain 11 (or 10) transmembrane segments (172, 231). Likewise, the hydrophobicity profile of PcMtr predicts 11 transmembrane helices (data not shown).

**Transport characteristics.** To determine the substrate specificity of PcMtr, the uptake of 14 radioactively labeled amino acids was tested in *S. cerevisiae* M4276 expressing *PcMTR* and in control cells, containing the empty vector yex-C (Fig. 1). The amino acids were used at 25 μM. PcMtr showed specificity for aromatic amino

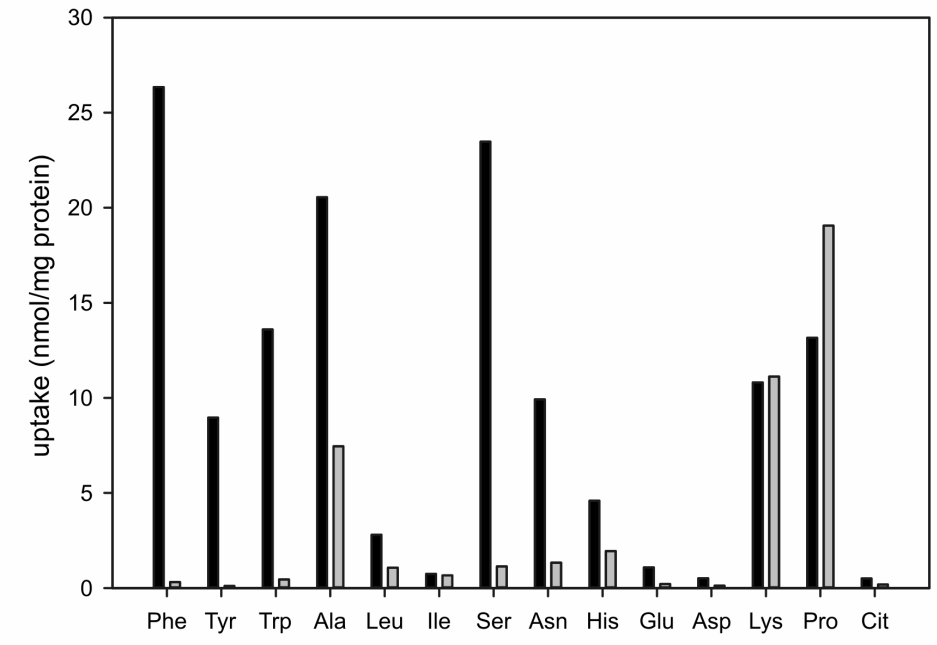



**Figure 1.** Uptake of amino acids in *S. cerevisiae* M4276 expressing *PcMTR* (solid bars) or in control cells containing the empty vector (grey bars). Uptake experiments were performed with cells grown to exponential phase on minimal proline medium. Expression of *PcMTR* was induced with 0.2 mM $CuSO_4$, 5 h before harvesting. The $^{14}C$-labeled L-amino acids were present at 25 μM concentration and the uptake levels were determined after 3 minutes incubation. The results shown are the means of three independent experiments.

acids, together with the neutral, aliphatic amino acids alanine, asparagine and serine. Uptake of the basic amino acid lysine was not increased upon expression of *PcMTR*, whereas the uptake of the acidic amino acids glutamate and aspartate was only slightly increased. This indicates that charged amino acids are poor (or not at all) substrates for PcMtr. The reduced level of proline uptake in cells overexpressing *PcMTR* is unclear, but this might relate to a reduced expression of endogenous proline permease(s) upon the overexpression of *PcMTR* while cells are grown on a medium with proline as nitrogen source Therefore, we cannot determine if PcMTR catalyzes the uptake of proline. Surprisingly, uptake of L-citrulline at 25 µM by *PcMTR* expressing cells was relatively low as compared to the other substrates. Citrulline

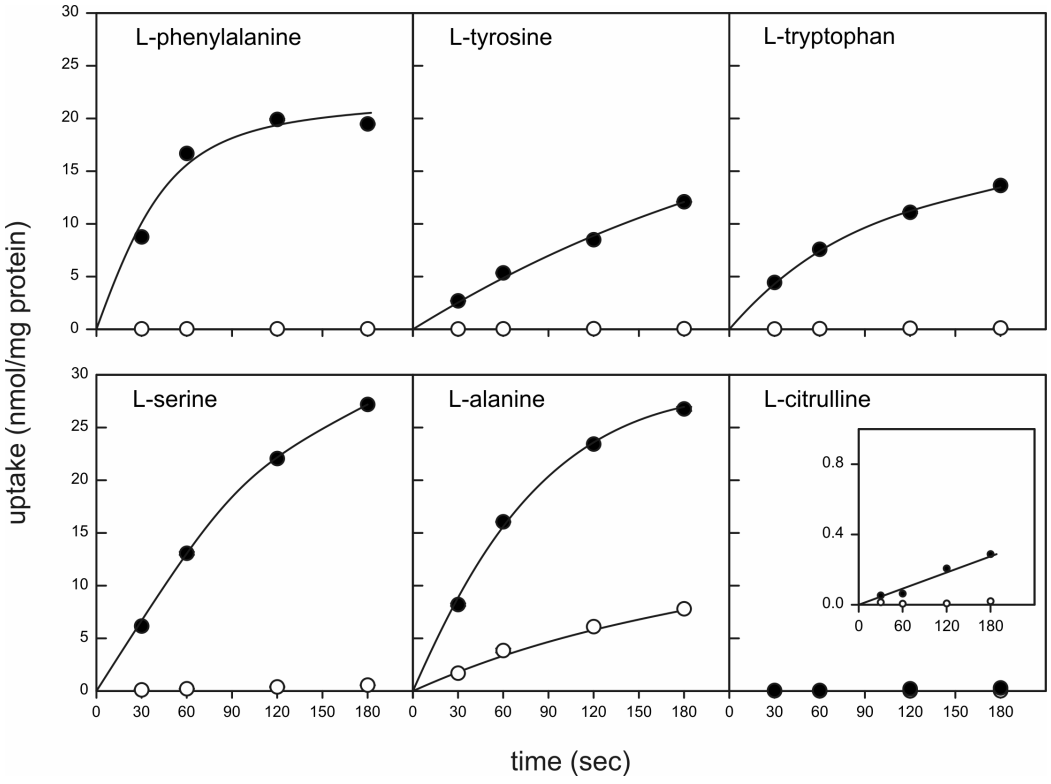



**Figure 2.** Time-dependent uptake of neutral and aromatic amino acids by *S. cerevisiae* M4276 cells expressing *PcMTR* (solid circles) or in control cells containing the empty vector (open circles). (A) L-phenylalanine, (B) L-tyrosine, (C) L-tryptophane, (D) L-serine, (E) L-alanine, (F) L-citrulline. $^{14}$C-labeled L-amino acids were present at 25 µM concentration, except for L-citrulline uptake, which was measured at 2.5 mM (F) or at 25 µM concentration (F, inset). Details are as described in the legend to Figure 1 and the Materials and Methods section.

uptake was also measured at 2.5 mM, which is close to the concentration of 2.3 mM used in the plates during the selection procedure of *S. cerevisiae* M4276 transformants (Fig. 2, last panel). Herein, the uptake by cells expressing *PcMTR* was 2 orders of magnitude higher than at 25 μM. This shows that PcMtr has a low-affinity for citrulline, but is capable of transporting citrulline with high capacity into the cell which explains why these cells can grow on citrulline as a sole nitrogen source (not shown). The substrate specificity of PcMtr corresponds with that reported for Mtr of *N. crassa* (112, 190), with phenylalanine and tryptophan as preferred substrates (Fig. 2).

To further characterize the substrate specificity of PcMtr, the inhibitory ability of a ten-fold excess of unlabeled amino acids on the uptake of $^{14}$C-L-phenylalanine via PcMtr was measured (Fig. 3). Strikingly, tyrosine that differs from phenylalanine by the presence of a hydroxyl group in the benzyl-side chain is a relatively poor competitor as compared to most neutral amphipatic amino acids. The affinity ($K_m$) for

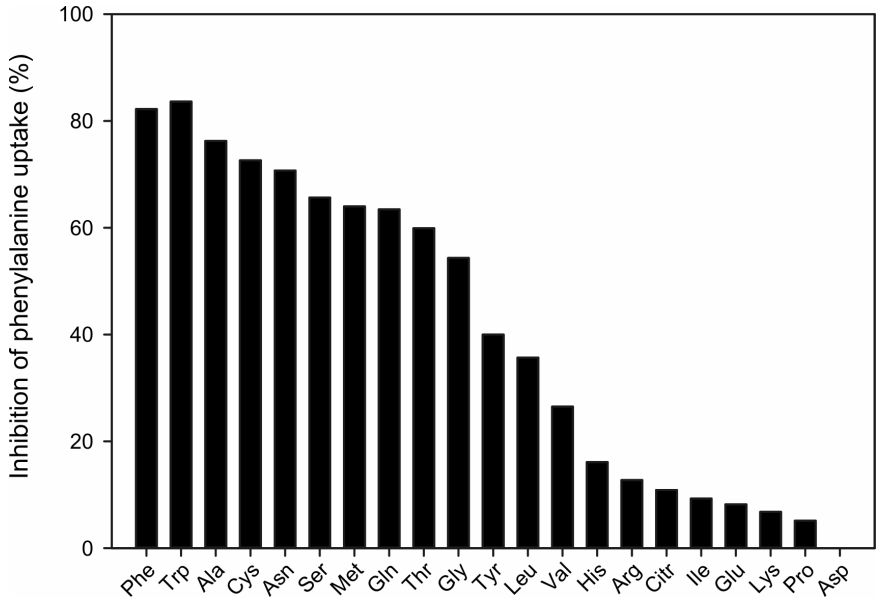



**Figure 3.** Substrate specificity of PcMtr as scored by the effect of an excess of unlabeled amino acids on the uptake of $^{14}$C-L-phenylalanine by *S. cerevisiae* strain M4276 expressing *PcMTR*. L-phenylalanine was present at a final concentration of 25 μM, and the indicated unlabeled amino acids were present in a ten-fold excess. The uptake was determined after 3 min and the results shown are the means of three independent experiments.

aromatic amino acid transport was determined, as well as the inhibitory constants ($K_i$) on phenylalanine transport (Table 1). Because of high background level of alanine uptake by endogenous permeases and the apparently very low affinity of PcMtr for citrulline ($K_i$ on phenylalanine transport > 10mM), it was not possible to determine the $K_m$ for uptake of these substrates. Except for the presence of a neutral side chain, amino acid substrates of PcMtr do not share any common features. Substrates include large and small (phenylalanine and alanine), and hydrophilic or hydrophobic (serine and tryptophan) amino acids, and the transport affinity is in the range of 30 to 150 μM (Table 1). As uptake measurements were done in a high affinity mode, amino acids, apart from citrulline, that are possibly transported, but with low affinity may not have been identified as such.

**Table 1.** Affinity ($K_m$) and inhibitory ($K_i$) constants for PcMtr mediated amino acid uptake.

| Amino acid | Kinetic parameter | |
|---|---|---|
| | $K_m$ (μM) | $K_i$ (μM)* |
| Phenylalanine | 28 | - |
| Tyrosine | 200 | n.d. |
| Tryptophan | 42 | 38 |
| Serine | n.d. | 110 |
| Alanine | n.d. | 130 |
| Citrulline | n.d. | >10,000 |

* $K_i$ values were estimated from the inhibitory effect of the indicated amino acids on the uptake of L-phenylalanine as described in the Materials and Methods section. n.d., not determined.

**Distribution of Mtr and AAP type permeases in fungi.** To determine the abundance of Mtr type of transporters in fungi, completed fungal genome sequences were scored for the presence of AAP and Mtr type of transporters. Using the BLAST search tool (BlastX or BlastP (2)), 5 of the 12 fungal genomes contained a single Mtr homolog (more than 50% amino acid identity). Remarkably, *Fusarium graminearum* contains 5 homologs, and the similarity among these sequences varies from 51 – 60%

amino acid sequence identity. The yeasts *S. cerevisiae, Candida albicans*, and *Eremothecium gossypii,* the fission yeast *Schizosaccharomyces pombe*, and the basidiomycetes *Cryptococcus neoformans* and *Coprinus cinereus*, do not contain an Mtr homolog (Table 2). An alignment and phylogenetic tree of Mtr homologs show that the proteins of *P. chrysogenum*, *A. nidulans* and *A. fumigatus* cluster together (Fig. 4). The divergence among Mtr homologs does not follow the phylogenetic evolutionary tree as PcMtr is more homologous to the Mtr homolog of the basidiomycete *Ustilago maydis* than of the ascomycete *N. crassa*. Also, the 5 Mtr homologs of *F. graminearum* do not cluster together. In addition, the number of AAP family members was determined in the various fungal genomes using BlastP. In all cases except *A. nidulans*, a clearly distinguishable group of proteins shows significant

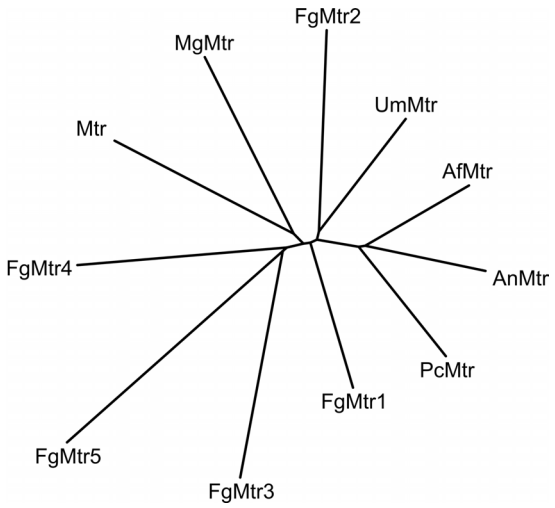



**Figure 4.** Phylogenetic relationship between fungal Mtr homologs. Mtr homologs were identified in the completed fungal genomes using BlastP and the *Neurospora crassa* Mtr ("NcMtr" in figure, accession number EAA33125) as a probe: *Fusarium graminearum* (FgMtr 1-5, accession numbers EAA78089, EAA78322, EAA72970, EAA71441 and EAA76673, respectively); *Magnaporthe grisea* (MgMtr, accession number EAA56571); *Ustilago maydis* (UmMtr, accession number EAK81441); *Aspergillus fumigatus* (AfMtr, deduced by BlastX against the *A. fumigatus* database (TIGR) and in silico analysis of DNA sequence of contig 5172); *Aspergillus nidulans* (AnMtr, accession number EAA58464). The alignment and tree were created using ClustalX and the neighbor joining method, version 3.6 from the PHYLIP package (Joe Felsenstein, Dep. of Genome Sciences, University of Washington).

homology with established AAP family members (E-values lower than $10^{-25}$). Their number ranges from 8 in the basidiomycete *U. maydis* to more than 18 in *A. nidulans*, *F. graminearum*, *S. cerevisiae* and *C. albicans* (Table 2). This indicates that in many fungi AAP-like transporters represent the major class of transporters while Mtr type amino acid permeases are rare and not universally present in fungal genomes.

**Table 2.** Distribution of Mtr homologs and AAP type permeases in fungal genomes. Genome databases were obtained from NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=euk), except for the *Podospora anserina* genome, which was obtained from the *P. anserina* Genome Project, Institut de Génétique et Microbiologie, Université de Paris-Sud (http://podospora.igmors.u-psud.fr/index.html), and the *Aspergillus fumigatus* genome, which was obtained from the *A. fumigatus* Genome Database of The Institute for Genomic Research (TIGR, http://www.tigr.org/tdb/e2k1/afu1).

| | | Number of transporters | | Genome size |
|---|---|---|---|---|
| | | Mtr homologs | AAP members | (Mb) |
| ascomycetes | *A. nidulans* | 1 | 20-23 | 31 |
| | *F. graminearum* | 5 | 23 | 40 |
| | *A. fumigatus* | 1 | n.d.* | 32 |
| | *P. anserina* | 1 | n.d. | 34 |
| | *N. crassa* | 1 | 9 | 40 |
| | *M. grisea* | 1 | 14 | 40 |
| | *S. cerevisiae* | 0 | 18 | 12 |
| | *E. gossypii* | 0 | 15 | 9.2 |
| | *C. albicans* | 0 | 18-20 | 16 |
| | *S. pombe* | 0 | 12 | 14 |
| basidiomycetes | *U. maydis* | 1 | 8 | 20 |
| | *C. cinereus* | 0 | n.d. | 38 |
| | *C. neoformans* | 0 | n.d. | 20 |

*n.d., not determined. The genomes are not annotated.

## Discussion

Here, we describe the cloning and characterization of PcMtr, an amino acid permease of *P. chrysogenum* that exhibits specificity for aromatic and neutral aliphatic amino acids. Complementation of citrulline uptake in *S. cerevisiae* M4276 yielded only PcMtr, and not the expected general amino acid permease (or Gap). Nevertheless, the *P. chrysogenum* Gap was recently shown to transport citrulline [31], implying that unlike in *S. cerevisiae*, multiple pathways exist in *P. chrysogenum* for the uptake of citrulline. PcMtr has a relatively broad specificity, as substrates include hydrophobic and hydrophilic as well as bulky and small residues. Amino acids with a charged side chain are not transported.

PcMtr and the functionally and structurally homologous Mtr transporter of *Neurospora crassa* [6,14], are not related to the abundant family of AAP transporters [2], a subfamily of the APC family [12]. Instead they represent a small class of their own which can be subdivided in the AAAP family that includes auxin and amino acid permeases in plants and animals and vacuolar amino acid transporters in *S. cerevisiae* (231). The homology in the amino acid sequences of PcMtr and its homologs with other AAAP family members is low (less than 25% identity), but they share the common hydropathy profile that suggests the presence of 11 transmembrane segments (231). Filamentous fungi appear to contain only a single gene that specifies an AAAP family member, although *F. graminearum* is exceptional in that it contains 5 homologs. However, Mtr-like transporters are lacking in some yeast and filamentous fungi. None of these systems has been studied but considering the fact that the AAP-like amino acid transporters are relatively abundant in filamentous fungi (8-20 different systems), it seems likely that the Mtr type transporters are involved in a specific function such as the uptake of a signaling molecule. This would provide a possible explanation that the wheat pathogen *F. graminearum*, contains 5 Mtr homologs. In particular expression analysis of these transporters may shed more light on their possible physiological function and significance.

## Acknowledgments

*S. cerevisiae* strain M4276 was kindly provided by M.C. Kielland-Brandt (Department of Physiology, Carlsberg Laboratory, Copenhagen, Denmark). This work was supported by the European Community (grant no. QLK3-CT-1999-00729).

# Chapter 5

## Random mutagenesis of *PcDIP5* and selection for improved α-aminoadipate transport

Hein Trip, Melchior E. Evers and Arnold J. M. Driessen

## Abstract

*Saccharomyces cerevisiae* is unable to utilize α-aminoadipate as a primary nitrogen source unless *LYS2* or *LYS5* is disrupted. In *S. cerevisiae* strain M4276, deficient in uptake of acidic amino acids, the *LYS2* gene was deleted, resulting in strain M4600. The acidic amino acid permease gene of *Penicillium chrysogenum*, *PcDIP5*, was expressed in M4600 to allow uptake and utilization of α-aminoadipate as primary nitrogen source. In an attempt to improve the uptake capacity of PcDip5 for α-aminoadipate, a random mutagenesis was performed by error-prone PCR. Cells of strain M4600 were transformed with the mutagenized *PcDIP5* and were selected for faster growth on plates with a low concentration of α-aminoadipate. Mutant strains were selected with faster growth characteristics but these did not relate to an improved α-aminoadipate transport activity.

## Introduction

Penicillin biosynthesis in *Penicillium chrysogenum*, which starts with the condensation of three amino acid precursors L-α-aminoadipate, L-cysteine and L-valine, to form the tripeptide ACV (1), is stimulated by external addition of α-aminoadipate (59, 81). The uptake of α-aminoadipate is mediated by active transport into the cells via the acidic amino acid permease PcDip5 and the general amino acid permease PcGap1 (208). PcDip5 is a high affinity transporter of L-glutamate and L-aspartate ($K_m$ = approx. 35 μM), whereas α-aminoadipate is transported with low affinity ($K_m$ = approx. 800 μM). PcGap1 can transport all amino acids and has a higher affinity for α-aminoadipate ($K_m$ = 230 μM).

In this work, we attempt to improve PcDip5 for α-aminoadipate transport by a random mutagenesis and a positive selection strategy. An improvement of the transport affinity might be beneficial to the penicillin production capacity as it would allow more efficient savaging of extracellular α-aminoadipate, i.e., a better retention in the cell. Little is known about structure-function relationships in members of the AAP family, to which PcDip5 belongs (6, 208). A site-directed mutagenesis study on the major proline permease gene *prnB* of *Aspergillus nidulans* has implicated two residues that are present in TMS 6 to be important for substrate selectivity or binding (201). In a random mutagenesis strategy on *CAN1*, the arginine permease gene of *S. cerevisiae*, mutants were selected that had acquired the ability to mediate citrulline uptake. These mutations were located in transmembrane segments 3 and 10, as well as in cytoplasmic and extracellular loops. Most of the mutations are at conserved positions in the AAPs family, but there is no evidence that they are directly involved in substrate recognition, i.e., they may affect the transport activity and specificity in an indirect way (160). Considering the large difference in affinity of PcDip5 for α-aminoadipate and glutamate but at the same time the similarity in structure of these substrates, we envision the possibility to alter PcDip5 in such a way that its affinity or capacity for α-aminoadipate transport is increased. For positive selection of improved transport, an assay may be employed in which growth is dependent on α-aminoadipate uptake via *PcDIP5*. In earlier work, PcDip5 was characterized by the functional expression of *PcDIP5* in *S. cerevisiae* M4276, which lacks the genes encoding the acidic amino acid permease *DIP5* and the general amino acid permease

*GAP1* (159, 208). To select for improved α-aminoadipate transport, a library of randomly mutagenized *PcDIP5* can be expressed in *S. cerevisiae* M4276 and cells may be selected for faster growth on plates with a limiting amount of α-aminoadipate as sole nitrogen source. However, to enable *S. cerevisiae* to use α-aminoadipate as a nitrogen source, either *LYS2* or *LYS5*, together encoding α-aminoadipate reductase, has to be disrupted (28, 233). α-Aminoadipate is an intermediate of the lysine biosynthesis pathway and is converted into α-aminoadipate semialdehyde by the activity of α-aminoadipate reductase. Normally, external addition of α-aminoadipate is toxic for *S. cerevisiae*, probably due to an increase of the intracellular α-aminoadipate semialdehyde concentration which might be toxic to the cells. If *LYS2* or *LYS5* is deleted and α-aminoadipate is added to the medium, α-aminoadipate semialdehyde is no longer formed, α-aminoadipate will accumulate and will be shuttled into a transaminase pathway. Probably, the amino group of α-aminoadipate is transferred to α-ketoglutarate to form α-ketoadipate and glutamate, of which the latter will go into nitrogen assimilation. Hereby, α-aminoadipate serves as primary nitrogen source, while cells are now lysine auxotroph and have to be provided with lysine for protein synthesis (233). Here, we describe the construction of a new, *LYS2* negative and acidic amino acid uptake deficient strain of *S. cerevisiae* and the use of it for positive selection of PcDip5 mutants improved in α-aminoadipate transport.

## Material and methods

**Strains and media.** The strains used in this study are *S. cerevisiae* M4276 (*MAT*α, *ura3*, *Δgap1*, *Δdip5*) (159) and M4600 (*MAT*α, *ura3*, *Δgap1*, *Δdip5*, *Δlys2*), which was directly derived from strain M4276 by the deletion of the *LYS2* gene. Cells were grown in minimal medium containing 0.1 % L-proline (MP) (159), glutamate (0.5 g/L) or α-aminoadipate at indicated concentrations as nitrogen source and supplemented with uracil (20 mg/L) when needed. For growth of strain M4600, 0.3 g/L lysine was added to complement for lysine auxotrophy. YPG (1 % yeast extract, 2 % peptone and 2 % glucose) was used for non-selective growth. *E. coli* DH5a was used for all cloning steps, carried out as described in (174).

**Deletion of LYS2 in S. cerevisiae M4276.** A LYS2 deletion cassette was constructed by using 2 oligonucleotides, one containing 60 bp of the 5' end of LYS2 and 20 bp of the 5' end of the zeocin resistance cassette/gene: ATGACTAACGAAAAGGTCTGGATAGAGAAGTTGGATAATCCAACTCTTTC AGTGTTACCAGGTCATAGCTGTTTCCGATCC, and one containing 60 bp of the 3' end of LYS2 and 20 bp of the 3' end of the zeocin resistance cassette: TTAAGCTGCGGAGCTTCCACGAGCACCAGCACCTGAAGCAACTAGACTTA TTTGCGCTGCTCACATGTTGGTCTCCAGC. A PCR was performed using the plasmid pREMI-Z (217) containing the zeocin resistance cassette, as template DNA. The 4.3 kb product was gel-purified and transformed to S. cerevisiae M4276 cells, according to Gietz et al. (62). Cells were selected for zeocin resistance on YPG plates containing 100 mg/L zeocin. Colonies appeared after two days and were transferred to liquid YPG medium for DNA isolation. Deletion of LYS2 was tested by PCR using oligonucleotides annealing to the 5' flanking region of LYS2 (forward primer) and the zeocin resistance gene (backward primer), as well as oligonucleotides annealing to LYS2, amplifying an internal LYS2 fragment.

The M4276 *lys2* strain, denoted M4600, was transformed with yEX-PcDIP5 (208) to enable uptake of α-aminoadipate, and with yEX-C, the empty vector, as a control. Transformants were tested on minimal medium plates containing 1 g/L L/D-α-aminoadipate, 600 mg/L L-lysine and 0.2 mM $CuSO_4$ for induction of expression of *PcDIP5* (208). Cells containing yEX-PcDIP5 were able to grow, whereas the control cells were not. To show that lysine in the medium was not used as a primary nitrogen source instead of α-aminoadipate, M4600 cells were streaked on plates containing 600 mg/L L-lysine as sole nitrogen source.

**Random mutagenesis of PcDIP5 by error prone PCR.** Random mutagenesis was carried out basically as described by Leemhuis et al. (109). The PcDIP5 gene (plus additional 200 bp plasmid DNA up- and downstream PcDIP5) was amplified from yEX-PcDIP5 (208) using the following primers: Dipmut F, GGTACCGGTCCGAATTCC and Dipmut R, GGCCTGTTTACTCACAGGC. The PCR mixture contained the following: 10 mM of dATP, dCTP, dGTP and dTTP, 1 X Taq DNA polymerase buffer (Roche), 2 mM MgCl, 0.2 mM $MnCl_2$, 0.2 μM of each primer, 2 units Taq polymerase (Roche) and 50 ng template DNA in a total volume of

50 μL. The PCR reaction was performed for 30 cycles: 30 sec at 94°C, 40 sec at 55°C and 2 min at 72°C. The PCR product was analyzed by gel-electroforesis and purified and digested with XhoI, located 27 bp downstream the startcodon, and NotI, located 136 bp downstream the stopcodon of PcDIP5. The resulting 1.8 kb fragment was ligated in the linearized yEX-C vector resulting from XhoI and NotI digested yEX-PcDIP5. For optimal ligation conditions, 5 % PEG-8000 (final conc.) was used in the ligation mixture. Electrocompetent XL1-Blue cells (Stratagene) were transformed with purified DNA from the ligation mixture, yielding approx. 120,000 transformants. Plasmid DNA was isolated for transformation of S. cerevisiae M4600.

**Transformation of M4600 and selection for improved growth at limiting α-aminoadipate concentration.** *S. cerevisiae* M4600 was transformed according to Gietz et al (62), resulting in at least 100.000 transformants. Cells were spread on minimal medium plates containing 0.1 g/L L/D-α-aminoadipate, 0.3 g/L L-lysine and 0.2 mM $CuSO_4$ for induction of *PcDIP5* expression. Plates were incubated for 10 days at 30°C. Colonies of apparent larger size were transferred to fresh plates with the same medium and plates were incubated for 10 days at 30°. Plasmid DNA was isolated from these clones by standard methods, transformed to *E. coli* DH5α for amplification and finally reintroduced into stain M4600 and also M4276. M4600 transformants were plated on minimal medium as described above, i.e. with 0.1 g/L L/D-α-aminoadipate as primary nitrogen source. M4276 transformants were used in a transport assay to directly test α-aminoadipate uptake.

**Transport assays.** Strain M4276 was transformed with plasmid DNA obtained from 8 M4600 transformants showing increased growth on plates with a limiting concentration of α-aminoadipate as primary nitrogen source. Transformants were grown overnight in minimal proline (MP) medium, then diluted 10 times in fresh MP medium, supplemented with 0.2 mM $CuSO_4$ for induction of the copper inducible promoter *pCUP1*, upstream *PcDIP5*. After 5 hours of incubation, cells were harvested by centrifugation, washed once with minimal medium without a nitrogen source and resuspended in the same medium. Transport assays using $^3$H-labelled L/D-α-aminoadipate were carried out as described by Trip et al., 2002 (209) using concentrations of 25 μM up to 1 mM.

## Results

**Deletion of *LYS2* in *S. cerevisiae* M4276.** In earlier work, transport of α-aminoadipate, glutamate and aspartate by PcDip5 was studied in *S. cerevisiae* strain M4276 *(Δgap1, Δdip5*) by functional expression of the *Penicillium chrysogenum DIP5* (*PcDIP5*) and transport assays using radioactively labeled amino acids (159, 208). Since these assays are performed on a minute scale, the growth inhibiting or even toxic effect of externally added α-aminoadipate to *S. cerevisiae* cells (28) does not interfere with the results. Here we aimed to screen a library of PcDip5 mutants for increased α-aminoadipate transport. At limiting α-aminoadipate concentration, growth of *S. cerevisiae* is dependent on the α-aminoadipate uptake capacity via PcDip5. Under conditions that transport is limiting, improved transport characteristics may lead to faster growth. In order to allow growth of *S. cerevisiae* on α-aminoadipate as primary nitrogen source, the *LYS2* gene needs to be disrupted in strain M4276. Since *LYS2* encodes the α-aminoadipate reductase, an enzyme of the lysine biosynthesis pathway, cells lacking this enzyme are lysine auxotroph and need to be provided with lysine (233). *S. cerevisiae* M4276 was transformed with a *LYS2* deletion cassette, containing the zeocin resistance gene as a selectable marker and two 60 bp flanking regions of *LYS2*. Zeocin resistant colonies were tested for the *LYS2* deletion by PCR using appropriate primers and appeared all to be *LYS2*$^-$. In addition, cells were tested phenotypically by plating on minimal medium plates, supplemented with uracil, with and without lysine. The zeocin resistant cells did not grow without lysine, whereas control cells (M4276) grew normally (Fig. 2A). The new strain, denoted M4600, was tested for its ability to use α-aminoadipate as a primary nitrogen source. Cells were transformed with yEX-PcDIP5 and with yEX-C (empty vector) as a negative control. Transformants were plated on minimal medium containing 1 g/L L/D-α-aminoadipate, 0.3 g/L L-lysine and 0.2 mM $CuSO_4$ for induction of *PcDIP5* expression. Cells containing yEX-PcDip5 grew normally, whereas cells containing the empty vector did not grow at all (Fig. 1). To test whether the lysine in the medium was not used as the primary nitrogen source, cells of M4600 were plated on minimal medium containing 0.6 g/L L-lysine as a sole nitrogen source. Very poor growth was observed, even after 5 days, indicating that lysine is not a good nitrogen source for *S. cerevisiae.* This result can also be taken as evidence that the presence of lysine does

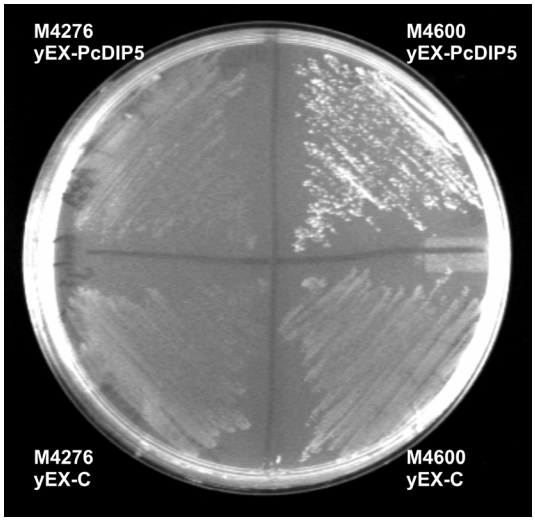



**Figure 1.** Growth of *Saccharomyces cerevisiae* strain M4600 (M4276 with deleted *LYS2* gene) with α-aminoadipate as primary nitrogen source is rescued by the heterologous expression of *PcDIP5*. Plates contain minimal medium with 1 g/L D/L α-aminoadipate, 0.6 g/L L-lysine to complement for lysine auxotrophy of strain M4600 and 0.2 mM $CuSO_4$ for *PcDIP5* expression. In M4276 yEX-PcDIP5 (upper left part), α-aminoadipate is taken up via PcDip5, but will be toxic to the cells. With the *LYS2* deletion, M4600 yEX-PcDIP5 can take up and assimilate α-aminoadipate and grow (upper right part). Without expression of *PcDIP5* (M4600 yEX-C, right, below), cells cannot take up α-aminoadipate and cannot grow on α-aminoadipate as primary nitrogen source.

not significantly interfere with α-aminoadipate as a primary nitrogen source (Fig. 2B). Using low concentrations of lysine (30 mg/L which should be sufficient to satisfy the lysine auxotrophy according to Zaret et al., 1985 (233)) resulted in strongly reduced growth (Fig. 2A). This might be explained by a reduced uptake of lysine due to the absence of the general amino acid permease in strain M4600, although the basic amino acid transport systems (Can1, Lyp1 and Alp1) which are still present in this strain may substitute for the Gap1 deficiency.

**Random mutagenesis of PcDIP5 by error prone PCR.** In order to generate a mutant of PcDip5 with improved α-aminoadipate transport capacity or affinity, random mutagenesis was applied using error prone PCR. A mutant PcDip5 library was created using 0.2 mM $MnCl_2$ in an otherwise standard PCR. Ligation of the PCR

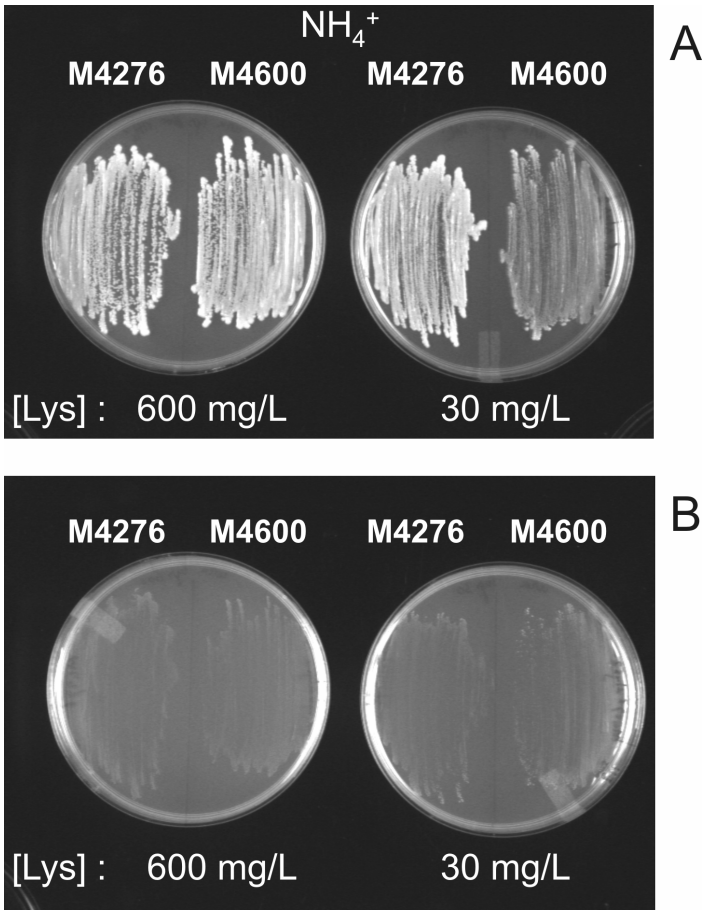



**Figure 2.** Lysine auxotrophy in *S. cerevisiae* M4600 (M4276 with deleted *LYS2* gene). (A) *S. cerevisiae* M4600 needs a relatively high concentration of externally added lysine to fully complement for lysine auxotrophy. Plates contain 5 g/L $NH_4SO_4$ as nitrogen source and 30 and 600 mg/L L-lysine. (B) Lysine is a poor nitrogen source for *S. cerevisiae* M4600. After 5 days of incubation, hardly any growth was observed with up to 0.6 g/L of L-lysine as a sole nitrogen source.

product in the expression vector yEX-C and transformation to *E. coli* resulted in approx. 120,000 clones. Plasmid DNA was isolated and transformed to *S. cerevisiae* M4600, yielding approximately 100,000 transformants. To test the effect of the mutagenesis on the activity of PcDip5, transformants were tested for their ability to grow on plates with minimal medium and 0.5 g/L L-glutamate as sole nitrogen source. Of 50 tested clones, 12 did not grow at all or very slowly on this medium, indicating that about 75 % of the mutant PcDip5 clones retained normal glutamate transport activity.

**Selection for improved α-aminoadipate uptake.** *S. cerevisiae* M4600 was transformed with the mutant library of PcDip5, resulting in about 100,000 transformants. Cells were plated on minimal medium containing 0.05 g/L L/D-α-aminoadipate, 0.3 g/L L-lysine and 0.2 mM $CuSO_4$, and plates were incubated for 10 days. A number of colonies with larger size than the majority of colonies appeared and these were transferred to fresh plates for isolation of individual clones. From these clones, plasmid DNA was isolated and transformed back to strain M4600 as well as M4276. Strain M4600 transformed with the isolated plasmid DNA, however, did not show an enhanced growth in comparison with M4600 expressing wild-type *PcDIP5*. Likewise, M4276 cells transformed with the same plasmid did not show increased α-aminoadipate uptake when tested at 25 μM and 1 mM concentration.

## Discussion

The acidic amino acid permease PcDip5 shows a high affinity for aspartate and glutamate ($K_m$, 35 μM), but a low affinity for α-aminoadipate ($K_m$, 800 μM), although the latter only differs from glutamate in having a side chain one that is one $CH_2$-group longer. This chapter describes an attempt to randomly mutagenize PcDip5 and select for improved α-aminoadipate transport using a plate assay in which growth of *S. cerevisiae* is dependent on α-aminoadipate transport via PcDip5. For this purpose, a new strain of *S. cerevisiae* was constructed, i.e., M4600. This strain is derived from strain M4276, deficient in uptake of acidic amino acids, by deleting *LYS2*, encoding aminoadipate reductase. This deletion blocks lysine biosynthesis, but enables the cells to use α-aminoadipate as a primary nitrogen source. In this strain, a library of randomly mutagenized *PcDIP5* was expressed and cells were grown on plates with a limiting concentration of α-aminoadipate as primary nitrogen source. However, PcDip5 clones isolated from colonies with a clearly larger size than the vast majority of colonies, did not show increased α-aminoadipate uptake after further analysis. The larger size of colonies may have been due to non-specific, possibly spontaneous mutations that provide the cells a growth advantage on the specific minimal medium. In general, selection on basis of colony size as an indicator for rate of transport of a growth limiting substrate might be too indirect.

In this work we tried to improve PcDip5 for α-aminoadipate transport by random mutagenesis with two intentions, firstly to improve α-aminoadipate (re)uptake in *P. chrysogenum* and secondly to gain inside in structure/function relationships in AAPs. For this purpose, a random mutagenesis approach seems to be preferred provided that a suitable screening method can be applied in which the desired change in transport characteristic leads to a much more distinct phenotype than a difference in colony size. A more rational approach is site-directed mutagenesis, but currently it is unclear what residues should be targeted for such an approach. A first step could be the construction of chimaeric genes, in which parts of genes of AAPs with different substrate specificity are combined. This may lead to a better insight in what regions/domains of the transporter define substrate specificity.

## Acknowledgement

We thank Dr. J. Kiel for valuable discussions.

# Chapter 6

## Summary and concluding remarks

### Summary

**Amino acid transport in *Penicillium chrysogenum*.** Fungi are capable of accumulating amino acids from their environment and use them as nitrogen and/or carbon source or as building blocks for protein or peptide synthesis. Amino acid uptake is mediated by a large set of permeases that differ in substrate specificity and regulation. Transport mostly occurs as proton symport and is thus is driven by the proton gradient (78, 181). Nearly all amino acid permeases identified so far comprise one family, named the AAP (amino acid permease) family (6). This family is distinct from two distantly related methionine/cysteine permeases of *Saccharomyces cerevisiae* (Mup1 and Mup2 (90)), and the unrelated Mtr type permeases of *Neurospora crassa* and *Penicillium chrysogenum* (46, 190) (see below). The AAP family and Mup1 and Mup2 are subdivided in the large and ubiquitous family of the amino acid/polyamine/organocation (APC) secondary transporters (91). The number of AAPs varies between organisms, and is 18 in *S. cerevisiae* (6, 91), approximately 20 in *Aspergillus nidulans* and 8 in *Ustilago maydis* (Trip et al, unpublished work). The relatively large number of amino acid permeases enables cells to fine tune specificity and selectivity to the differing environments in order to transport the preferred amino acids from a complex mixture.

Based on physiological and kinetic studies, nine different amino acid transport systems were identified in *Penicillium chrysogenum* (82). However, this number may be an underestimation due to overlapping substrates specificities and different regulation patterns. Since *P. chrysogenum* is closely related to *A. nidulans*, it may contain a similar large number of AAPs for which the function remains to be elucidated. In this thesis work, three amino acid permeases belonging to the AAP family and a fourth amino acid permease unrelated to AAPs have been cloned and characterized by means of functional expression in *S. cerevisiae* M4276. This strain lacks Gap1, the main general amino acid permease and Dip5, the acidic amino acid permease. Therefore, it shows a dramatically reduced ability to transport most amino

acids, while it is defective in the uptake of acidic amino acids. ArlP was cloned by means of a degenerated primer strategy which was based on conserved sequence motifs in fungal AAP transport systems. ArlP is specific for aromatic amino acids and leucine which it transports with moderate affinity ($K_m$, 350 μM) (209). PcDip5 is the acidic amino acid permease. It is homologous to Dip5 of *S. cerevisiae*, and was cloned after complementation of the previously mentioned *S. cerevisiae* strain M4276. Dip5 transports glutamate and aspartate with high affinity ($K_m$, 35 μM) while α-aminoadipate is recognized with low affinity ($K_m$, 800 μM). PcGap1, the general amino acid permease which is homologous to Gap1 of *S. cerevisiae* and that transports all amino acids, was cloned using a degenerated primer strategy based on regions specifically conserved in (putative) fungal general amino acid permeases (208). A fourth permease, PcMtr, was cloned after complementation of citrulline uptake in *S. cerevisiae* M4276. This cloning strategy was expected to yield the general amino acid permease, but instead a system was obtained that is homologous to Mtr of *Neurospora crassa.* Both transport aromatic and neutral aliphatic amino acids. They are not members of the APC family, but of the auxin and amino acid permease (AAAP) family (231), comprising members that vary strongly in amino acid sequence, but share a common topology of 11 transmembrane segments, instead of the typical 12 transmembrane segment topology of most APC transporters (91, 231).

**Uptake of α-aminoadipate uptake.** The acidic amino acid α-aminoadipate is one of the three amino acid precursors of penicillin, together with cysteine and valine. In the cytosol, they are condensed by ACV synthetase to form the tripeptide L-α-aminoadipyl-L-cysteinyl-D-valine (ACV), which is transformed into isopenicillin N (IPN) by IPN synthase. IPN enters the microbodies by a so far undefined transport mechanism whereupon the α-aminoadipate moiety is replaced by phenylacetic acid to form penicillin G. The latter is secreted by the cell. The intracellular concentration of α-aminoadipate, but not of cysteine and valine (92, 115) appears limiting for the rate of ACV formation and therefore also for the overall penicillin synthesis rate. α-aminoadipate is an intermediate of the lysine biosynthesis pathway, but is also channeled into the penicillin biosynthesis pathway. The intracellular concentration of α-aminoadipate can be increased by blocking the enzymatic step that leads α-aminoadipate conversion in the lysine route (26), or by the external addition of α-

aminoadipate (59, 81). This leads to increased levels of penicillin production. Uptake of α-aminoadipate is mediated by the acidic amino acid permease PcDip5, as well as the general amino acid permease PcGap1 (this thesis). PcDip5 transports glutamate and aspartate with high affinity ($K_m$, 35 μM) whereas α-aminoadipate is transported with a lower affinity only ($K_m$, 800 μM). The general amino acid permease transports all amino acids and shows a somewhat higher affinity for α-aminoadipate than PcDip5 ($K_m$, 230 μM). Both transporters transport α-aminoadipate only in the L-configuration. α-Aminoadipate transport via PcGap1 is strongly inhibited (approx. 95%) by a ten-fold excess of leucine, whereas PcDip5 mediated transport is barely affected by the presence of leucine. This phenomenon was used to discriminate between PcGap1-and PcDip5-mediated α-aminoadipate uptake in high penicillin yielding *P. chrysogenum* strains. When α-aminoadipate is added at 25 μM to penicillin producing mycelium, PcGap1 and PcDip5 contribute almost equally to the uptake of α-aminoadipate.

The activity of amino acid transporters in fungi is regulated at the transcriptional and post-transcriptional level. In this work, the expression of the *PcGAP1* and *PcDIP5* genes in *P. chrysogenum* growing in media with different nitrogen and carbon sources was tested by semi-quantitative RT-PCR. Expression of *PcGAP1* was low when ammonia was present, while expression was high when glutamate or urea was used as sole nitrogen source. *PcDIP5* showed a similar expression pattern, although *PcGAP1*, and not *PcDIP5*, was moderately expressed when serine, a substrate of PcGap1 and not of PcDip5, was the sole nitrogen source. In penicillin producing medium which contains urea and glutamate as nitrogen source and lactose as carbon source, expression of *PcGAP1* and *PcDIP5* was at an intermediate level. So, both *PcGAP1* and *PcDIP5* seem to be under nitrogen catabolite repression (NCR) and are expressed when cells are grown in the presence of urea (a poor nitrogen source) or glutamate as sole nitrogen source (this thesis).

**AAP family permeases and Mtr homologs.** With the availability of an increasing number of completed fungal genome sequences (most of them ascomycetes), the abundance of AAPs and Mtr homologs in fungi, could be investigated. Usually, a distinct group of AAPs was found in each fungus, varying in number from 8 in *U. maydis* to approximately 20 in *A. nidulans*. In an evolutionary

tree, AAPs appear to cluster in groups specific for basic amino acids, acidic amino acids, and proline. This family also contains less well defined groups of general amino acid permeases and permeases specific for neutral amino acids (this thesis). In most filamentous fungi, one clear, distinct Mtr homolog (with more than 50% amino acid identity) is present, with the exception of *Fusarium graminearum*, which has 5 Mtr homologs (this thesis). The physiological role of these Mtr homologs remains to be elucidated. However, rather than play a general role in amino acid uptake, these systems may fulfill a function in the uptake of signaling molecules during differentiation. It will be of interest to test this hypothesis by the selective deletion of Mtr transporters in fungi. On the other hand, a *N. crassa* mutant in which the *mtr* gene was inactivated showed a strongly reduced uptake level of neutral amino acids when the cells were grown on minimal medium with ammonia as nitrogen source (190). This would suggest that Mtr fulfills a role in amino acid uptake, although a role in the uptake of signaling molecules under more physiological conditions cannot be excluded.

**Random mutagenesis of PcDip5.** The AAP family of transporters has not been studied in detail. Therefore, little is known about the structure/function relationships in AAPs nor is it clear which residues contribute to the binding sites to determine substrate selectivity. Alignments of AAPs show the presence of strongly conserved regions and residues, while some regions seem to be specifically conserved in AAPs with similar substrate specificity as for instance for the arginine (160) and a proline permease (201). However, none of these residues seem to be directly involved in substrate selectivity or binding. We have undertaken an attempt to use random mutagenesis and selection to improve the affinity or capacity of PcDip5 for α-aminoadipate transport. A special constructed strain of *S. cerevisiae*, able to utilize α-aminoadipate as primary nitrogen source but lacking the transporter to take it up from the medium, was transformed with a mutant library of *PcDIP5* and over 100,000 transformants were spread on plates in which a low concentration of α-aminoadipate was limiting for growth. Colonies of larger size were further analyzed, but none of the clones showed an increased α-aminoadipate transport. Apparently, this selection procedure which makes use of colony size is insufficiently reliable to perform such a screening. As more AAP family members are being characterized, amino acids positions that are conserved specifically among a group of transporters with similar

substrate specificity may lead to a better definition of possible amino acid targets which can be modified by site-directed mutagenesis approach. This may lead to the identification of residues that are directly involved in substrate binding and selectivity.

**Biotechnological application of the α-aminoadipate transporter.** In the last decade, the study on the structure and functioning of non-ribosomal peptide synthetases, such as ACV synthetase, has made major progress (24, 108). In different fungi, a number of non-ribosomal peptide synthetases are found some of which are involved in the formation of a special class of peptides named peptaibols (31). These are helical peptides varying in length from 5 to 20 amino acids of which generally a large proportion is made up by α-aminoisobutyric acid residues (Aib), from which the name pept**aib**ol was derived. These peptides were found to have different interesting effects - often antibiotic activity against bacteria and fungi, but also neuroleptic effects in mice (169) – probably due to their membrane disturbing effect by the formation of oligomeric ion-channel assemblies (31). Non-ribosomal peptide synthetases consist of a series of linked modules, in which each module is responsible for the recognition, activation and incorporation of one residues into the peptide chain (161, 229). A potential application of these peptide synthetases is to recombining modules by genetic engineering, thereby creating new peptides with novel properties. The possibility of recombining the modules has been investigated in the European project Eurofung (for designing and improving health- and food-related production processes using filamentous fungal cell factories) using ACV synthetase of *Penicillium chrysogenum* as a model system. The replacement of the α-aminoadipate specific module by a new module, possibly originating from another organism and specific for a different (natural or unnatural) amino acid, could theoretically generate new tripeptides. In order to introduce unnatural amino acids, efficient production of such peptides would require exogenous availability of such compounds. By means of active uptake a sufficiently high intracellular concentration may be obtained in order to obtain an effective peptide synthesis. In most cases, the substrate will not passively diffuses across the cytoplasmic membrane, and because of its broad substrate specificity, engineered variants of the general amino acid permease may provide a mechanism to allow entry of such compounds into the cell.

One substrate suggested for a recombinant ACV synthetase is pipecolic acid. It is a six-carbon cyclic imino acid and serves as a substrate for some non-ribosomal peptide and polyketide synthetases in for example *Streptomyces hygroscopicus* (144). The pipecolic acid binding and activating unit that is involved in the synthesis of the immunosuppressant immunomycin in *S. hygroscopicus* may replace the α-aminoadipate specific module of ACV synthetase in *P. chrysogenum*. By this strategy, a new synthetase may be generated that catalyzes the formation of a pipecolyl-cysteinyl-valine tripeptide. For this purpose, pipecolic should be present in the cytosol at a sufficiently high concentration. In *P. chrysogenum*, pipecolic acid is derived from the lysine biosynthesis pathway. It can be formed from α-aminoadipate in three consecutive steps (137, 138). In a saccharopine reductase knock-out mutant strain ($SR^-$), lysine biosynthesis is abolished and pipecolic acid accumulates intracellularly. Through the external supply of α-aminoadipate, the production of pipecolic acid in this strain could be further increased (138). Feeding may be more effective when an α-aminoadipate transporter like PcDip5 or PcGap1 is overexpressed. For this purpose, the *PcDIP5* and *PcGAP1* genes were cloned behind the strong pcbC promoter. These constructs were transformed into a wild-type, low penicillin producing *P. chrysogenum* strain, and in the case of PcDip5, a moderate increase in uptake of α-aminoadipate was observed (H. Trip, unpublished data). The effect on pipecolic acid formation in a saccharopine reductase knock-out strain is under investigation by the group of Prof. Martín (Léon, Spain). In this respect, an increased production of pipecolic acid is also of interest because it is a precursor for the indolizidine alkaloid swansonine, produced by *Metarhizium anisopliae*. This compound exhibits anti-tumor activity (42, 84) motivating the development of efficient production strategies.

**Concluding remarks**

Amino acid transporters from *P. chrysogenum* can be functionally expressed and characterized with respect to substrate specificity and kinetics in *S. cerevisiae*. The uptake of α-aminoadipate in *P. chrysogenum* via PcDip5 and PcGap1 could be assayed and their individual contribution to α-aminoadipate uptake in high yielding *P. chrysogenum* strains was determined using selective inhibition of PcGap1 by leucine. The exact role of these transporters during penicillin production, however, remains to

be established. As α-aminoadipate is derived from the lysine biosynthesis pathway, the transporters may play a role in the maintenance of a high intracellular concentration of α-aminoadipate, i.e., as to prevent cellular leakage of this compound. However, to address this question, a single and double, *PcDIP5*/*PcGAP1* knock-out mutant of *P. chrysogenum* needs to be constructed. Although this was attempted, the technical difficulty and low efficiency of homologous recombination in P. *chrysogenum* currently hampers such a genetic analysis.

# Chapter 7

**Nederlandse samenvatting**

Penicilline en daarvan afgeleide verbindingen zijn nog steeds de meest gebruikte antibiotica op de wereld: tezamen maken ze meer dan de helft uit van de totale wereldmarkt van antibiotica. De ontdekking van penicilline staat op naam van Alexander Fleming, een professor in de bacteriologie in het St. Mary's ziekenhuis in Londen. Hij verrichtte ondermeer onderzoek aan de ziekteverwekkende bacterie *Staphylococcus aureus*. In 1928, teruggekomen op zijn laboratorium na een vakantie, trof hij een schimmelinfectie aan op een van de voedingsbodems waarop hij *Staphylococcus aureus* kweekte. Hij zag dat in de buurt van de schimmel geen bacteriën groeiden en realiseerde dat dit geen mislukt experiment was, maar een interessant fenomeen. De schimmel werd geïsoleerd en bleek een stof uit te scheiden die verantwoordelijk was voor het bacteriedodende effect. Omdat de schimmel een ondersoort van de klasse van *Penicillium* was, noemde Fleming de werkzame stof penicilline. In de eerste tien jaar na de publicatie werd weinig aandacht geschonken aan deze ontdekking, totdat in 1938 de professoren Howard Florey and Ernst Chain in Oxford de therapeutische effecten van penicilline op muizen onderzochten en ook een methode beschreven hoe penicilline geproduceerd kon worden. Na hun publicatie in 1940 werd het belang van penicilline onderkend en er werd besloten dat de studies zich zo snel mogelijk moesten richten op het gebruik van deze verbinding voor mensen. Dit gebeurde voor het eerst succesvol in 1941. Tegelijkertijd raakte de farmaceutische industrie in de Verenigde Staten geïnteresseerd in de productie van penicilline op grotere schaal. Vanwege de grote successen tijdens de eerste klinische toepassingen, werd de farmaceutische industrie in 1943 door het "War Production Board" in de VS aangemoedigd om de productie van penicilline op te voeren. Er werd een *Penicillium* soort gevonden, *Penicillium chrysogenum*, die meer penicilline produceerde dan Flemings *Penicillium notatum*. Penicilline kon op grote schaal geproduceerd en gezuiverd worden en aan mensen met een bacteriële infectie worden toegediend. Nog voor het eind van de Tweede Wereldoorlog lag de productiecapaciteit hoog genoeg voor de behandeling van 100.000 patiënten per jaar. In december 1945 kregen Fleming, Florey en Chain de Nobelprijs voor hun werk.

Na 1945 begonnen verschillende bedrijven met de productie van penicilline en werden pogingen ondernomen om de productie te verhogen door *Penicillium chrysogenum* te verbeteren. De huidige stammen produceren meer dan 10.000 keer zoveel penicilline als de oorspronkelijke *Penicillium notatum* stam die Fleming op de voedingsbodem aantrof. In de eerste decennia verliep stamverbetering door middel van klassieke methode van mutatie en selectie. Dit wil zeggen: het lukraak veroorzaken van mutaties ("willekeurige mutagenese") in het DNA, het genetische materiaal, door gebruik te maken van ultraviolette bestraling of giftige verbindingen zoals mosterdgas om vervolgens nakomelingen te selecteren die een verhoogde penicilline productie vertoonden. Deze methode werkte in het begin erg krachtig, maar begon tegen de jaren negentig een verzadigingsniveau te bereiken. Men besefte dat een rationele en meer gerichte benadering nodig was, waarbij het DNA niet willekeurig, maar veel specifieker veranderd zou moeten worden. Dit werd mogelijk gemaakt met de opkomst van de "recombinante DNA technologie". Sinds midden jaren 70 was het mogelijk om op gerichte wijze veranderingen aan te brengen in het DNA van organismen. Om deze vorm van genetische modificatie te gebruiken voor stamverbetering van *Penicillium chrysogenum* was kennis nodig van de processen die in de schimmel de penicilline synthese bewerkstelligen en moesten de genen die daarbij betrokken zijn worden geïdentificeerd. Omdat deze processen echter niet op zichzelf staan, maar in meer of mindere mate geïntegreerd zijn in het functioneren van de schimmel als geheel, is ook kennis daarvan op het kleinste, moleculaire niveau van belang. Dat wil zeggen, hoe leeft de schimmel, hoe groeit het, hoe vermenigvuldigt het zich, hoe reageert het op zijn leefmilieu, wat eet het, hoe neemt het zijn voedingsstoffen tot zich, enzovoort. Wat beschreven staat in dit proefschrift maakt deel uit van dit onderzoek en gaat over de vraag hoe *Penicillium chrysogenum* een bepaalde groep van bouwstenen en voedingstoffen opneemt uit zijn omgeving, namelijk aminozuren. Voor daar dieper op in te gaan, eerst wat algemenere informatie.

Schimmels vormen een aparte groep organismen in de levende wereld die is opgedeeld in drie domeinen: bacteriën, archaea en eukaryoten. Bacteriën en archaea bestaan uit één cel en hebben geen celkern. Eukaryoten kunnen één- of meercellig zijn, maar worden gekenmerkt door de aanwezigheid van een celkern en andere organellen (door een membraan omgeven structuren die speciale functies vervullen) binnen de cel. De eukaryoten zijn ook weer opgedeeld in een aantal rijken zoals

dieren, planten en ook schimmels. Planten halen energie uit zonlicht, terwijl de overige eukaryoten dat moeten halen uit voedingsstoffen die opgenomen moeten worden uit de omgeving. Veel schimmels zijn meercellig, maar toch relatief simpel van opbouw in vergelijking met dieren en planten. Om die reden vallen schimmels onder de noemer "micro-organismen". Er zijn ééncellige schimmels, de gisten (waaronder bakkersgist en biergist) en de meercellige, filamenteuze schimmels. Het woord filamenteus slaat op het feit dat ze ketens van cellen vormen die zichtbaar zijn als witte draden, hyfen, die samen het mycelium genoemd worden. Daarnaast vormen ze speciale structuren bedoeld voor de verspreiding van sporen (te vergelijken met de zaden van planten). Bij bepaalde ondersoorten zijn deze structuren beter bekend als paddestoelen. Bij andere soorten zijn de structuren minder groot, zoals conidia: hyfen die vanuit het oppervlak de lucht in groeien en waarbij aan het uiteinde sporen gevormd worden. Dit is soms zichtbaar als een groene of zwarte kleur op bedorven voedsel. *Penicillium chrysogenum* is een filamenteuze schimmel en produceert het antibioticum penicilline. De reden dat sommige organismen (niet alleen schimmels) antibiotica uitscheiden is waarschijnlijk om mogelijke concurrenten in de omgeving te doden en daardoor de aanwezige voedingstoffen voor zichzelf te kunnen houden. De opname van voedingsstoffen van buiten naar binnen de cel gebeurt door speciale eiwitten die aanwezig zijn in de celmembraan. Alle cellen in alle organismen worden omgeven door een membraan die een aantal essentiële functies heeft:

- Het vormen van een flexibele barrière tussen het binnenste van de cel en de buitenomgeving. Deze is ondoorlaatbaar voor de meeste stoffen.
- De membraan bevat eiwitten die belangrijke functies vervullen, zoals het transporteren van voedingsstoffen van buiten naar binnen de cel en de uitscheiding van afvalproducten.
- Het in stand houden van een elektrische potentiaal verschil (spanning), en een concentratie verschil van ionen en moleculen aan weerszijden van de membraan. Dit wordt samen de electrochemische potentiaal genoemd en vormt de drijvende kracht voor energie-vragende processen zoals het hierboven genoemde transport.

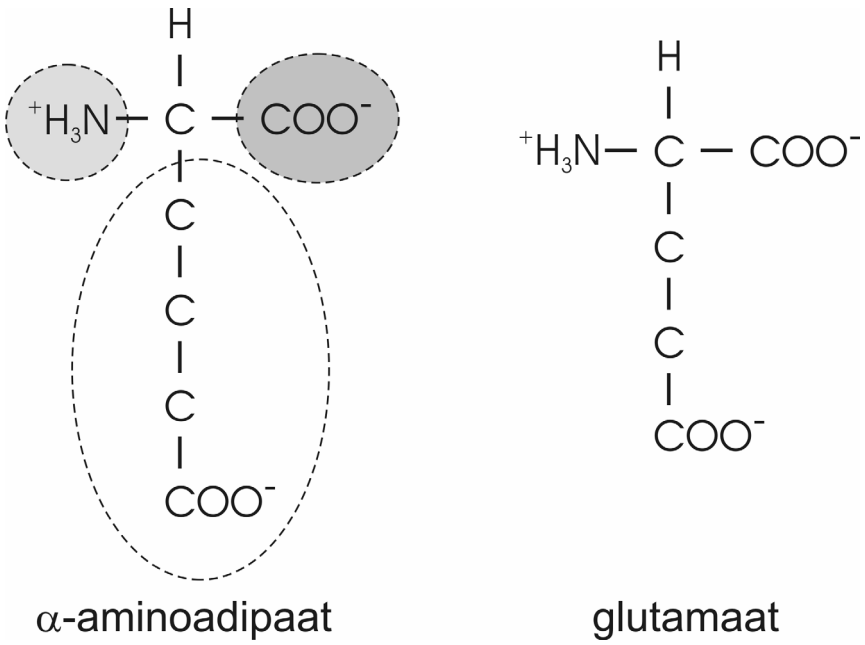
De membraan wordt gevormd door een dubbele laag vetachtige moleculen (lipiden). In deze laag zitten eiwitten die nodig zijn voor het transport van stoffen van buiten

naar binnen of andersom, voor de communicatie van cellen met de omgeving, voor de verankering van uitwendige structuren aan de membraan, en andere functies. Voor het transport van kleine moleculen door de membraan zijn er ruwweg drie mechanismen te onderscheiden:

1. gefaciliteerde diffusie
2. primair transport
3. secundair transport

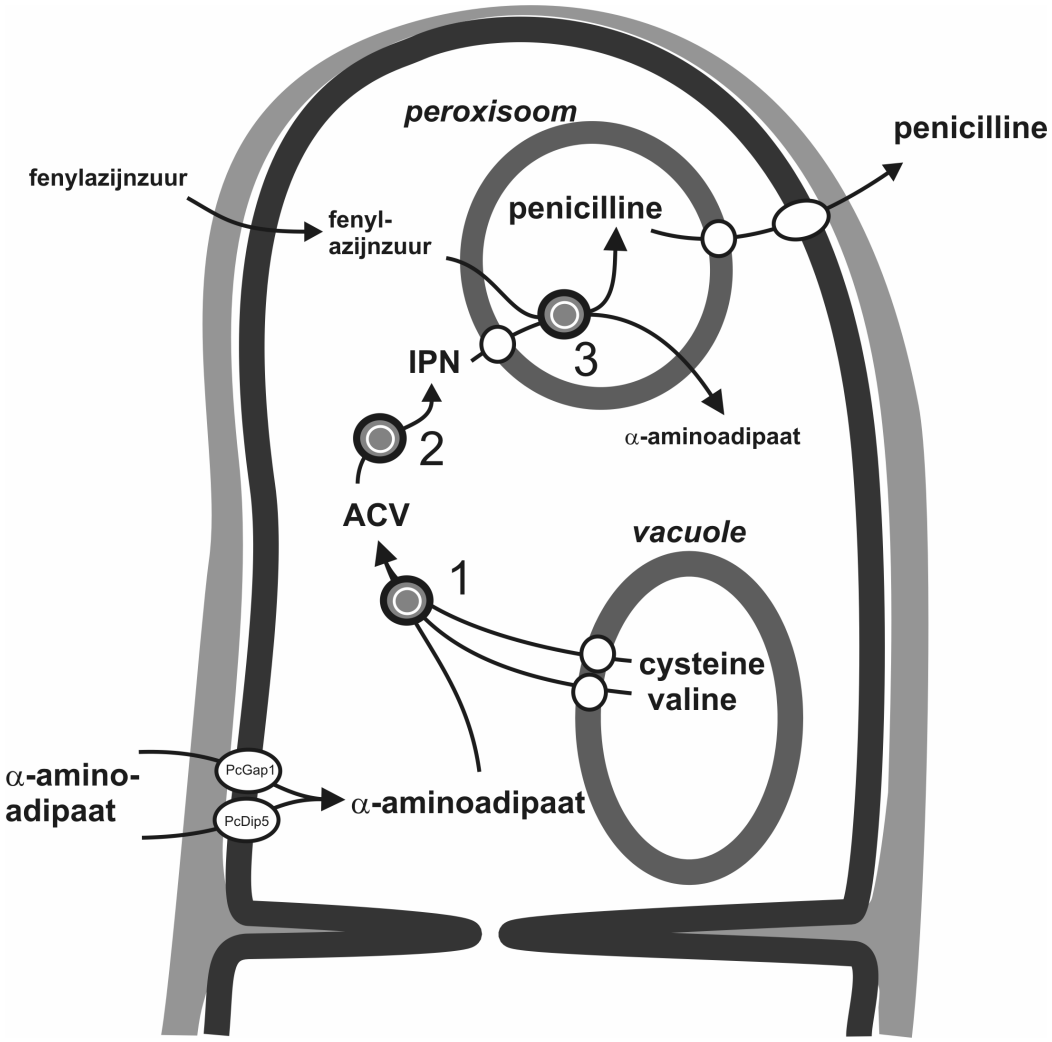
Deze drie mechanismen onderscheiden zich van elkaar door de manier waarop het transport aangedreven wordt. Wanneer een verbinding opgenomen wordt waarvan de concentratie in de cel hoger is dan erbuiten, dan kost dit energie. Bij secundair transport wordt deze energie geleverd door de gelijktijdige verplaatsing van (meestal) een ion (een geladen deeltje) door de membraan via hetzelfde transporteiwit. Hierdoor kunnen stoffen opgenomen worden waarvan de concentratie in de cel al vele malen hoger is dan buiten de cel. De opname van aminozuren uit de omgeving gebeurt in schimmels via secundair transport. De drijvende kracht wordt geleverd door de electrochemische potentiaal van protonen over de membraan. Protonen zijn positief geladen waterstofdeeltjes ($H^+$) en hebben buiten de cel een hogere concentratie dan binnen de cel. Daarnaast is de electrische potentiaal buiten de cel positief ten opzichte van binnen de cel. Hierdoor werkt er een kracht op de protonen die aan hen trekt (de proton motive force) . De opname van een aminozuur molecuul kost energie. Door beide processen te koppelen, dat wil zeggen de naar binnen gerichte beweging van het proton en het de opname van het aminozuur, is het mogelijk om aminozuren efficiënt op te nemen uit de buitenomgeving.

Aminozuren zijn moleculen die gekenmerkt worden door een zogenaamde aminogroep (amino) en carboxylgroep (zuur) (Fig. 1). In eiwitten, die bestaan uit ketens van aminozuurmoleculen, komen 20 verschillende aminozuren voor en daarnaast bestaan er nog een paar andere die relatief weinig voorkomen. Aminozuren bevatten zowel koolstofatomen als stikstofatomen. Beide elementen zijn nodig in de cel en aminozuren kunnen dus als “koolstofbron” en “stikstofbron” dienen. Daarnaast kan er door verbranding of “catabolisme” energie uit aminozuren gehaald worden. De uit de omgeving opgenomen aminozuren kunnen ook direct gebruikt worden voor de synthese van eiwitten. Er zijn ook andere moleculen in de cel waarvoor aminozuren




**Figuur 1.** Schematische weergave van twee aminozuurmoleculen: α-aminoadipaat en glutamaat. Atomen zijn als letters weergegeven. C = koolstof, O = zuurstof, N = stikstof en H = waterstof. De aminogroep is aangegeven in de lichtgrijze cirkel, de zure carboxylgroep is aangegeven met de donkergrijze ellips. De zijketen van α-aminoadipaat is in aangegeven in de grote ellips. De zijketen verschilt voor elk aminozuur.

gebruikt worden. Zo ook het penicilline molecuul. De synthese van penicilline in de cel gebeurt in drie stappen (Fig. 2). Eerst worden drie aminozuren aan elkaar gekoppeld: α-aminoadipaat, cysteïne en valine. Het product is een tripeptide genaamd ACV, een afkorting van L-α-**a**minoadipyl-L-**c**ysteïnyl-D-**v**aline. In de tweede stap wordt er een ringvormige structuur gevormd uit cysteïne en valine waardoor de zogenaamde β-lactam structuur. Dit is een karakteristieke structuur voor penicilline en de daarvan afgeleide antibiotica. Als laatste stap wordt de α-aminoadipaat groep vervangen door een fenylazijnzuur molecuul (niet een aminozuur) waarbij gebruik gemaakt wordt van een chemische geactiveerde vorm van het fenylazijnzuur. Hierbij wordt het uiteindelijke molecuul penicilline gevormd (penicilline G). Dit wordt over de celmembraan getransporteerd en uitgescheiden in de omgeving.




**Figuur 2.** Schematische weergave van een cel aan het uiteinde van een hyfe van *Penicillium chrysogenum* met daarin aangegeven de drie stappen van de synthese van penicilline. 1. De koppeling van drie aminozuren α-aminoadipaat, cysteïne en valine tot het tripeptide ACV. 2. De omzetting van ACV in isopenicilline N (IPN). 3. De uitwisseling van α-aminoadipaat met fenylazijnzuur, waarbij het eindproduct penicilline gevormd wordt. De lichtgrijze buitenste laag om de cel geeft de celwand weer die de cel stevigheid geeft, maar doorlaatbaar is voor de meeste stoffen. De binnenste laag geeft de celmembraan weer, met daarin de twee aminozuurtransporters PcGap1 en PcDip5. Het peroxisoom en de vacuole (organellen) zijn ook omgeven door een membraan die ook transporteiwitten bevat. De eiwitten die betrokken zijn bij transport van penicilline en zijn precursors (voorlopers) zijn hier weergegeven als cirkels of ellipsen, maar zijn nog niet geïdentificeerd.

De snelheid waarmee penicilline wordt geproduceerd wordt mede bepaald door een aantal beperkende factoren ofwel "bottlenecks". Eén beperkende factor is de concentratie van α-aminoadipaat in de cel. α-Aminoadipaat behoort niet tot de groep van 20 natuurlijke aminozuren maar het is een tussenproduct in de route naar de synthese van het basische aminozuur lysine. Indien de productie van α-aminoadipaat in de cel verhoogd wordt, gaat de snelheid van penicilline productie omhoog. Dit is ook te bewerkstelligen door α-aminoadipaat van buiten toe te voegen waardoor de concentratie in de cel ook toeneemt. Dit betekent dat deze verbinding de cellen binnen gaat, waarschijnlijk via één of meerdere transporteiwitten. Het onderzoek beschreven in dit proefschrift heeft zich er op gericht om de eiwitten in de membraan te identificeren die betrokken zijn bij het transport van α-aminoadipaat. Vragen die daarbij zijn gesteld zijn: Hoe vindt dit transport plaats? En zijn deze transporter(s) belangrijk voor het in standhouden van een hoge concentratie van α-aminoadipaat in de cel? Het is namelijk eerder aangetoond dat de aanwezigheid van transporteiwitten soms nodig is om bepaalde moleculen die uit de cel gelekt zijn weer terug de cel in te transporteren, zodat de concentratie in de cel op peil blijft. Om het α-aminoadipaat transport te beschrijven is getracht de genen op te sporen die coderen voor de desbetreffende transporteiwitten.

Eiwitten bestaan uit lange ketens van aminozuren die ruimtelijk op een bepaalde manier opgevouwen zijn. Ze worden gecodeerd door genen, dat wil zeggen, een gen bevat de informatie hoe de volgorde van de aminozuren in een eiwit moet zijn. Genen bestaan uit DNA, het erfelijke materiaal dat in elke cel zit en doorgegeven wordt van generatie op generatie. DNA bestaat uit lange ketens van vier verschillende nucleïnezuren, waarvan de volgorde bepalend is voor de uiteindelijke aminozuurvolgorde van het eiwitmolecuul. Het DNA van de schimmel *Penicillium chrysogenum* bevat ongeveer 10.000 genen, het DNA van een mens bevat tussen de 30.000 en 40.000. In bakkersgist (*Saccharomyces cerevisiae*), een ééncellige schimmel waarvan al veel bekend is, is het aminozuur transport veel beter bestudeerd dan in *Penicillium chrysogenum*. Er zijn ongeveer 22 genen geïdentificeerd die coderen voor aminozuurtransporteiwitten. Aminozuren zijn in te delen in een aantal groepen op basis van hun karakteristieken, zoals hydrofobe (= waterafstotende) aminozuren (bijvoorbeeld leucine); neutrale aminozuren (bijvoorbeeld alanine); zure aminozuren (bijvoorbeeld glutamaat); basische aminozuren (bijvoorbeeld lysine) en

aromatische aminozuren (bijvoorbeeld tyrosine). Er is aangetoond dat er transporteiwitten zijn die één of een beperkt aantal verschillende aminozuren kunnen transporteren. Er zijn echter ook transporteiwitten die een groot aantal verschillende aminozuren kunnen transporteren en deze beschikken dus over een brede specificiteit. Er is één algemene aminozuurtransporter die alle aminozuren kan transporteren, genaamd Gap1 (**g**eneral **a**mino acid **p**ermease, permease is een andere benaming voor een transporteiwit). Hoeveel aminozuurtransporters er aanwezig zijn in *P. chrysogenum* is niet bekend, maar studies in het verleden toonden aan dat er waarschijnlijk net als in *S. cerevisiae*, één algemene aminozuur permease of Gap is, en een aantal meer specifieke transporters, waaronder één die in staat is om enkel de zure aminozuren glutamaat en aspartaat te transporteren. Er zijn echter nooit genen van aminozuurtransporteiwitten gekloneerd en geïdentificeerd in *P. chrysogenum*. α-Aminoadipaat is een zuur aminozuur dat qua structuur sterke gelijkenis vertoond met glutamaat. De hypothese in dit werk was daarom dat α-aminoadipaat door hetzelfde transportsysteem getransporteerd zou kunnen worden dat verantwoordelijk is voor de opname van glutamaat. Er zijn twee transportsystemen die over deze eigenschap kunnen beschikken, namelijk het zure aminozuurtransportsysteem en het algemene aminozuurtransportsysteem. *S. cerevisiae* en *P. chrysogenum* zijn beide schimmels in de klasse van ascomyceten en die relatie is terug te zien in de overeenkomsten tussen de genen van beide schimmels die coderen voor vergelijkbare functies. Die overeenkomst is gebruikt om de genen te isoleren uit *P. chrysogenum* die coderen voor het zure en algemene aminozuurtransportsysteem.

Het eerste hoofdstuk van het proefschrift is een algemene introductie over de penicilline biosynthese in *Penicillium chrysogenum* met de nadruk op de onderverdeling van de biosynthese processen in verschillende celcompartimenten en het belang van transportprocessen. De verschillende processen betrokken bij penicilline biosynthese vinden plaats in verschillende compartimenten (Figuur 2). Deze compartimenten zijn: 1. het cytoplasma. Dit is de vloeistof in de cel waarin veel reacties en andere processen plaatsvinden. Hiertoe behoren ook de eerste twee stappen van de penicilline biosynthese. 2. Het peroxisoom (of feitelijk meerdere peroxisomen). Dit is een organel dat omgeven is door een membraan waarbinnen een aantal specifieke reacties plaatsvinden, waaronder de laatste stap van de penicilline biosynthese. 3. De vacuole. Dit is een organel omgeven door een membraan, waarbinnen overbodige eiwitten afgebroken worden en waarin bepaalde aminozuren

opgeslagen worden, waaronder cysteïne en valine en mogelijk ook α-aminoadipaat, één van de bouwstenen die gebruikt worden bij de synthese van het penicilline molecuul. Deze zogenaamde compartimentalisatie betekent dat het penicilline molecuul of de precursors door een membraan van het ene naar het andere compartiment getransporteerd moeten worden. Deze transportprocessen, de excretie van penicilline over de celmembraan naar de omgeving en de opname van voeding- en bouwstoffen uit de omgeving door de celmembraan naar binnen staan beschreven in hoofdstuk 1.

In hoofdstuk 2 staat beschreven hoe in de zoektocht naar het transportsysteem voor zure aminozuren, een ander gen, *ArlP* genoemd, gekloneerd en gekarakteriseerd is. *ArlP* codeert voor een transporteiwit dat aromatische aminozuren en ook leucine de cel in transporteert. Het gen is geïsoleerd uit *P. chrysogenum* en overgebracht (getransformeerd) naar een bepaalde specifieke stam van *Saccharomyces cerevisiae* (M4276) waarin twee aminozuur-transportsystemen ontbreken. Vaak kunnen genen van een organisme overgebracht worden naar een ander organisme en kan het daarin dezelfde functie uitoefenen. Dat kan nuttig zijn wanneer zo'n eigenschap of functie makkelijker bestudeerd kan worden dan in het oorspronkelijke organisme. Dat is hier ook het geval: aminozuurtransport kan makkelijker in de gist *S. cerevisiae* dan in *P. chrysogenum* bestudeerd worden. De giststam M4276 mist twee transportergenen, namelijk één die codeert voor de algemene aminozuurtransporter (*GAP1*) en één die codeert voor het zure aminozuurtransportsysteem (*DIP5*). Door deze genetische veranderingen is deze stam niet in staat om glutamaat, aspartaat en α-aminoadipaat op te nemen. Ook de opname van de meeste andere aminozuren is sterk verlaagd door het ontbreken van Gap1. Door *ArlP* uit *P. chrysogenum* tot expressie te brengen, dat wil zeggen, het gen vertaald te laten worden in een functioneel transporteiwit in *S. cerevisiae* M4276, kan de opname van aminozuren die door het desbetreffende eiwit getransporteerd kunnen worden, sterk omhoog gaan. Die opname kan gemeten worden met behulp van radioactief gemerkte aminozuren. In dit geval bleek de opname van aromatische aminozuren en leucine sterk verhoogd, maar niet dat van glutamaat, aspartaat of α-aminoadipaat.

In het derde hoofdstuk staat beschreven hoe de genen voor het zure aminozuurtransportsysteem (*PcDIP5*) en voor het algemene aminozuurtransportsysteem (*PcGAP1*) van *P. chrysogenum* gekloneerd zijn. Beide zijn bestudeerd door middel van expressie in *S. cerevisiae* M4276 en testen van

opname van aminozuren. PcDip5 blijkt zeer goed glutamaat en aspartaat te kunnen transporteren. Ook α-aminoadipaat wordt getransporteerd, echter de opname van deze verbinding treedt op met een snelheid die ongeveer tien keer zo langzaam is dan dat van glutamaat. Ook PcGap1 transporteert α-aminoadipaat, en daarnaast ook alle andere aminozuren. Vervolgens is gekeken in welke mate beide transporteiwitten bijdragen aan de opname van α-aminoadipaat in cellen van *P. chrysogenum* die zijn gekweekt onder omstandigheden waarbij penicilline geproduceerd wordt. Beide blijken een ongeveer even groot aandeel te hebben in α-aminoadipaat opname.

In het vierde hoofdstuk staat een aminozuurtransportsysteem beschreven waarvan het gen min of meer toevalligerwijs gekloneerd is. Dit transportsysteem genaamd PcMtr transporteert aromatische en neutrale aminozuren, maar het is vooral interessant omdat het qua structuur niet lijkt op de typische aminozuurtransportsystemen uit schimmels. Deze vormen één familie bestaande uit nauw verwante transporteiwitten. PcMtr valt daarbuiten en is meer verwant met een soortgelijk transportsysteem uit een andere schimmel, namelijk het Mtr eiwit van *Neurospora crassa*. Net zoals de genetische code van het totale DNA van de mens sinds een paar jaar bekend is, is dit ook uitgezocht voor een aantal schimmels. Die van *Penicillium chrysogenum* is daarentegen nog niet bekend. Aan de hand van de DNA databases kon afgeleid worden dat de meeste schimmels beschikken over een groot aantal (8 – 20) van de typische aminozuurtransportsystemen maar daarnaast beschikken ze vaak ook over één exemplaar van een aminozuurtransportsysteem dat behoort tot de Mtr familie. Deze eiwitten spelen vermoedelijk een heel specifieke rol in de cel, bijvoorbeeld bij de opname van signaalmoleculen. Echter is het is niet duidelijk waarom deze groep eiwitten zo wijdverbreid voorkomt in schimmel en wat hun exacte functie is.

Het vijfde hoofdstuk beschrijft een poging om het zure aminozuurtransportsysteem van *P. chrysogenum*, PcDip5, zodanig te veranderen dat het beter in staat is om α-aminoadipaat te transporteren. Er zijn daarom "willekeurig" mutaties aangebracht in het gen, waardoor het eiwit veranderde eigenschappen zou kunnen krijgen. In verreweg de meeste gevallen zijn zulke mutaties negatief voor de functie van het eiwit, maar soms kan er een subtiele verandering plaats vinden die een gewenst effect heeft. Uiteindelijk is dit niet gevonden in het geval van α-

aminoadipaat transport wat mogelijk samenhangt met het selectiesysteem dat is toegepast om dergelijke verbeterde transporteiwitten te vinden.

Samenvattend kan worden gezegd dat met het hier gepresenteerde werk een bijdrage is geleverd aan het in kaart brengen van de aminozuurtransportsystemen van *Penicillium chrysogenum*. Het aantal genen coderend voor aminozuurtransportsysteem in verschillende schimmels is vastgesteld met behulp van hun complete genetische codes. Meer specifiek is aangetoond welke transporteiwitten betrokken zijn bij de opname van het aminozuur α-aminoadipaat, een bouwsteen die gebruikt wordt tijdens de biosynthese van penicilline in *P. chrysogenum*.

# References


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

2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman**. 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403-410.

3. **Alvarez, E., J. M. Cantoral, J. L. Barredo, B. Diez, and J. F. Martín**. 1987. Purification to homogeneity and characterization of acyl coenzyme A:6-aminopenicillanic acid acyltransferase of Penicillium chrysogenum. Antimicrob. Agents Chemother. **31**:1675-1682.

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

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

6. **Andre, B.** 1995. An overview of membrane transport proteins in Saccharomyces cerevisiae. Yeast **11**:1575-1611.

7. **Aplin, R. T., J. E. Baldwin, S. C. Cole, J. D. Sutherland, and M. B. Tobin**. 1993. On the production of alpha, beta-heterodimeric acyl-coenzyme A: isopenicillin N-acyltransferase of Penicillium chrysogenum. Studies using a recombinant source. FEBS Lett. **319**:166-170.

8. **Aplin, R. T., J. E. Baldwin, P. L. Roach, C. V. Robinson, and C. J. Schofield**. 1993. Investigations into the post-translational modification and mechanism of isopenicillin N:acyl-CoA acyltransferase using electrospray mass spectrometry. Biochem. J. **294**:357-363.

9. **Arst, H. N., Jr. and D. J. Cove**. 1969. Methylammonium resistance in Aspergillus nidulans. J. Bacteriol. **98**:1284-1293.

10. **Banuelos, O., J. Casqueiro, S. Steidl, S. Gutierrez, A. Brakhage, and J. F. Martín**. 2002. Subcellular localization of the homocitrate synthase in Penicillium chrysogenum. Mol. Genet. Genomics **266**:711-719.

11. **Barredo, J. L., P. van Solingen, B. Diez, E. Alvarez, J. M. Cantoral, A. Kattevilder, E. B. Smaal, M. A. Groenen, A. E. Veenstra, and J. F. Martín**. 1989. Cloning and characterization of the acyl-coenzyme A: 6-

aminopenicillanic-acid-acyltransferase gene of Penicillium chrysogenum. Gene **83**:291-300.

12. **Beck, T., A. Schmidt, and M. N. Hall**. 1999. Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. J. Cell Biol. **146**:1227-1238.

13. **Benko, P. V., T. C. Wood, and I. H. Segel**. 1967. Specificity and regulation of methionine transport in filamentous fungi. Arch. Biochem. Biophys. **122**:783-804.

14. **Benko, P. V., T. C. Wood, and I. H. Segel**. 1969. Multiplicity and regulation of amino acid transport in *Penicillium chrysogenum*. Arch. Biochem. Biophys. **129**:498-508.

15. **Bernard, F. and B. Andre**. 2001. Genetic analysis of the signalling pathway activated by external amino acids in Saccharomyces cerevisiae. Mol. Microbiol. **41**:489-502.

16. **Bhattacharjee, J. K.** 1985. alpha-Aminoadipate pathway for the biosynthesis of lysine in lower eukaryotes. Crit Rev. Microbiol. **12**:131-151.

17. **Biswas, S., M. Roy, and A. Datta**. 2003. N-acetylglucosamine-inducible CaGAP1 encodes a general amino acid permease which co-ordinates external nitrogen source response and morphogenesis in Candida albicans. Microbiology **149**:2597-2608.

18. **Bovenberg, R. A. L., Driessen, A. J. M., Schuurs, T. A., Nieboer, M., van den Berg, M. A., Konings, W. N., and Westerlaken, I.** 2001. International Patent WO0132904

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

20. **Breitling, R., O. Sharif, M. L. Hartman, and S. K. Krisans**. 2002. Loss of compartmentalization causes misregulation of lysine biosynthesis in peroxisome-deficient yeast cells. Eukaryot. Cell **1**:978-986.

21. **Brown, C. E. and A. H. Romano**. 1969. Evidence against necessary phosphorylation during hexose transport in Aspergillus nidulans. J. Bacteriol. **100**:1198-1203.

22. **Burzlaff, N. I., P. J. Rutledge, I. J. Clifton, C. M. Hensgens, M. Pickford, R. M. Adlington, P. L. Roach, and J. E. Baldwin**. 1999. The reaction cycle of isopenicillin N synthase observed by X-ray diffraction. Nature **401**:721-724.

23. **Busch, S., H. B. Bode, A. A. Brakhage, and G. H. Braus**. 2003. Impact of the cross-pathway control on the regulation of lysine and penicillin biosynthesis in Aspergillus nidulans. Curr. Genet. **42**:209-219.

24. **Byford, M. F., J. E. Baldwin, C. Y. Shiau, and C. J. Schofield**. 1997. The Mechanism of ACV Synthetase. Chem. Rev. **97**:2631-2650.

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

26. **Casqueiro, J., S. Gutierrez, O. Banuelos, M. J. Hijarrubia, and J. F. Martín**. 1999. Gene targeting in Penicillium chrysogenum: disruption of the lys2 gene leads to penicillin overproduction. J. Bacteriol. **181**:1181-1188.

27. **Chang, Y. D. and R. C. Dickson**. 1988. Primary structure of the lactose permease gene from the yeast Kluyveromyces lactis. Presence of an unusual transcript structure. J. Biol. Chem. **263**:16696-16703.

28. **Chattoo, B. B., F. Sherman, D. A. Azubalis, T. A. Fjellstedt, D. Mehnert, and M. Ogur**. 1979. Selection of lys2 mutants of the yeast Saccharomyces cerevisiae by the utilization of α-aminoadipate. Genetics **93**:51-65.

29. **Chen, E. J. and C. A. Kaiser**. 2002. Amino acids regulate the intracellular trafficking of the general amino acid permease of Saccharomycescerevisiae. Proc. Natl. Acad. Sci. U. S. A **99**:14837-14842.

30. **Christensen, L. H., C. M. Henriksen, J. Nielsen, J. Villadsen, and M. Egel-Mitani**. 1995. Continuous cultivation of Penicillium chrysogenum. Growth on glucose and penicillin production. J. Biotechnol. **42**:95-107.

31. **Chugh, J. K. and B. A. Wallace**. 2001. Peptaibols: models for ion channels. Biochem. Soc. Trans. **29**:565-570.

32. **Cohen, G., A. Argaman, R. Schreiber, M. Mislovati, and Y. Aharonowitz**. 1994. The thioredoxin system of Penicillium chrysogenum and its possible role in penicillin biosynthesis. J. Bacteriol. **176**:973-984.

33. **Cooney, C. L. and F. Acevedo**. 1977. Theoretical conversion yields for penicillin synthesis. Biotechnol. Bioeng. **19**:1449-1462.

34. **Cooper R.D.G.** 1993. The enzymes involved in biosynthesis of pencillin and cephalosporin; their structure and function. Bioorg. Medic. Chem. **1**:1-17.

35. **Crawford, L., A. M. Stepan, P. C. McAda, J. A. Rambosek, M. J. Conder, V. A. Vinci, and C. D. Reeves**. 1995. Production of cephalosporin intermediates by feeding adipic acid to recombinant Penicillium chrysogenum strains expressing ring expansion activity. Biotechnology (N. Y. ) **13**:58-62.

36. **Cubero, B., D. Gomez, and C. Scazzocchio**. 2000. Metabolite repression and inducer exclusion in the proline utilization gene cluster of Aspergillus nidulans. J. Bacteriol. **182**:233-235.

37. **Dansen, T. B., K. W. Wirtz, R. J. Wanders, and E. H. Pap**. 2000. Peroxisomes in human fibroblasts have a basic pH. Nat. Cell Biol. **2**:51-53.

38. **De Boer, M., J. P. Bebelman, P. M. Goncalves, J. Maat, H. Van Heerikhuizen, and R. J. Planta**. 1998. Regulation of expression of the amino acid transporter gene BAP3 in Saccharomyces cerevisiae. Mol. Microbiol. **30**:603-613.

39. **De Lucas, J. R., S. Valenciano, A. I. Dominguez, G. Turner, and F. Laborda**. 1997. Characterization of oleate-nonutilizing mutants of Aspergillus nidulans isolated by the 3-amino-1,2,4-triazole positive selection method. Arch. Microbiol. **168**:504-512.

40. **DeBusk, R. M. and A. G. DeBusk**. 1980. Physiological and regulatory properties of the general amino acid transport system of Neurospora crassa. J. Bacteriol. **143**:188-197.

41. **Demain, A. L.** 1957. Inhibition of penicillin formation by lysine. Arch. Biochem. Biophys. **67**:244-246.

42. **Dennis, J. W.** 1986. Effects of swainsonine and polyinosinic:polycytidylic acid on murine tumor cell growth and metastasis. Cancer Res. **46**:5131-5136.

43. **Dhar, M. M. and A. W. Khan**. 1971. Formation of antibiotics. Nature **233**:182-184.

44. **Dickson, R. C. and K. Barr**. 1983. Characterization of lactose transport in Kluyveromyces lactis. J. Bacteriol. **154**:1245-1251.

45. **Didion, T., B. Regenberg, M. U. Jørgensen, M. C. Kielland-Brandt, and H. A. Andersen**. 1998. The permease homologue Ssy1p controls the expression of amino acid and peptide transporter genes in Saccharomyces cerevisiae. Mol. Microbiol. **27**:643-650.

46. **Dillon, D. and D. Stadler**. 1994. Spontaneous mutation at the mtr locus in neurospora: the molecular spectrum in wild-type and a mutator strain. Genetics **138**:61-74.

47. **Dobeli, H. and J. Nuesch**. 1980. Regulatory properties of O-acetyl-L-serine sulfhydrylase of Cephalosporium acremonium: evidence of an isoenzyme and its importance in cephalosporin C biosynthesis. Antimicrob. Agents Chemother. **18**:111-117.

48. **Drew, S. W. and A. L. Demain**. 1973. Methionine control of cephalosporin C formation. Biotechnol. Bioeng. **15**:743-754.

49. **Dubois, E. and M. Grenson**. 1979. Methylamine/ammonia uptake systems in saocharomyces cerevisiae: multiplicity and regulation. Mol. Gen. Genet. **175**:67-76.

50. **Eriksen, S. H., B. Jensen, I. Schneider, S. Kaasgaard, and J. Olsen**. 1995. Uptake of phenoxyacetic acid by Penicillium chrysogenum. Appl. Microbiol. Biotechnol. **42**:945-950.

51. **Eriksen, S. H., T. B. Soderblom, B. Jensen, and J. Olsen**. 1998. Uptake of phenylacetic acid by two strains of Penicillium chrysogenum. Biotechnol. Bioeng. **60**:310-316.

52. **Fawcett, P. and E. P. Abraham**. 1975. Delta-(alpha-aminoadipyl)cysteinylvaline synthetase. Methods Enzymol. **43:471-3.**:471-473.

53. **Fernandez, E., F. Lombo, C. Mendez, and J. A. Salas**. 1996. An ABC transporter is essential for resistance to the antitumor agent mithramycin in the producer Streptomyces argillaceus. Mol. Gen. Genet. **251**:692-698.

54. **Fernandez-Canon, J. M., A. Reglero, H. Martinez-Blanco, M. A. Ferrero, and J. M. Luengo**. 1989. Phenylacetic acid transport system in Penicillium chrysogenum Wis 54-1255: molecular specificity of its induction. J. Antibiot. (Tokyo) **42**:1410-1415.

55. **Fiermonte, G., L. Palmieri, S. Todisco, G. Agrimi, F. Palmieri, and J. E. Walker**. 2002. Identification of the mitochondrial glutamate transporter. Bacterial expression, reconstitution, functional characterization, and tissue distribution of two human isoforms. J. Biol. Chem. **277**:19289-19294.

56. **Forsberg, H. and P. O. Ljungdahl**. 2001. Sensors of extracellular nutrients in Saccharomyces cerevisiae. Curr. Genet. **40**:91-109.

57. **Foster, B. A., S. M. Thomas, J. A. Mahr, F. Renosto, H. C. Patel, and I. H. Segel**. 1994. Cloning and sequencing of ATP sulfurylase from Penicillium chrysogenum. Identification of a likely allosteric domain. J. Biol. Chem. **269**:19777-19786.

58. **Friedrich, C. G. and A. L. Demain**. 1977. Homocitrate synthase as the crucial site of the lysine effect on penicillin biosynthesis. J. Antibiot. (Tokyo) **30**:760-761.

59. **Friedrich, C. G. and A. L. Demain**. 1978. Uptake and metabolism of alpha-aminoadipic acid by Penicillium chrysogenum Wis 54-1255. Arch. Microbiol. **119**:43-47.

60. **Gaber, R. F., K. Ottow, H. A. Andersen, and M. C. Kielland-Brandt**. 2003. Constitutive and hyperresponsive signaling by mutant forms of Saccharomyces cerevisiae amino acid sensor Ssy1. Eukaryot. Cell **2**:922-929.

61. **Geraghty, M. T., D. Bassett, J. C. Morrell, G. J. Gatto, Jr., J. Bai, B. V. Geisbrecht, P. Hieter, and S. J. Gould**. 1999. Detecting patterns of protein distribution and gene expression in silico. Proc. Natl. Acad. Sci. U. S. A **96**:2937-2942.

62. **Gietz, D., A. St Jean, R. A. Woods, and R. H. Schiestl**. 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20**:1425.

63. **Gilstring, C. F. and P. O. Ljungdahl**. 2000. A method for determining the in vivo topology of yeast polytopic membrane proteins demonstrates that Gap1p fully integrates into the membrane independently of Shr3p. J. Biol. Chem. **275**:31488-31495.

64. **Giots, F., M. C. Donaton, and J. M. Thevelein**. 2003. Inorganic phosphate is sensed by specific phosphate carriers and acts in concert with glucose as a nutrient signal for activation of the protein kinase A pathway in the yeast Saccharomyces cerevisiae. Mol. Microbiol. **47**:1163-1181.

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

66. **Gouka, R. J., W. van Hartingsveldt, R. A. Bovenberg, C. M. van Zeijl, C. A. van den Hondel, and R. F. van Gorcom**. 1993. Development of a new transformant selection system for Penicillium chrysogenum: isolation and characterization of the P. chrysogenum acetyl-coenzyme A synthetase gene (facA) and its use as a homologous selection marker. Appl. Microbiol. Biotechnol. **38**:514-519.

67. **Grauslund, M., T. Didion, M. C. Kielland-Brandt, and H. A. Andersen**. 1995. BAP2, a gene encoding a permease for branched-chain amino acids in Saccharomyces cerevisiae. Biochim. Biophys. Acta **1269**:275-280.

68. **Grenson, M.** 1983. Inactivation-reactivation process and repression of permease formation regulate several ammonia-sensitive permeases in the yeast Saccharomyces cerevisiae. Eur. J. Biochem. **133**:135-139.

69. **Grenson, M., C. Hou, and M. Crabeel**. 1970. Multiplicity of the amino acid permeases in Saccharomyces cerevisiae. IV. Evidence for a general amino acid permease. J. Bacteriol. **103**:770-777.

70. **Hackette, S. L., G. E. Skye, C. Burton, and I. H. Segel**. 1970. Characterization of an ammonium transport system in filamentous fungi with methylammonium-14C as the substrate. J. Biol. Chem. **245**:4241-4250.

71. **Hahn, M., U. Neef, C. Struck, M. Gottfert, and K. Mendgen**. 1997. A putative amino acid transporter is specifically expressed in haustoria of the rust fungus Uromyces fabae. Mol. Plant Microbe Interact. **10**:438-445.

72. **Hein, C. and B. Andre**. 1997. A C-terminal di-leucine motif and nearby sequences are required for NH4(+)-induced inactivation and degradation of the general amino acid permease, Gap1p, of Saccharomyces cerevisiae. Mol. Microbiol. **24**:607-616.

73. **Hein, C., J. Y. Springael, C. Volland, R. Haguenauer-Tsapis, and B. Andre**. 1995. NPl1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. Mol. Microbiol. **18**:77-87.

74. **Henriksen, C. M., J. Nielsen, and J. Villadsen**. 1998. Cyclization of alpha-aminoadipic acid into the the delta-lactam 6-oxo-piperidine-2-carboxylic acid by Penicillium chrysogenum. J. Antibiot. (Tokyo) **51**:99-106.

75. **Herschbach, G. J. M., C. P. van der Beek, and P. W. M. van Dijck**. 1984. The penicillins: properties, biosynthesis, and fermentation, p. 45-140. *In* E. J. Vandamme (ed.), Biotechnology of industrial antibiotics. Dekker,M., New York.

76. **Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagoloff**. 1986. Yeast/E. coli shuttle vectors with multiple unique restriction sites. Yeast **2**:163-167.

77. **Hillenga, D. J.** Transport processes in penicillin biosynthesis. 1999. PhD Thesis. University of Groningen, The Netherlands.

78. **Hillenga, D. J., H. J. Versantvoort, A. J. M. Driessen, and W. N. Konings**. 1996. Basic amino acid transport in plasma membrane vesicles of Penicillium chrysogenum. J. Bacteriol. **178**:3991-3995.

79. **Hillenga, D. J., H. J. Versantvoort, A. J. M. Driessen, and W. N. Konings**. 1996. Sulfate transport in Penicillium chrysogenum plasma membranes. J. Bacteriol. **178**:3953-3956.

80. **Hoffmann, W.** 1985. Molecular characterization of the CAN1 locus in Saccharomyces cerevisiae. A transmembrane protein without N-terminal hydrophobic signal sequence. J. Biol. Chem. **260**:11831-11837.

81. **Hönlinger, C. and C. P. Kubicek**. 1989. Regulation of delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine and isopenicillin N biosynthesis in Penicillium chrysogenum by the alpha-aminoadipate pool size. FEMS Microbiol. Lett. **53**:71-75.

82. **Horák, J.** 1986. Amino acid transport in eucaryotic microorganisms. Biochim. Biophys. Acta **864**:223-256.

83. **Horák, J.** 1997. Yeast nutrient transporters. Biochim. Biophys. Acta **1331**:41-79.

84. **Humphries, M. J., K. Matsumoto, S. L. White, and K. Olden**. 1986. Oligosaccharide modification by swainsonine treatment inhibits pulmonary colonization by B16-F10 murine melanoma cells. Proc. Natl. Acad. Sci. U. S. A **83**:1752-1756.

85. **Hunter, D. R. and I. H. Segel**. 1971. Acidic and basic amino acid transport systems of Penicillium chrysogenum. Arch. Biochem. Biophys. **144**:168-183.

86. **Hunter, D. R. and I. H. Segel**. 1973. Control of the general amino acid permease of Penicillium chrysogenum by transinhibition and turnover. Arch. Biochem. Biophys. **154**:387-399.

87. **Indiveri, C., A. Tonazzi, I. Stipani, and F. Palmieri**. 1999. The purified and reconstituted ornithine/citrulline carrier from rat liver mitochondria catalyses a second transport mode: ornithine+/H+ exchange. Biochem. J. **341**:705-711.

88. **Ingolia, T. D. and S. W. Queener**. 1989. Beta-lactam biosynthetic genes. Med. Res. Rev. **9**:245-264.

89. **Iraqui, I., S. Vissers, F. Bernard, J. O. de Craene, E. Boles, A. Urrestarazu, and B. Andre**. 1999. Amino acid signaling in Saccharomyces cerevisiae: a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the AGP1 gene, which encodes a broad-specificity amino acid permease. Mol. Cell Biol. **19**:989-1001.

90. **Isnard, A. D., D. Thomas, and Y. Surdin-Kerjan**. 1996. The study of methionine uptake in Saccharomyces cerevisiae reveals a new family of amino acid permeases. J. Mol. Biol. **262**:473-484.

91. **Jack, D. L., I. T. Paulsen, and M. H. Saier**. 2000. The amino acid/polyamine/organocation (APC) superfamily of transporters specific for amino acids, polyamines and organocations. Microbiology **146**:1797-1814.

92. **Jaklitsch, W. M., W. Hampel, M. Rohr, C. P. Kubicek, and G. Gamerith**. 1986. alpha-Aminoadipate pool concentration and penicillin biosynthesis in strains of Penicillium chrysogenum. Can. J. Microbiol. **32**:473-480.

93. **Jauniaux, J. C. and M. Grenson**. 1990. GAP1, the general amino acid permease gene of Saccharomyces cerevisiae. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. Eur. J. Biochem. **190**:39-44.

94. **Jauniaux, J. C., M. Vandenbol, S. Vissers, K. Broman, and M. Grenson**. 1987. Nitrogen catabolite regulation of proline permease in Saccharomyces cerevisiae. Cloning of the PUT4 gene and study of PUT4 RNA levels in wild-type and mutant strains. Eur. J. Biochem. **164**:601-606.

95. **Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgins, and T. Gibson**. 1998. Multiple sequence alignment with Clustal X. Trends in Biochemical Science **23**:403-405.

96. **Jethwaney, D., M. Hofer, R. K. Khaware, and R. Prasad**. 1997. Functional reconstitution of a purified proline permease from Candida albicans: interaction with the antifungal cispentacin. Microbiology **143**:397-404.

97. **Jørgensen, H., J. Nielsen, J. Villadsen, and H. Mollgaard**. 1995. Analysis of penicillin V biosynthesis during fed-batch cultivations with a high-yielding strain of Penicillium chrysogenum. Appl. Microbiol. Biotechnol. **43**:123-130.

98. **Kitamoto, K., K. Yoshizawa, Y. Ohsumi, and Y. Anraku**. 1988. Dynamic aspects of vacuolar and cytosolic amino acid pools of Saccharomyces cerevisiae. J. Bacteriol. **170**:2683-2686.

99. **Klasson, H., G. R. Fink, and P. O. Ljungdahl**. 1999. Ssy1p and Ptr3p are plasma membrane components of a yeast system that senses extracellular amino acids. Mol. Cell Biol. **19**:5405-5416.

100. **Kleinkauf, H. and H. Von Döhren**. 1987. Biosynthesis of peptide antibiotics. Annu. Rev. Microbiol. **41**:259-289.

101. **Kleinkauf, H. and H. Von Döhren**. 1995. The nonribosomal peptide biosynthetic system--on the origins of structural diversity of peptides, cyclopeptides and related compounds. Antonie Van Leeuwenhoek **67**:229-242.

102. **Kleinkauf, H. and H. Von Döhren**. 1996. A nonribosomal system of peptide biosynthesis. Eur. J. Biochem. **236**:335-351.

103. **Koo, K. and W. D. Stuart**. 1991. Sequence and structure of mtr, an amino acid transport gene of Neurospora crassa. Genome **34**:644-651.

104. **Kruckeberg, A. L.** 1996. The hexose transporter family of Saccharomyces cerevisiae. Arch. Microbiol. **166**:283-292.

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

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

107. **Lara, F., M. R. del Carmen, G. Vazquez, and S. Sanchez**. 1982. Induction of penicillin biosynthesis by L-glutamate in penicillium chrysogenum. Biochem. Biophys. Res. Commun. **105**:172-178.

108. **Lautru, S. and G. L. Challis**. 2004. Substrate recognition by nonribosomal peptide synthetase multi-enzymes. Microbiology **150**:1629-1636.

109. **Leemhuis, H., H. J. Rozeboom, M. Wilbrink, G. J. Euverink, B. W. Dijkstra, and L. Dijkhuizen**. 2003. Conversion of cyclodextrin glycosyltransferase into a starch hydrolase by directed evolution: the role of alanine 230 in acceptor subsite +1. Biochemistry **42**:7518-7526.

110. **Lemmens, M., K. Verheyden, P. Van Veldhoven, J. Vereecke, G. P. Mannaerts, and E. Carmeliet**. 1989. Single-channel analysis of a large conductance channel in peroxisomes from rat liver. Biochim. Biophys. Acta **984**:351-359.

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

112. **Lester, G.** 1965. Genetic control of amino acid permeability in Neurospora crassa. J. Bacteriol. **91**:677-684.

113. **Liras, P.** 1999. Biosynthesis and molecular genetics of cephamycins. Cephamycins produced by actinomycetes. Antonie Van Leeuwenhoek **75**:109-124.

114. **Litzka, O., B. K. Then, B. J. Van Den, S. Steidl, and A. A. Brakhage**. 1999. Transcriptional control of expression of fungal beta-lactam biosynthesis genes. Antonie Van Leeuwenhoek **75**:95-105.

115. **López-Nieto, M., F. R. Ramos, J. M. Luengo, and J. F. Martín**. 1985. Characterization of the biosynthesis in vivo of α-aminoadipyl-cysteinyl-valine in *Penicillium chrysogenum*. Appl. Microbiol. Biotechnol. **22**:343-351.

116. **Ludewig, U., N. von Wiren, and W. B. Frommer**. 2002. Uniport of NH4+ by the root hair plasma membrane ammonium transporter LeAMT1;1. J. Biol. Chem. **277**:13548-13555.

117. **Luengo, J. M.** 1995. Enzymatic synthesis of hydrophobic penicillins. J. Antibiot. (Tokyo) **48**:1195-1212.

118. **Luengo, J. M., G. Revilla, M. J. Lopez, J. R. Villanueva, and J. F. Martín**. 1980. Inhibition and repression of homocitrate synthase by lysine in Penicillium chrysogenum. J. Bacteriol. **144**:869-876.

119. **Madi, L., S. A. McBride, L. A. Bailey, and D. J. Ebbole**. 1997. rco-3, a gene involved in glucose transport and conidiation in Neurospora crassa. Genetics **146**:499-508.

120. **Magasanik, B. and C. A. Kaiser**. 2002. Nitrogen regulation in Saccharomyces cerevisiae. Gene **290**:1-18.

121. **Marger, M. D. and M. H. Saier, Jr.** 1993. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. Trends Biochem. Sci. **18**:13-20.

122. **Margolis-Clark, E., I. Hunt, S. Espinosa, and B. J. Bowman**. 2001. Identification of the gene at the pmg locus, encoding system II, the general amino acid transporter in Neurospora crassa. Fungal. Genet. Biol. **33**:127-135.

123. **Mark, C. G. and A. H. Romano**. 1971. Properties of the hexose transport systems of Aspergillus nidulans. Biochim. Biophys. Acta **249**:216-226.

124. **Marobbio, C. M., A. Vozza, M. Harding, F. Bisaccia, F. Palmieri, and J. E. Walker**. 2002. Identification and reconstitution of the yeast mitochondrial transporter for thiamine pyrophosphate. EMBO J. **21**:5653-5661.

125. **Martín, J. F.** 1998. New aspects of genes and enzymes for beta-lactam antibiotic biosynthesis. Appl. Microbiol. Biotechnol. **50**:1-15.

126. **Martín, J. F., J. Casqueiro, K. Kosalkova, A. T. Marcos, and S. Gutierrez**. 1999. Penicillin and cephalosporin biosynthesis: mechanism of carbon catabolite regulation of penicillin production. Antonie Van Leeuwenhoek **75**:21-31.

127. **Martín, J. F. and P. Liras**. 1989. Enzymes involved in penicillin, cephalosporin and cephamycin biosynthesis. Adv. Biochem. Eng Biotechnol. **39**:153-187.

128. **Marzluf, G. A.** 1993. Regulation of sulfur and nitrogen metabolism in filamentous fungi. Annu. Rev. Microbiol. **47**:31-55.

129. **Mathison, L., C. Soliday, T. Stepan, T. Aldrich, and J. Rambosek**. 1993. Cloning, characterization, and use in strain improvement of the Cephalosporium acremonium gene cefG encoding acetyl transferase. Curr. Genet. **23**:33-41.

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

131. **Minambres, B., H. Martinez-Blanco, E. R. Olivera, B. Garcia, B. Diez, J. L. Barredo, M. A. Moreno, C. Schleissner, F. Salto, and J. M. Luengo**. 1996. Molecular cloning and expression in different microbes of the DNA encoding Pseudomonas putida U phenylacetyl-CoA ligase. Use of this gene to improve the rate of benzylpenicillin biosynthesis in Penicillium chrysogenum. J. Biol. Chem. **271**:33531-33538.

132. **Monahan, B. J., J. A. Fraser, M. J. Hynes, and M. A. Davis**. 2002. Isolation and characterization of two ammonium permease genes, meaA and mepA, from Aspergillus nidulans. Eukaryot. Cell **1**:85-94.

133. **Monahan, B. J., S. E. Unkles, I. T. Tsing, J. R. Kinghorn, M. J. Hynes, and M. A. Davis**. 2002. Mutation and functional analysis of the Aspergillus nidulans ammonium permease MeaA and evidence for interaction with itself and MepA. Fungal. Genet. Biol. **36**:35-46.

134. **Mukherjee, P. K. and R. Prasad**. 1998. Purified arginine permease of Candida albicans is functionally active in a reconstituted system. Yeast **14**:335-345.

135. **Muller, W. H., R. A. Bovenberg, M. H. Groothuis, F. Kattevilder, E. B. Smaal, L. H. van der Voort, and A. J. Verkleij**. 1992. Involvement of microbodies in penicillin biosynthesis. Biochim. Biophys. Acta **1116**:210-213.

136. **Muller, W. H., T. P. van der Krift, A. J. Krouwer, H. A. Wosten, L. H. van der Voort, E. B. Smaal, and A. J. Verkleij**. 1991. Localization of the pathway of the penicillin biosynthesis in Penicillium chrysogenum. EMBO J. **10**:489-495.

137. **Naranjo, L., E. Martín de Valmaseda, O. Banuelos, P. Lopez, J. Riano, J. Casqueiro, and J. F. Martín**. 2001. Conversion of pipecolic acid into lysine in Penicillium chrysogenum requires pipecolate oxidase and saccharopine reductase: characterization of the lys7 gene encoding saccharopine reductase. J. Bacteriol. **183**:7165-7172.

138. **Naranjo, L., E. Martín de Valmaseda, J. Casqueiro, R. V. Ullan, M. Lamas-Maceiras, O. Banuelos, and J. F. Martín**. 2004. Inactivation of the lys7 gene, encoding saccharopine reductase in Penicillium chrysogenum, leads to accumulation of the secondary metabolite precursors piperideine-6-carboxylic acid and pipecolic acid from alpha-aminoadipic acid. Appl. Environ. Microbiol. **70**:1031-1039.

139. **Nehls, U., R. Kleber, J. Wiese, and R. Hampp**. 1999. Isolation and characterization of a general amino acid permease from the ectomycorrhizal fungus *Amanita muscaria*. New Phytologist **144**:343-349.

140. **Nelissen, B., R. De Wachter, and A. Goffeau**. 1997. Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of Saccharomyces cerevisiae. FEMS Microbiol. Rev. **21**:113-134.

141. **Nicolay, K., M. Veenhuis, A. C. Douma, and W. Harder**. 1987. A 31P NMR study of the internal pH of yeast peroxisomes. Arch. Microbiol. **147**:37-41.

142. Nielsen, J. Physiological engineering aspects of Penicillium chrysogenum. 1994. PhD Thesis. Center for Process Biotechnology, Lyngby, Denmark.

143. **Nielsen, J. and H. S. Jørgensen**. 1995. Metabolic control analysis of the penicillin biosynthetic pathway in a high-yielding strain of Penicillium chrysogenum. Biotechnol. Prog. **11**:299-305.

144. **Nielsen, J. B., M. J. Hsu, K. M. Byrne, and L. Kaplan**. 1991. Biosynthesis of the immunosuppressant immunomycin: the enzymology of pipecolate incorporation. Biochemistry **30**:5789-5796.

145. **Nuesch, J., J. Heim, and H. J. Treichler**. 1987. The biosynthesis of sulfur-containing beta-lactam antibiotics. Annu. Rev. Microbiol. **41:51-75.**:51-75.

146. **Omura, F., Y. Kodama, and T. Ashikari**. 2001. The N-terminal domain of the yeast permease Bap2p plays a role in its degradation. Biochem. Biophys. Res. Commun. **287**:1045-1050.

147. **Ostergaard, S., H. B. A. Theilgaard, and J. Nielsen**. 1998. Identification and purification of O-acetyl-L-serine sulphhydrylase in Penicillium chrysogenum. Appl. Microbiol. Biotechnol. **50**:663-668.

148. **Palmieri, F.** 1994. Mitochondrial carrier proteins. FEBS Lett. **346**:48-54.

149. **Palmieri, L., M. De, V, V. Iacobazzi, F. Palmieri, M. J. Runswick, and J. E. Walker**. 1997. Identification of the yeast ARG-11 gene as a mitochondrial ornithine carrier involved in arginine biosynthesis. FEBS Lett. **410**:447-451.

150. **Palmieri, L., B. Pardo, F. M. Lasorsa, A. del Arco, K. Kobayashi, M. Iijima, M. J. Runswick, J. E. Walker, T. Saheki, J. Satrustegui, and F. Palmieri**. 2001. Citrin and aralar1 are Ca(2+)-stimulated aspartate/glutamate transporters in mitochondria. EMBO J. **20**:5060-5069.

151. **Palmieri, L., H. Rottensteiner, W. Girzalsky, P. Scarcia, F. Palmieri, and R. Erdmann**. 2001. Identification and functional reconstitution of the yeast peroxisomal adenine nucleotide transporter. EMBO J. **20**:5049-5059.

152. **Palmieri, L., M. J. Runswick, G. Fiermonte, J. E. Walker, and F. Palmieri**. 2000. Yeast mitochondrial carriers: bacterial expression, biochemical identification and metabolic significance. J. Bioenerg. Biomembr. **32**:67-77.

153. **Pao, S. S., I. T. Paulsen, and M. H. Saier, Jr.** 1998. Major facilitator superfamily. Microbiol. Mol. Biol. Rev. **62**:1-34.

154. **Paulsen, I. T., M. K. Sliwinski, B. Nelissen, A. Goffeau, and M. H. Saier, Jr.** 1998. Unified inventory of established and putative transporters encoded within the complete genome of Saccharomyces cerevisiae. FEBS Lett. **430**:116-125.

155. **Pieniazek, N., P. P. Stepien, and A. Paszewski**. 1973. An Aspergillus nidulans mutant lacking cystathionine -synthase: identity of L-serine sulfhydrylase with cystathionine -synthase and its distinctness from O-acetyl-L-serine sulfhydrylase. Biochim. Biophys. Acta **297**:37-47.

156. **Purdue, P. E. and P. B. Lazarow**. 2001. Peroxisome biogenesis. Annu. Rev. Cell Dev. Biol. **17**:701-752.

157. **Regenberg, B., L. During-Olsen, M. C. Kielland-Brandt, and S. Holmberg**. 1999. Substrate specificity and gene expression of the amino-acid permeases in Saccharomyces cerevisiae. Curr. Genet. **36**:317-328.

158. **Regenberg, B. and J. Hansen**. 2000. GAP1, a novel selection and counter-selection marker for multiple gene disruptions in Saccharomyces cerevisiae. Yeast **16**:1111-1119.

159. **Regenberg, B., S. Holmberg, L. D. Olsen, and M. C. Kielland-Brandt**. 1998. Dip5p mediates high-affinity and high-capacity transport of L-glutamate and L-aspartate in Saccharomyces cerevisiae. Curr. Genet. **33**:171-177.

160. **Regenberg, B. and M. C. Kielland-Brandt**. 2001. Amino acid residues important for substrate specificity of the amino acid permeases Can1p and Gnp1p in Saccharomyces cerevisiae. Yeast **18**:1429-1440.

161. **Reiber, K., T. Neuhof, J. H. Ozegowski, H. von Döhren, and T. Schwecke**. 2003. A nonribosomal peptide synthetase involved in the biosynthesis of ampullosporins in Sepedonium ampullosporum. J. Pept. Sci. **9**:701-713.

162. **Reifenberger, E., E. Boles, and M. Ciriacy**. 1997. Kinetic characterization of individual hexose transporters of Saccharomyces cerevisiae and their relation to the triggering mechanisms of glucose repression. Eur. J. Biochem. **245**:324-333.

163. **Reifenberger, E., K. Freidel, and M. Ciriacy**. 1995. Identification of novel HXT genes in Saccharomyces cerevisiae reveals the impact of individual hexose transporters on glycolytic flux. Mol. Microbiol. **16**:157-167.

164. **Renosto, F., R. L. Martín, and I. H. Segel**. 1987. ATP sulfurylase from Penicillium chrysogenum. Molecular basis of the sigmoidal velocity curves induced by sulfhydryl group modification. J. Biol. Chem. **262**:16279-16288.

165. **Renosto, F., P. A. Seubert, and I. H. Segel**. 1984. Adenosine 5'-phosphosulfate kinase from Penicillium chrysogenum. Purification and kinetic characterization. J. Biol. Chem. **259**:2113-2123.

166. **Reumann, S., M. Bettermann, R. Benz, and H. W. Heldt**. 1997. Evidence for the Presence of a Porin in the Membrane of Glyoxysomes of Castor Bean. Plant Physiol **115**:891-899.

167. **Reumann, S., E. Maier, R. Benz, and H. W. Heldt**. 1996. A specific porin is involved in the malate shuttle of leaf peroxisomes. Biochem. Soc. Trans. **24**:754-757.

168. **Reumann, S., E. Maier, H. W. Heldt, and R. Benz**. 1998. Permeability properties of the porin of spinach leaf peroxisomes. Eur. J. Biochem. **251**:359-366.

169. **Ritzau, M., S. Heinze, K. Dornberger, A. Berg, W. Fleck, B. Schlegel, A. Hartl, and U. Grafe**. 1997. Ampullosporin, a new peptaibol-type antibiotic from Sepedonium ampullosporum HKI-0053 with neuroleptic activity in mice. J. Antibiot. (Tokyo) **50**:722-728.

170. **Roberg, K. J., S. Bickel, N. Rowley, and C. A. Kaiser**. 1997. Control of amino acid permease sorting in the late secretory pathway of Saccharomyces cerevisiae by SEC13, LST4, LST7 and LST8. Genetics **147**:1569-1584.

171. **Robin, J., M. Jakobsen, M. Beyer, H. Noorman, and J. Nielsen**. 2001. Physiological characterisation of Penicillium chrysogenum strains expressing the expandase gene from Streptomyces clavuligerus during batch cultivations. Growth and adipoyl-7-aminodeacetoxycephalosporanic acid production. Appl. Microbiol. Biotechnol. **57**:357-362.

172. **Russnak, R., D. Konczal, and S. L. McIntire**. 2001. A family of yeast proteins mediating bidirectional vacuolar amino acid transport. J. Biol. Chem. **276**:23849-23857.

173. **Saier, M. H., Jr., J. T. Beatty, A. Goffeau, K. T. Harley, W. H. Heijne, S. C. Huang, D. L. Jack, P. S. Jahn, K. Lew, J. Liu, S. S. Pao, I. T. Paulsen, T. T. Tseng, and P. S. Virk**. 1999. The major facilitator superfamily. J. Mol. Microbiol. Biotechnol. **1**:257-279.

174. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, N.Y.

175. **Scarborough, G. A.** 1970. Sugar transport in Neurospora crassa. J. Biol. Chem. **245**:1694-1698.

176. **Scarborough, G. A.** 1970. Sugar transport in Neurospora crassa. II. A second glucose transport system. J. Biol. Chem. **245**:3985-3987.

177. **Schafer, H., K. Nau, A. Sickmann, R. Erdmann, and H. E. Meyer**. 2001. Identification of peroxisomal membrane proteins of Saccharomyces cerevisiae by mass spectrometry. Electrophoresis **22**:2955-2968.

178. **Schmidt, A., M. N. Hall, and A. Koller**. 1994. Two FK506 resistance-conferring genes in Saccharomyces cerevisiae, TAT1 and TAT2, encode amino acid permeases mediating tyrosine and tryptophan uptake. Mol. Cell Biol. **14**:6597-6606.

179. **Schneider, R. P. and W. R. Wiley**. 1971. Kinetic characteristics of the two glucose transport systems in Neurospora crassa. J. Bacteriol. **106**:479-486.

180. **Schneider, R. P. and W. R. Wiley**. 1971. Regulation of sugar transport in Neurospora crassa. J. Bacteriol. **106**:487-492.

181. **Seaston, A., C. Inkson, and A. A. Eddy**. 1973. The absorption of protons with specific amino acids and carbohydrates by yeast. Biochem. J. **134**:1031-1043.

182. **Sewell, A. K., F. Yokoya, W. Yu, T. Miyagawa, T. Murayama, and D. R. Winge**. 1995. Mutated yeast heat shock transcription factor exhibits elevated basal transcriptional activation and confers metal resistance. J. Biol. Chem. **270**:25079-25086.

183. **Skye, G. E. and I. H. Segel**. 1970. Independent regulation of cysteine and cystine transport in Penicillium chrysogenum. Arch. Biochem. Biophys. **138**:306-318.

184. **Slayman, C. L. and C. W. Slayman**. 1974. Depolarization of the plasma membrane of Neurospora during active transport of glucose: evidence for a proton-dependent cotransport system. Proc. Natl. Acad. Sci. U. S. A **71**:1935-1939.

185. **Sophianopoulou, V. and G. Diallinas**. 1993. AUA1, a gene involved in ammonia regulation of amino acid transport in Saccharomyces cerevisiae. Mol. Microbiol. **8**:167-178.

186. **Sophianopoulou, V. and G. Diallinas**. 1995. Amino acid transporters of lower eukaryotes: regulation, structure and topogenesis. FEMS Microbiol. Rev. **16**:53-75.

187. **Sophianopoulou, V. and C. Scazzocchio**. 1989. The proline transport protein of Aspergillus nidulans is very similar to amino acid transporters of Saccharomyces cerevisiae. Mol. Microbiol. **3**:705-714.

188. **Sophianopoulou, V., T. Suarez, G. Diallinas, and C. Scazzocchio**. 1993. Operator derepressed mutations in the proline utilisation gene cluster of Aspergillus nidulans. Mol. Gen. Genet. **236**:209-213.

189. **Springael, J. Y. and B. Andre**. 1998. Nitrogen-regulated ubiquitination of the Gap1 permease of Saccharomyces cerevisiae. Mol. Biol. Cell **9**:1253-1263.

190. **Stadler, D. R.** 1966. Genetic control of the uptake of amino acids in Neurospora. Genetics **54**:677-685.

191. **Stanbrough, M. and B. Magasanik**. 1995. Transcriptional and posttranslational regulation of the general amino acid permease of Saccharomyces cerevisiae. J. Bacteriol. **177**:94-102.

192. **Stanbrough, M. and B. Magasanik**. 1996. Two transcription factors, Gln3p and Nil1p, use the same GATAAG sites to activate the expression of GAP1 of Saccharomyces cerevisiae. J. Bacteriol. **178**:2465-2468.

193. **Struck, C., M. Ernst, and M. Hahn**. 2002. Characterization of a developmentally regulated amino acid transporter (AAT1p) of the rust fungus *Uromyces fabae*. Molecular Plant Pathology **3**:23-30.

194. **Struck, C., E. Mueller, H. Martin, and G. Lohaus**. 2004. The *Uromyces fabae* UfAAT3 gene encodes a general amino acid permease that prefers uptake of in planta scarce amino acids. Molecular Plant Pathology **5**:183-189.

195. **Stuart, W. D., K. Koo, and S. J. Vollmer**. 1988. Cloning of mtr, an amino acid transport gene of Neurospora crassa. Genome **30**:198-203.

196. **Sulter, G. J., I. J. van de Klei, J. Schanstra, W. Harder, and M. Veenhuis**. 1991. Ethanol metabolism in a peroxisome-deficient mutant of the yeast *Hansenula polymorpha*. FEMS Microbiol. Lett. **82**:297-302.

197. **Sychrova, H. and M. R. Chevallier**. 1993. Cloning and sequencing of the Saccharomyces cerevisiae gene LYP1 coding for a lysine-specific permease. Yeast **9**:771-782.

198. **Sychrova, H. and J. L. Souciet**. 1994. CAN1, a gene encoding a permease for basic amino acids in Candida albicans. Yeast **10**:1647-1651.

199. **Takagi, H., M. Shichiri, M. Takemura, M. Mohri, and S. Nakamori**. 2000. Saccharomyces cerevisiae sigma 1278b has novel genes of the N-

acetyltransferase gene superfamily required for L-proline analogue resistance. J. Bacteriol. **182**:4249-4256.

200. **Tanaka, J. and G. R. Fink**. 1985. The histidine permease gene (HIP1) of Saccharomyces cerevisiae. Gene **38**:205-214.

201. **Tavoularis, S. N., U. H. Tazebay, G. Diallinas, M. Sideridou, A. Rosa, C. Scazzocchio, and V. Sophianopoulou**. 2003. Mutational analysis of the major proline transporter (PrnB) of Aspergillus nidulans. Mol. Membr. Biol. **20**:285-297.

202. **Tazebay, U. H., V. Sophianopoulou, B. Cubero, C. Scazzocchio, and G. Diallinas**. 1995. Post-transcriptional control and kinetic characterization of proline transport in germinating conidiospores of Aspergillus nidulans. FEMS Microbiol. Lett. **132**:27-37.

203. **Tazebay, U. H., V. Sophianopoulou, C. Scazzocchio, and G. Diallinas**. 1997. The gene encoding the major proline transporter of Aspergillus nidulans is upregulated during conidiospore germination and in response to proline induction and amino acid starvation. Mol. Microbiol. **24**:105-117.

204. **Then Bergh, K. and A. A. Brakhage**. 1998. Regulation of the Aspergillus nidulans penicillin biosynthesis gene acvA (pcbAB) by amino acids: implication for involvement of transcription factor PACC. Appl. Environ. Microbiol. **64**:843-849.

205. **Tobin, M. B., M. D. Fleming, P. L. Skatrud, and J. R. Miller**. 1990. Molecular characterization of the acyl-coenzyme A:isopenicillin N acyltransferase gene (penDE) from Penicillium chrysogenum and Aspergillus nidulans and activity of recombinant enzyme in Escherichia coli. J. Bacteriol. **172**:5908-5914.

206. **Torres, N. V., J. M. Riol-Cimas, M. Wolschek, and C. P. Kubicek**. 1996. Glucose transport by *Aspergillus niger*: the low-affinity carrier is only formed during growth on high glucose concentrations. Appl. Microbiol. Biotechnol. **44**:790-794.

207. **Treichler, H. J., M. Liersch, J. Nuësch, and H. Döbeli**. 1979. Role of sulfur metabolism in cephalosporin C and penicillin biosynthesis, p. 97-104. *In* O. K. Sebek and A. L. Laskin (eds.), Genetics of industrial microorganisms. American Society for Microbiology, Washington D.C.

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

209. **Trip, H., M. E. Evers, W. N. Konings, and A. J. M. Driessen**. 2002. Cloning and characterization of an aromatic amino acid and leucine permease of Penicillium chrysogenum. Biochim. Biophys. Acta **1565**:73-80.

210. **Ullan, R. V., G. Liu, J. Casqueiro, S. Gutierrez, O. Banuelos, and J. F. Martín**. 2002. The cefT gene of Acremonium chrysogenum C10 encodes a putative multidrug efflux pump protein that significantly increases cephalosporin C production. Mol. Genet. Genomics **267**:673-683.

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

212. **van de Kamp, M., E. Pizzinini, A. Vos, T. R. van der Lende, T. A. Schuurs, R. W. Newbert, G. Turner, W. N. Konings, and A. J. M. Driessen**. 1999. Sulfate transport in Penicillium chrysogenum: cloning and characterization of the sutA and sutB genes. J. Bacteriol. **181**:7228-7234.

213. **van de Kamp, M., T. A. Schuurs, A. Vos, T. R. van der Lende, W. N. Konings, and A. J. M. Driessen**. 2000. Sulfur regulation of the sulfate transporter genes sutA and sutB in Penicillium chrysogenum. Appl. Environ. Microbiol. **66**:4536-4538.

214. **van der Lende, T. R., P. Breeuwer, T. Abee, W. N. Konings, and A. J. M. Driessen**. 2002. Assessment of the microbody luminal pH in the filamentous fungus Penicillium chrysogenum. Biochim. Biophys. Acta **1589**:104-111.

215. **van der Lende, T. R., K. M. van de, M. Berg, K. Sjollema, R. A. Bovenberg, M. Veenhuis, W. N. Konings, and A. J. M. Driessen**. 2002. delta-(L-alpha-Aminoadipyl)-L-cysteinyl-D-valine synthetase, that mediates the first committed step in penicillin biosynthesis, is a cytosolic enzyme. Fungal. Genet. Biol. **37**:49-55.

216. **van der Rest, M. E., Y. de Vries, B. Poolman, and W. N. Konings**. 1995. Overexpression of Mal61p in Saccharomyces cerevisiae and characterization of maltose transport in artificial membranes. J. Bacteriol. **177**:5440-5446.

217. **van Dijk, R., K. N. Faber, A. T. Hammond, B. S. Glick, M. Veenhuis, and J. A. Kiel**. 2001. Tagging Hansenula polymorpha genes by random integration of linear DNA fragments (RALF). Mol. Genet. Genomics **266**:646-656.

218. **van Roermund, C. W., Y. Elgersma, N. Singh, R. J. Wanders, and H. F. Tabak**. 1995. The membrane of peroxisomes in Saccharomyces cerevisiae is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. EMBO J. **14**:3480-3486.

219. **van der Klei, I. J., W. Harder, and M. Veenhuis**. 1991. Methanol metabolism in a peroxisome-deficient mutant of Hansenula polymorpha: a physiological study. Arch. Microbiol. **156**:15-23.

220. **van der Klei, I. J. and M. Veenhuis**. 2002. Peroxisomes: flexible and dynamic organelles. Curr. Opin. Cell Biol. **14**:500-505.

221. **Vandenbol, M., J. C. Jauniaux, and M. Grenson**. 1989. Nucleotide sequence of the Saccharomyces cerevisiae PUT4 proline-permease-encoding gene: similarities between CAN1, HIP1 and PUT4 permeases. Gene **83**:153-159.

222. **Vandenbol, M., J. C. Jauniaux, and M. Grenson**. 1990. The Saccharomyces cerevisiae NPR1 gene required for the activity of ammonia-sensitive amino acid permeases encodes a protein kinase homologue. Mol. Gen. Genet. **222**:393-399.

223. **Vasseur, V., M. Van Montagu, and G. H. Goldman**. 1995. Trichoderma harzianum genes induced during growth on Rhizoctonia solani cell walls. Microbiology **141**:767-774.

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

225. **Visser, W. F., C. W. van Roermund, H. R. Waterham, and R. J. Wanders**. 2002. Identification of human PMP34 as a peroxisomal ATP transporter. Biochem. Biophys. Res. Commun. **299**:494-497.

226. **Vousden, W. and Turner, G.** 2001. Localisation of ACV synthetase of *Aspergillus nidulans*. Fungal Genetics Conference, **48:**467.

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

228. **Wiame, J. M., M. Grenson, and H. N. Arst, Jr.** 1985. Nitrogen catabolite repression in yeasts and filamentous fungi. Adv. Microb. Physiol **26**:1-88.

229. **Wiest, A., D. Grzegorski, B. W. Xu, C. Goulard, S. Rebuffat, D. J. Ebbole, B. Bodo, and C. Kenerley**. 2002. Identification of peptaibols from Trichoderma virens and cloning of a peptaibol synthetase. J. Biol. Chem. **277**:20862-20868.

230. **Wipf, D., M. Benjdia, M. Tegeder, and W. B. Frommer**. 2002. Characterization of a general amino acid permease from Hebeloma cylindrosporum. FEBS Lett. **528**:119-124.

231. **Young, G. B., D. L. Jack, D. W. Smith, and M. H. Saier, Jr.** 1999. The amino acid/auxin:proton symport permease family. Biochim. Biophys. Acta **1415**:306-322.

232. **Zaret, K. S. and F. Sherman**. 1982. DNA sequence required for efficient transcription termination in yeast. Cell **28**:563-573.

233. **Zaret, K. S. and F. Sherman**. 1985. alpha-Aminoadipate as a primary nitrogen source for Saccharomyces cerevisiae mutants. J. Bacteriol. **162**:579-583.

234. **Zhang, J., S. Wolfe, and A. L. Demain**. 1992. Biochemical studies on the activity of delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase from Streptomyces clavuligerus. Biochem. J. **283**:691-698.