PROTEOMIC ANALYSIS OF THE PEROXISOMAL MEMBRANE OF *PENICILLIUM CHRYSOGENUM*

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PROTEOMIC ANALYSIS OF THE PEROXISOMAL MEMBRANE OF *PENICILLIUM CHRYSOGENUM*

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chapter 1

TRANSPORT PROCESSES DURING BIOSYNTHESIS OF β-LACTAM ANTIBIOTICS IN *PENICILLIUM CHRYSOGENUM*

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INTRODUCTION

Alexander Fleming, a microbiologist working at St. Mary's Hospital in London, was the first to discover the antibacterial properties of penicillin. On one of his petri dishes with *Staphylococcus aureus* he noticed that cells growing around the mold became transparent (Fleming, 1929). Filtered broth from the mould showed marked inhibitory, bactericidal and bacteriolytic properties against many pathogenic bacteria. The mold was identified as a member of genus *Penicillium* (Fleming, 1929) secreting a compound with antibacterial activity. The term "antibiotic" was defined by Selman Waksman, in 1941, the head of the team of scientist that discovered a range of antibiotics including streptomycin and neomycine. According to Waksman, antibiotics are substances produced by, or semisynthetic substances partially derived from, microorganisms, that are able to inhibit or kill another organism in a diluted solution (Harris, 2008). Till now more than 20,000 antibiotics have been discovered, although a limited number actually makes it into a pharmaceutical product.

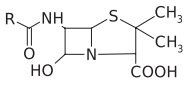
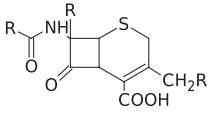
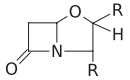
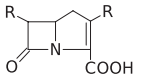
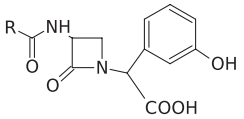
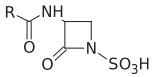
Penicillin, the antibiotic discovered by Fleming belongs to the group of β-lactam antibiotics. These now cover more than 65% of the world market of antibiotics with more than 50 products. Based on their chemical structure β-lactams can be divided into 5 groups: penams, ceph-3-ems, clavams, carbapenems and monolactams (see Table 1). The characteristic feature of the last two classes, which are carbapenems and monolactams, is their broad antimicrobial spectrum and resistance to β-lactamases. The best-characterized groups are: penicillins, cephalosporins and cephamycins. The common characteristic of β-lactam antibiotics is a four-membered β-lactam ring structure, except for monolactams, which possess a single ring structure. As shown in Table 1, the ability to synthesize β-lactams is wide spread in the nature. Hydrophilic cephalosporins are produced by some fungi, but also by Gram-positive and Gram-negative bacteria. Hydrophobic penicillins are produced as secondary metabolites by filamentous fungi only.

The term secondary metabolite is used for a broad range of compounds, which share some general characteristics (Vining, 1992)

- a wide range of chemical structures and biological activities
- they are not essential for growth and are strain-specific
- derived from primary intermediates and metabolites by unique biosynthetic pathways; commonly these pathways consist of multiple enzymes with promiscuous substrate specificity, catalyze complex reactions that are energetically expensive
- biosynthesis is directed by an organized set of genes associated with special regulatory mechanisms that control both the timing and level of gene expression. These control mechanisms are well integrated with the physiology of the producing organism.

Secondary metabolites can have antibiotic, antiviral, anti-tumor, and immunosuppressive activities. Others are siderophores needed to capture metal ions such as Fe^{2+}. However, some of the secondary metabolites are too toxic to be used as therapeutics (for instance mycotoxins), and are able to induce mutagenesis or possess carcinogenic properties.

Table 1. Overview of natural classes of β-lactams and producing microorganisms, selected examples. Table adapted from (Brakhage et al., 2004).

			Bacteria	
Classes of natural β-lactams	Antibiotics	Fungi	Gram +	Gram -
Penam 	Penicillins	Penicillium chrysogenum P. notatum Aspergillus nidulans		
Ceph-3-em 	Cephalosporins Cephamycins Cephabacins Chitinovrins	Acremonium chrysogenum (syn. Cephalosporium acremonium) Paecilomyces persinicus	Streptomyces clavuligerus Nocardia lactamdurans	Flavobacterium sp. *Lysobacter lactamgenus*
Clavam 	Clavulanic acid		Streptomyces clavuligerus	
Carbapenem 	Thienamycins Olivanic acid Epithienamycins		Streptomyces clavuligerus S. olivaceus	Erwinia carotovora Seratia sp.
Monolactam 	Nocardicines		*Nocardia uniformis* *subsp. tsuyamanensis*	
	Monobactams			*Agrobacterium radiobacter* *Pseudomonas acidophila*

Genetic and biochemical studies on the biosynthesis of β-lactams by filamentous fungi (Hillenga et al., 1996) has been comprehensive during the last decade. The biosynthetic pathway meets the general characteristics of secondary metabolite formation as quoted above (Koetsier et al., 2009; van den Berg et al., 2008). However, β-lactam production is not only the domain of filamentous fungi. The number of prokaryotic microorganisms able to synthesize β-lactams is continuously increasing.

After the success of penicillins in the infection treatment, a lot of effort was focused on the discovery of new antibiotics. The narrow spectrum of penicillins against pathogens has led to the development of wide spectrum penicillins, but at the same time resistance of microorganisms against β-lactams developed rapidly. This led to the search for antimicrobial derivatives of penicillins, which could treat wider range of infections and overcome the resistance mechanisms. Most of the β-lactams produced recently are semi-synthetically made, which means that the β-lactam core structure is derived from a fermentative process whereupon an enzyme-catalyzed or chemical attachment of a side chain or other modification is realized.

Since the discovery of antimicrobial properties of penicillin, the low yielding *Penicillium chrysogenum* strain NRRL1951 has been subjected to an intense classical strain improvement program (CSI). Herein, rounds of mutagenesis and selection led to improved strains with superb fermentation characteristics and β-lactam yield. The production level of naturally produced penicillin by industrial strains during the last 60 years has increased more than 40,000 times compared to the *P. notatum* strain discovered by Fleming (van den Berg et al., 2008).

Although the basic enzymology of the β-lactam biosynthesis pathway is very well described, still many aspects of the molecular mechanisms responsible for biosynthesis remain unknown. Here, I will discuss the biosynthetic pathway with a focus on the transport processes and the compartmentalization of the process.

BIOSYNTHESIS OF THE β-LACTAM PRECURSORS

The biosynthesis of β-lactams starts with the condensation of three precursor amino acids: L-α-aminoadipate, L-cysteine and L-valine into the tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (LLD-ACV) by ACV synthetase (ACVS). These precursors are withdrawn from the amino acid biosynthetic pathways.

Synthesis of L-α-aminoadipate

L-α-aminoadipate is an intermediate of the L-lysine biosynthesis pathway (Aharonowitz et al., 1992; Casqueiro et al., 2002). L-lysine can be synthesized via two different biosynthetic pathways (Bhattacharjee, 1985). Bacteria, lower fungi and green plants synthesize L-lysine through diaminopimelic acid pathway. In yeast, fungi, euglenoids and thermophilic bacteria (*Thermus thermophilus*) synthesis occurs via L-α-aminoadipate pathway (Casqueiro

et al., 2002). Extensive genetic and biochemical studies of L-α-aminoadipate pathway were performed in *Saccharomyces cerevisiae* and these studies form a paradigm for the enzymology of the α-aminoadipate (AAA) pathway in other organisms. In *S. cerevisiae as well as in P. chrysogenum* four enzymes are involved in this pathway (Figure 1).

In *S. cerevisiae* two of the enzymes are localized in microbodies or peroxisomes, i.e., single membrane enclosed organelles in the cell with an alkaline luminal pH that also contain some of the key enzymes of the β-lactam biosynthetic pathway as will be discussed below. The microbody localized enzymes of the lysine biosynthetic pathway are Lys1, the *saccharopine dehydrogenase* and Lys4, a *homoaconitase*. They were identified by the use of a context sensitive motif search (CoSMoS) for peroxisome-associated genes in *S. cerevisiae* (Geraghty et al., 1999). Lys1 and Lys4 possess a C-terminal peroxisomal targeting signal (PTS-1); their peroxisomal localization was confirmed by cellular studies using GFP fusion proteins. Studies on the role of microbodies in the lysine biosynthesis revealed that a *Pex12* deficient mutant of *S. cerevisiae* is unable to form microbodies (Breitling et al., 2002). Five genes of *S. cerevisiae* involved in L-lysine biosynthesis are significantly up-regulated: Lys1 (*saccharopine dehydrogenase)*, Lys4 (*homoaconitase)*, Lys 20 (*homocitrate synthase*), Lys12 (*homoisocitrate dehydrogenase*) and Lys 9 (another *saccharopine dehydrogenase*).

Although in *S. cerevisiae* the lysine biosynthetic pathway is partially localized in microbodies, subcellular localization studies in *P. chrysogenum* indicate that it is cytosolic. A GFP fusion of *homocitrate synthase* expressed in *P. chrysogenum* show mainly a cytosolic localization, with a minor localization in mitochondria (Bañuelos et al., 2002). Also in *Aspergillus fumigatus, homocitrate synthase* fused to EGFP was found to be exclusively localized to the cytosol (Schobel et al., 2010).

In L-lysine biosynthesis, first the condensation of acetyl-CoA and α-ketoglutarate into homocitrate occurs, catalyzed by *homocitrate synthase*. In subsequent isomerization and oxidative decarboxylation steps, homocitrate is converted into L-α-aminoadipate via iso-homocitrate, homoisocitrate and α-ketoadipic acid. L-α-aminoadipate is further converted into α-aminoadipate-δ-semialdehyde, saccharopine and L-lysine (Figure 1).

Synthesis of L-cysteine

L-cysteine is derived from sulfate, which is actively taken up by the cells by the SutB transport that catalyze a H^+-sulphate symport reaction (Figure 2). Sulfate is then converted into adenosine–5-phosphosulfate (APS) in a reaction catalyzed by *ATP sulfurase*. Next, APS is activated into 3-phospho-adenosine-5-phosphosulfate (PAPS) by *APS-kinase*. This reaction is followed by reduction of PAPS to sulfite by *PAPS reductase* and further reduction to sulfide by *sulfide reductase* (Marzluf, 1997; van de Kamp et al., 1999).

L-cysteine can be synthesized from sulfide via two different pathways: the transsulfuration and the sulfhydrylation pathway. In *P. chrysogenum*, sulfide is converted into cysteine via the cytosolic transsulfuration pathway (Nuesch et al., 1987). Herein, L-cysteine is

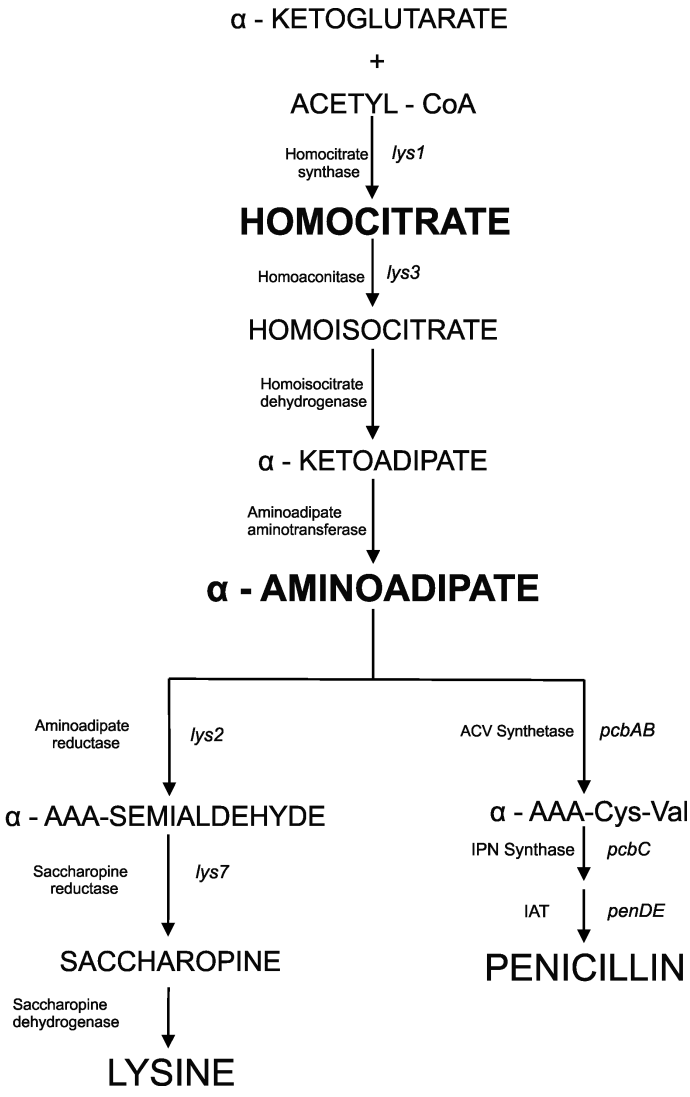


Figure 1. Lysine biosynthesis pathway in *P. chrysogenum*. L-α-aminoadipate is a branch point where the lysine and penicillin biosynthesis pathways separate. Adapted based on (Naranjo et al., 2001; Teves et al., 2009).

formed by cleavage of L-cystathionine derived from the L-homocysteine, which is formed from L-methionine or *O*-acetyl-L-homoserine. In the sulfhydrylation pathway L-serine is acetylated to O-acetyl-*L*-serine by *O-acetyl-L-serine sulfhydrylase* (OASS) (Foglino et al., 1995; Jørgensen et al., 1995). Based on a stoichiometric metabolic model, it is predicted that when cysteine would be synthesized via direct sulfhydrylation pathway, the yield of penicillin biosynthesis on glucose might be 20% higher (Jørgensen et al., 1995).

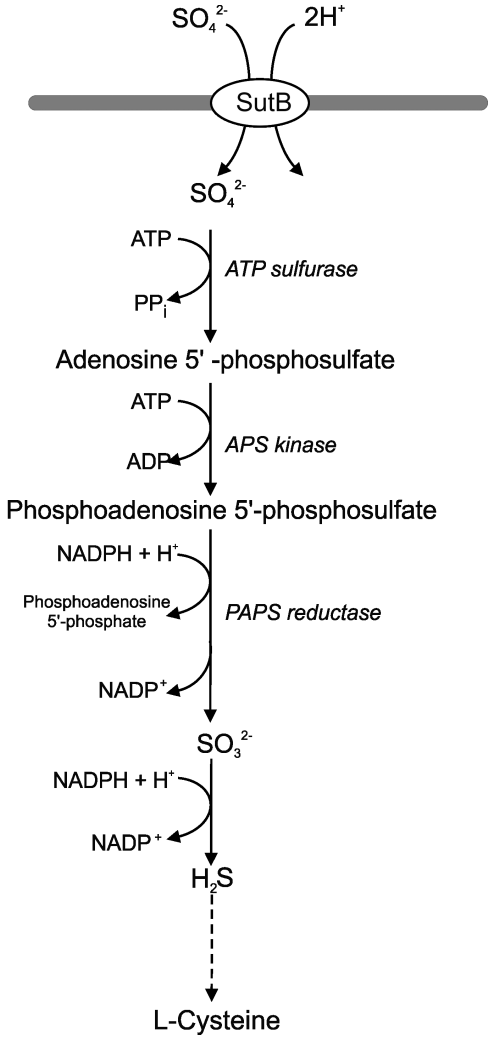


Figure 2. The transsulfuration pathway for the biosynthesis of L-cysteine in *P. chrysogenum*.

In the filamentous fungi, *Aspergillus nidulans* and *Cephalosporium acremonium*, both the direct sulfhydrylation and transsulfuration pathway are present. In *A. nidulans* the direct sulfhydrylation pathway is utilized. This was further exploited during the strain improvement of *C. acremonium* (Grynberg et al., 2000; Liu et al., 2001; Nuesch et al., 1987). In *P. chrysogenum* only the transsulfuration pathway appears to be present. Mutants of OAH-sulfhydrylase (O-acetyl-L-homoserine sulfhydrylase) were not able to grow on the inorganic sources of sulfur (Nuesch et al., 1987).

The analysis of *P. chrysogenum* genome and transcriptional studies show that all genes encoding enzymes involved in the synthesis of L-cysteine starting from the early

steps of sulfate reduction, serine synthesis and enzymes of the transsulfuration pathway are transcribed at a high level in a high yielding *P. chrysogenum* strain, independent on the presence of the penicillin side chain precursor phenylacetic acid (van den Berg et al., 2008). Similar results were obtained by proteomic analysis of industrial strains of *P. chrysogenum* (Jami et al., 2010). The cysteine synthase is already overexpressed in the early producing Wis54-1255 strain as comparing to the parental strain. This is also the case with the ASP-78 strain (high producer, Antibioticos, Spain) where additionally an increase of the *cystathionine* β-*synthase* (involved in the transsulfuration) was observed (Jami et al., 2010). These finding indicate that the cysteine biosynthetic pathway has undergone some major improvement during the classical strain improvement process resulting in the high penicillin yielding *P. chrysogenum* strains.

Synthesis of valine

In the biosynthesis of valine, four enzymes are involved. The pathway starts with the condensation of pyruvate with hydroxyethyl thiamine pyrophosphate into α-acetolactate catalyzed by *acetohydroxy acid synthase*. *Acetohydroxy acid isomeroreductase* transforms α-acetolactate into dihydroxy-isovalerate. Next, *dihydroxy acid dehydrase* converts dihydroxyisovalerate *into* α-ketoisovalerate, which is further converted by *glutamate transaminase* into L-valine. The synthesis of valine occurs in mitochondria, and thus the L-valine must be transported in the cytosol to be available for β-lactam biosynthesis (Kubicek-Pranz and Kubicek, 1991). Similar as for the L-cysteine pathway, expression of the genes encoding enzymes involved in the synthesis of valine is PAA independent, and are transcribed at a high level in a high penicillin yielding strain of *P. chrysogenum* (van den Berg et al., 2008)J.

PENICILLIN BIOSYNTHESIS

Figure 3 shows a schematic overview of the enzymatic steps involved in penicillin biosynthesis and the subcellular localization of the associated enzymes in *P. chrysogenum*. Biosynthesis of ß-lactam antibiotics starts in the cytosol with the condensation of three amino acids L-α-aminoadipic acid, L-cysteine and L-valine into δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV), in a reaction catalyzed by the δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS). Next, the ß-lactam ring is formed (Isopenicillin N, IPN) in a reaction catalyzed by isopenicillin N synthase (IPNS). The next steps of penicillin biosynthesis occur in microbodies. First, a carboxyl acid precursor for ß-lactam biosynthesis is activated into a CoA derivative by a CoA ligase. In penicillin G and V biosynthesis, the precursors are phenylacetic acid (PAA) or phenoxyacetic acid (POA), respectively. These are added to the growth medium and that are activated by phenylacetyl CoA ligase (PCL), although other CoA ligases may contribute as well. In the final step, side chain exchange reaction occurs, wherein the

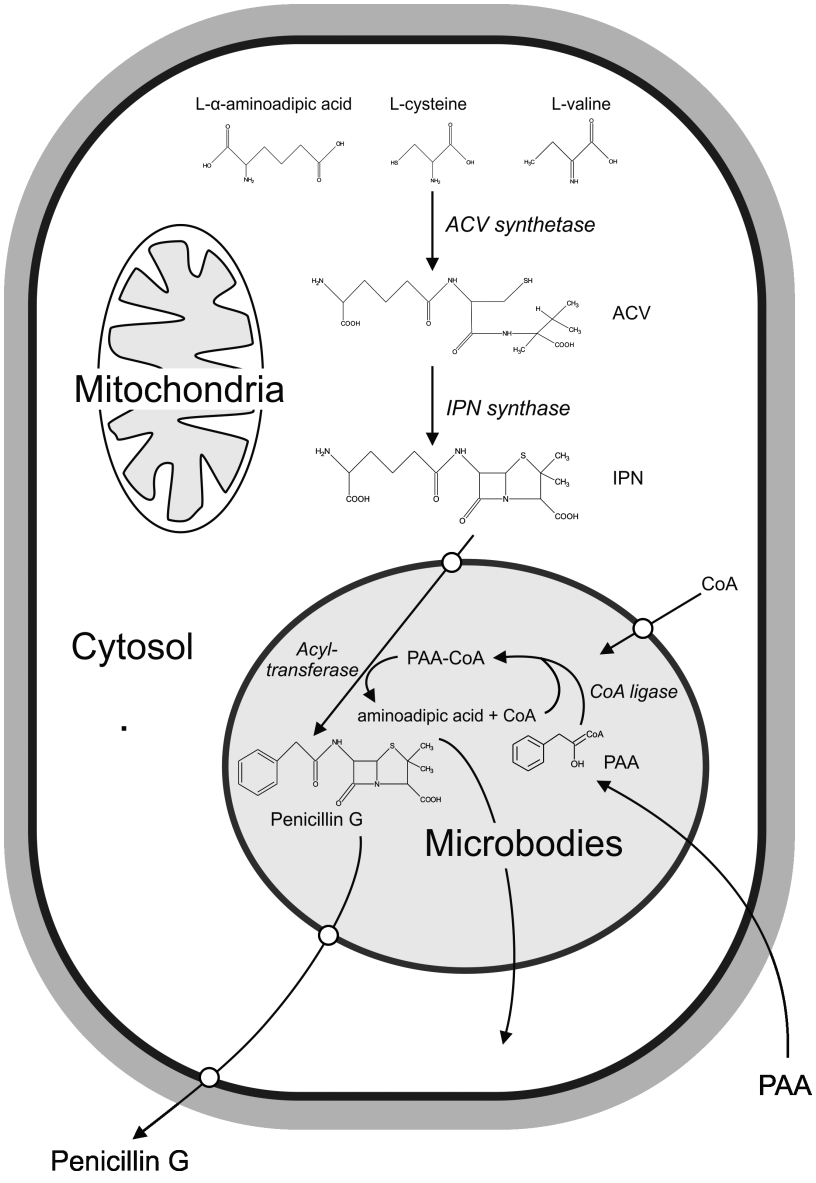


Figure 3. Cellular localization of the penicillin biosynthetic pathway. δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) and isopenicillin N synthase are localized in the cytosol (C), isopenicillin N enters the microbody/peroxisome (P). PCL (phenylacetyl CoA-ligase) and IAT (acyl-CoA isopenicillin N acyltransferase) are also localized in peroxisome. PAA is the abbreviation for phenylacetic acid.

aminoadipate moiety of isopenicillin N is replaced by the side chain precursor. This step is catalyzed by isopenicillin N-acyl transferase (IAT), also present in the microbody. This section discusses the function of the various enzymes in further detail.

L-α-aminoadipate-L-cysteine-D-valine synthetase

L-α-aminoadipate-L-cysteine-D-valine synthetase (ACVS) is a Type A, linear non-ribosomal peptide synthetase (NRPS). It is a three-module complex of 424 kDa (Figure 4). Each module comprises of cognate adenylation domain (A), peptidyl carrier domain (P) and condensation domain (C). The amino acids are first recognized and activated by cognate adenylation domain (A) and tethered as a thioester to 4'-phosphopantetheine of the peptidyl carrier domain (P). Next, the peptide bonds are formed in a reaction that involves the condensation domain (C). A specific feature is that L-α-aminoadipate is peptide-bond coupled at its δ-position to cysteine. Finally, the linear tripeptide is attached to the peptidyl carrier domain of the C-terminal module and released by the thioesterase domain (TE). At this point also the L-valine is epimerized to D-valine (Keller et al., 2005).

The mechanism tripeptide formation is the same for the synthesis of penicillins and cephalosporins. ACVS is a cytosolic protein encoded by *pcbAB* gene. The cellular localization of this enzyme was previously an issue of debate as ACVS was initially shown to be a membrane bound protein, as it co-fractionated with vacuoles and Golgi vesicles (Müller et al., 1991) and a vacuolar localization was suggested (Kubicek-Pranz and Kubicek, 1991). The ACV synthetase is a relatively hydrophobic protein, but it

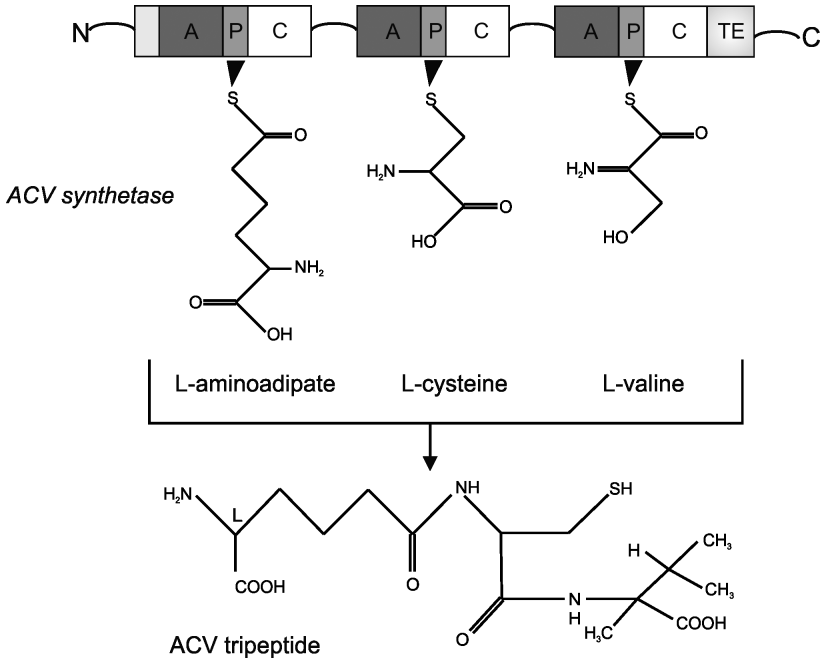


Figure 4. Model representation of the domain organization of the ACV synthase (a non-ribosomal peptide synthetase) that is responsible for the production of the tripeptide, L-α-aminoadipyl-L-cysteinyl-D-valine (Keller et al., 2005). A, C and TE represent the adenylation, condensation and termination/epimerization domains. P is the peptidyl carrier domain that binds the cognate amino acid.

contains no trans-membrane spanning domains. The analysis of amino acid sequence of *P. chrysogenum* ACV synthetase also did not show any targeting information for the endoplasmic reticulum or vacuole. The *P. chrysogenum* ACVS is highly instable and sensitive to proteolytic degradation and this severely interferes with cellular fractionation experiments (Etchegaray et al., 1997). By a combination of optimized protocols of cell lysis and immuno-gold electron microscopy analysis, the enzyme could be localization to the cytosol (van der Lende et al., 2002b). In *A. nidulans*, a fusion of ACVS with GFP localized to the cytosol (Evers et al., 2004). This localization is in accordance with pH optimum of the enzymatic activity. An acidic vacuolar pH as well as the highly proteolytic environment would not support the activity of ACVS (Evers et al., 2004).

Isopenicillin N synthase

Next, the tripeptide is modified and the bicyclic penicillin ring is formed. This reaction is catalyzed by isopenicillin N-synthase (IPNS), which is a mononuclear non-heme iron dependent oxidoreductase, found in fungi and bacteria that are producers of penicillins and cephalosporins (Baldwin and Abraham, 1988). In this reaction IPNS binds the ACV tripeptide. In the reaction four hydrogen atoms are extracted from the ACV tripeptide and a molecule of oxygen is used with the formation of two water molecules (Chen et al., 1989). This reaction results in the formation of the β-lactam and thiazolidine rings. IPNS requires the presence of the ferrous iron (Fe^{2+}) and molecular oxygen (Burzlaff et al., 1999; Cooper, 1993) for catalysis. Isopenicillin N synthase is a 38 kDa protein, encoded by the *ipnA* (*pcbC*) gene. This gene is part of the penicillin biosynthesis gene cluster. Based on the results of the cellular fractionation studies IPNS is a soluble, cytosolic enzyme (Müller et al., 1991; Tobin et al., 1990; van de Kamp et al., 1999). Thus, in the cytosol the tripeptide is transferred from ACVS to IPNS.

Acyl Coenzyme A: Isopenicillin N Acyltransferase

The last step in penicillin biosynthesis is the replacement of the L-α-aminoadipate moiety of isopenicillin N for a phenylacetyl or phenoxyacetyl group. This replacement is mediated by *acyl-coenzyme A: isopenicillin N acyltransferase* (IAT) that is a microbody localized enzyme (Müller et al., 1991; Sprote et al., 2009). In this reaction the relatively hydrophobic penicillin G or V are synthesized (Brakhage, 1998). IAT is encoded by *penDE* gene, which belongs to the penicillin biosynthesis gene cluster. IAT originates from a precursor of 40 kDa, which is post-translationally modified (acylation) and cleaved to yield a heterodimer, comprised of two subunits: the α-subunit of 11 kDa and β-subunit of 29 kDa (Aplin et al., 1993; Tobin et al., 1990). The β-subunit contains a C-terminal ARL sequence, which targets the IAT to the microbody matrix. Removal or mutation of this motif results in the mislocalization of IAT and abolished penicillin biosynthesis in *P. chrysogenum* (Müller et al., 1992).

CoA ligases

The hydrophobic precursors for β-lactam biosynthesis need to be activated into their respective CoA thioesters (Evers et al., 2004) before the IAT catalyzed exchange can take place. For the precursors PAA and POA, this reaction is catalyzed by phenylacetyl–CoA ligase (PCL). The optimal pH for the PCL activity is 8.5, which corresponds to the pH of the microbody matrix (van der Lende et al., 2002a, Koetsier et al., 2010). PCL belongs to the acyladenylate protein family. First, the acyl or aryl acids are activated to acyl-AMP or aryl-AMP in a reaction that requires ATP. Next, the carboxyl group is transferred to the thiol group of CoA and a thioester is formed while AMP is released (Turgay et al., 1992). Other members of the acyladenylate protein family are the non-ribosomal peptide synthetases (NRPS), fatty acid–CoA synthetases, luciferases and coumarate–CoA ligases (Turgay et al., 1992). PCL is encoded by the *pclA* gene, which does not belong to the penicillin biosynthesis cluster (Lamas-Maceiras et al., 2006). Amplification of the *pclA* gene led to a 8–fold increase in the phenylacetyl CoA ligase activity and a 35 % increase in the penicillin production (Lamas-Maceiras et al., 2006). Disruption of the *pclA* gene in the *P. chrysogenum* Wis54–1255, a low yielding strain, caused a 40 % decrease of penicillin production and a similar reduction of the cellular PAA-CoA ligase activity (Lamas-Maceiras et al., 2006). This suggests that other proteins are present with PAA-CoA ligase activity (Meijer et al., 2010a). Biochemical studies on PCL indicate that this enzyme has a higher activity for fatty acids than for POA and PAA. Besides, PCL does not contain an optimal binding site for POA or PAA. This suggests that PCL may have developed from an enzyme that was, or still is, involved in the activation of fatty acids during β-oxidation. Likely, years of evolution and improvement of industrial strains in the direction of a higher production of penicillin G could have recruited PCL into biosynthesis pathway of penicillins (Koetsier et al., 2009).

The *P. chrysogenum* genome possesses eight acyl-CoA ligases (Meijer et al., 2010a; van den Berg et al., 2008). All genes contain a peroxisomal targeting signal (PTS1) and the corresponding proteins localize to the microbody matrix. Out of eight identified genes, *aclA* is upregulated when the fungus is grown in the presence of adipic acid, a side chain precursor that is used in the fermentative production of cephalosporins, adipoyl-7-aminocephalosporanic acid (ad-7-ACA) or adipoyl-7-desacetoxyaminocephalosporanice acid (ad-7-ADCA) (Veiga et al., 2012). AclA is involved in the activation of saturated fatty acids, including adipic acid. Based on the penicillin titers in Δ*aclA*, Δ*pcl* and Δ*aclA*-Δ*pcl* deletion strains, AclA appears not to be involved in the penicillin biosynthesis. Since the *pcl* deletion strain of *P. chrysogenum* DS17690 still produces about 20 % of the penicillin G compared to the wild type (DS17690), one or more of the remaining identified acyl-CoA-ligase might exhibit activity towards PAA (Koetsier et al., 2010).

MICROBODIES

Microbodies are a key player in the process of the β-lactam biosynthesis as they harbor the last two key enzymes in biosynthesis, i.e., IAT and PCL. Microbodies are small organelles surrounded by a single membrane. They were mentioned for the first time by Rhodin in 1954 as a special type of cytoplasmic body, where a single membrane encloses a proteinaceus matrix, devoid of DNA (De Duve et al., 1966). Microbodies can differ in size from 200 to 800 nm in diameter and are present in the cells of all eukaryotic organisms (Müller et al., 1992). Based on their biochemical function, different classes of microbodies are described: peroxisomes, glycosomes, glyoxysomes, hydrogenosomes and Woronin bodies. Peroxisomes are involved in the utilization of hydrogen peroxide (De Duve et al., 1966), whereas glyoxysomes possess two unique enzymes of glyoxylate cycle, namely malate synthase and isocitrate synthase. Glyoxysomes are present in germinating oily seeds (Tolbert, 1971). Glycosomes are present in protozoan kinetoplastida, including pathogenic trypanosomatids as Trypanosoma and Leishmania. They harbor enzymes required for the survival of trypanosomes in the blood stream of their host (Coley et al., 2011; Michels et al., 2006). Hydrogenosomes compartmentalize the final steps of energy metabolism in the number anaerobic eukaryotes (Hackstein et al., 2001) and also show some relatedness with mitochondria. Woronin bodies are specialized peroxisomes characteristic only for filamentous fungi. They function as a plug for septal pores upon hyphal damage, to prevent a cytoplasmic leakage (Tenney et al., 2000). While most of these organelles differ in their types of metabolism and function within the cells, they utilize common mechanism of biogenesis. In the next section, the biogenesis of these organelles is discussed as well as the role of microbodies in β-lactam production.

All types of microbodies follow similar route of biogenesis, with some differences that are specific for each class of organelle. Yeast and fungal microbodies can be formed by *"de novo"* synthesis from the endoplasmic reticulum or they proliferate by fission from pre-existing organelles (Hettema et al., 2009; Thomas and Erdmann, 2005). In *P. chrysogenum*, microbodies are mainly formed in the metabolically active subapical tip of the hyphae, from which they are directly distributed to the freshly formed cells (Meijer et al., 2010b). Proteins involved in the formation of microbodies and the import of the matrix proteins are called peroxins which are encoded by the PEX genes (Kiel and van der Klei, 2009). In the yeast *Hansenula polymorpha*, these PEX genes are crucial for both pathways of microbody synthesis, *de novo* and by fission. Microbody biogenesis is fully inhibited when both pathways are blocked, i.e.by deletion of *PEX25* and *PEX11* respectively (Saraya et al., 2011).

Microbody biogenesis by fission

The microbody biogenesis by fission is a multistep process. It is initiated by the interaction of Pex11 with the membrane, what induces membrane curvature. This is

followed by elongation and constriction of the membrane (Bonekamp et al., 2013; Opaliński et al., 2011). Microbody division requires Dynamin Like Proteins (DLPs) such as Dnm1 and Vps1. These are GTPases involved in intracellular fission processes (Thomas and Erdmann, 2005). The DLPs form a ring like structures around constricted membrane, to mediate membrane division by GTP hydrolysis, leading to the formation of a new microbody. DLPs are mobilized to the microbodies (and mitochondria) by the Fis1 (all eukaryotes) and Mff (mammals only) proteins. These are membrane adaptor proteins, tail-anchored to the microbody (and the outer mitochondrial) membrane (Bonekamp et al., 2013; Opaliński et al., 2012; Opaliński et al., 2011; Schrader and Fahimi, 2008; Thomas and Erdmann, 2005)

The first DLP shown to be involved in the microbody fission is Vps1 of *Saccharomyces cerevisiae* (Hoepfner et al., 2001). Vps1 cooperates with Dnm1, which is also involved in the fission of mitochondria. These proteins are also present in *H. polymorpha* (Saraya et al., 2011), although only Dnm1 appears to be involved in the fission of microbodies (Nagotu et al., 2008). A hypothesis was proposed by Schrader, that in most eukaryotes a single Dnm1/Fis1 based system is involved in the fission of both organelles, microbodies and mitochondria, which provides a functional link between these organelles (Schrader and Fahimi, 2008). It is unknown whether the same holds for *P. chrysogenum* (Camöes et al., 2009), although overexpression of Dnm1 in *P. chrysogenum*, results in increased microbody numbers (Meijer et al., 2010b).

In *P. chrysogenum* three Pex11 family members have been identified, namely: Pex11, Pex11B and Pex11C, (Kiel et al., 2006). These all localize exclusively to the microbody membrane. Phylogetically, Pex11 and Pex11B are clustered on the same clade, whereas Pex11C shows a higher homology to Pex11C of *H. polymorpha*, located on the separate clade (Opaliński et al., 2012). Although, Pex11 and Pex11B have low sequence identity (22% identity, 35% similarity), their structures are highly similar, which suggests an identical topology and analogous function. Pex11 and Pex11B possess two N-terminal amphipathic helices and three C-terminally located hydrophobic regions (Opaliński et al., 2011). On the other hand, Pex11C shares only 17% similarity with Pex11, while also the location of amphipathic and hydrophobic regions differs significantly (Opaliński et al., 2012). The overproduction of Pex11 in *P. chrysogenum* leads to the massive proliferation of microbodies. Pex11 plays a key role in the peroxisome proliferation. Interestingly, the Δ*pex11* as well as triplicate Δ*pex11*Δ*pex11B*Δ*pex11C* mutants show organelles with a reduced size, which may represent immature microbodies. Importantly, this suggests a Pex11 independent mechanism of microbody formation. The additional deletion of *vps1* did not result in further significant changes in the number and size of the microbodies, which led the authors to suggest that *P. chrysogenum* may contain an additional route for peroxisomal formation. The microbodies present in the cells, lacking the key components of the fission machinery, are likely synthesized by the *de novo* pathway, which is independent from *Pex11* and *Vps1* (Opaliński et al., 2012).

Microbody biogenesis by formation de novo

The mechanism of *de novo* microbody formation from endoplasmatic reticulum is still largely unknown. In yeast *H. polymorpha* and *S. cerevisiae*, the *de novo* formation of microbodies requires Pex3, Pex19, Pex25 and RhoI, whereas higher eukaryotes also dependent on Pex16 (Kim et al., 2006; Opaliński et al., 2012; Saraya et al., 2011). Mutants with a disrupted function of those genes are devoid of microbodies. Importantly, in yeast and mammals, re-introduction of Pex3p, Pex19 and Pex16, restored microbody formation, which led to the postulation of *de novo* microbody formation (Faber et al., 2002; Geuze et al., 2003; Haan et al., 2006; Kim et al., 2006; Matsuzono et al., 1999; Muntau et al., 2000; South and Gould, 1999; Titorenko and Rachubinski, 2001). Newly synthetized Pex3, Pex16 and Pex19 are targeted to the ER membrane, from which new microbodies are developed (Hoepfner et al., 2005; Kim et al., 2006; Matsuzaki and Fujiki, 2008). Pex16 is a peroxisomal membrane protein, essential for microbody development in higher eukaryotes only. It was proposed that Pex16 regulates de novo microbody formation. Through its co-translationally insertion into the ER membrane, it may recruit other peroxisomal membrane proteins, e.g. Pex3 (Kim et al., 2006) and thereby control the process of the *de novo* synthesis.

Recently, the role of Pex3, Pex16 and Pex11 in *de novo* microbody synthesis in *P. chrysogenum* was investigated (Opaliński et al., 2012; Opaliński et al., 2011). Pex16 fused to GFP localizes to the microbody membrane. However, green fluorescence was also recorded to small organelle like structures tentatively assigned as early microbodies. The *P. chrysogenum* Δ*pex16* mutant however still possess partially functional microbodies, although matrix proteins were largely mislocalized to the cytosol (Opaliński et al., 2012). In this mutant also the level of Pex11 was decreased which implies that interference with the de novo synthesis may also affect fission and proliferation. Concluding, in *P. chrysogenum* Pex16 is involved in microbody development, however, it is not a key protein in this process, whereas Δ*pex3 P. chrysogenum* mutants were completely devoid of microbodies. Interestingly, unlike yeast (Kiel et al., 2006), PEX25 is not present in the genome of filamentous fungi. This suggests a different mechanism of *de novo* microbody formation compared to yeast (Opaliński et al., 2012; Saraya et al., 2011)

Role of microbodies in β-lactam biosynthesis

In *P. chrysogenum* cells, which have abnormal or which are devoid of microbodies, the penicillin titer is severely decreased or abolished (Meijer et al., 2010b; Opaliński et al., 2012). Additionally in *P. chrysogenum*, the mislocalization of IAT to the cytosol inhibits the penicillin synthesis (Müller et al., 1992). Similar observations were reported for *Aspergillus nidulans*, where the lack of functional microbodies led to the significant decrease of produced levels of penicillin. However, *A. nidulans* contains two variants of IAT, one of which localizes to the cytosol. Also, this fungus is able to produce penicillin when the microbody IAT is redirected to the cytosol (Sprote et al., 2009).

A comparison of different industrial strains shows that high producing strains of *P. chrysogenum* (DS17690) contain larger numbers of microbodies than low producing strain (NRRL1951) (van den Berg et al., 2008). Interestingly, in the low producing *P. chrysogenum* strain, the overexpression of peroxisome biogenesis gene Pex11 induced the massive proliferation of microbodies, which resulted in the 2-3 fold increase of the penicillin production. Similar observations were made when Pex14/17 was overexpressed in *P. chrysogenum* NRRL1951 (Kiel et al., 2005; Opaliński et al., 2012). However, the level of microbody localized enzymes (PCL and IAT) remained unchanged (Kiel et al., 2005). These findings led to the conclusion that the proliferation of the microbody membrane increases the efficiency of penicillin production by increasing the flux of penicillin related metabolites through the membrane. This would thus suggest that transport across the microbody membrane limits production. In this respect, the inhibition of autophagy, the main cellular process that recycles microbodies, led to the 2-fold increase of the microbody number, which also resulted in increased penicillin titer (Bartoszewska et al., 2011).

The β-lactam production activity has also been examined for a series of *PEX* deletion strains of *P. chrysogenum*, i.e., *pex11*, *pex16* and *pex3*. Whereas the Δ*pex11* mutant showed no apparent change, the Δ*pex3* and Δ*pex16* strains showed a 50% reduction in the penicillin G production compared to the parental strain. In these mutants, microbody matrix proteins are mislocalized to cytosol. These data indicate that microbodies are required for efficient penicillin biosynthesis (Bartoszewska et al., 2011; Opaliński et al., 2012). The latter is likely because of several reasons. The matrix of the microbodies provides an optimal environment for IAT and PCL as far a pH concerns. For both enzymes the pH optimum is alkaline, and they are inactive at a pH lower than 6. This is in accordance with the pH of microbody matrix in *P. chrysogenum* that was shown to be slightly alkaline (pH 7.0- 7.5) (van der Lende et al., 2002a). The pH of the microbodies in CHO cells is neutral (pH 6.9- 7.1) (Jankowski et al., 2001), alkaline in the yeast *S. cerevisiae* (pH 8.2) (van Roermund et al., 2004) and acidic in the yeast *H. polymorpha* (Nicolay et al., 1987) . Compartmentalization of the penicillin biosynthesis and location of the last two steps in the microbody may also maximize the flux because of other reasons. The confined environment of the small organelles may provide a means to obtain higher concentrations of enzymes and substrates involves, possibly resulting in substrate channeling between PCL and IAT. This might prevent a loss of intermediates and allow for the regulation of the biosynthesis pathway separate from the processes that occur in the cytosol. It should be emphasized that in *A. nidulans*, also cytosolic penicillin biosynthesis can take place (Sprote et al., 2009). *A. nidulans* contains two IAT enzymes, one localized in the microbody and one in the cytosol. Gene inactivation of microbody IAT results in a drop in productivity by 80 %. Currently, it is not known how the cytosolic IAT is supplied with the CoA activated side chains that are normally produced in the microbody. Possibly, *A. nidulans* also contains a cytosolic PCL-like enzyme.

TRANSPORT DURING BIOSYNTHESIS OF β-LACTAM ANTIBIOTICS

A key question is how β-lactams and intermediates are transported across the microbody and plasma membrane. However, because of the high degree of compartmentalization of the penicillin biosynthetic pathway, there is also a requirement for intracellular transport of metabolites and precursors. Our current insights in the mechanism of transport are discussed in the following section.

Uptake of carboxylic acid precursors

The most commonly used side chain precursors during the production of penicillins are PAA and POA. They are weak organic acids, which in their undissociated form are able to enter *P. chrysogenum* through passive diffusion (Hillenga et al., 1995). However, it has been suggested that in the pH range of 5 to 8, PAA is also actively transported from the medium into the cell (Fernandez-Canon et al., 1989). The differences observed in these studies may be related to the concentration of PAA used and a type of strains (high- and low- producer). At high concentration of precursor as used in typical fermentations, PAA readily enters a cell through a passive diffusion. However, at low concentrations, the accumulation of PAA is 10 times lower in the lower yielding strains as compared to the high yielding strains, which suggests the involvement of transport proteins. Alternatively, in the latter study, the capture of the PAA into its CoA derivative is monitored that mistakenly is taken as a measure of uptake. This is in particular a problem in uptake studies when dealing with highly membrane permeable metabolites. During the production of β-lactams, the side chain precursors are added in the high concentrations to the fermentation broth, as likely passive diffusion is the main way to enter the cells (Hillenga et al., 1995).

Studies on the expression responses of *P. chrysogenum* genes on the presence of PAA, showed that some of the transport related genes are upregulated under such conditions (Harris et al., 2009). Later, a perturbation experiment was performed with PAA (also with penicillin G) in chemostat cultures, where PAA was either pulsed in the chemostat or its concentration was increased linearly within time (Douma et al., 2011). In these experiments the *P. chrysogenum* DS17690 strain was used which is a high penicillin yielding strain. In both experiments dynamics of intracellular levels of PAA (and penicillin G) and oxygen were measured to get more insight into the mechanism and kinetics of transport processes. Results of both experiments suggest that undissociated form of PAA enters the cell by passive diffusion. Interestingly, after the pulse of PAA to the culture medium, a drop of culture pH was observed. This observation suggested that *P. chrysogenum*, as *S. cerevisiae*, contains indeed a weak cation exporter, enabling secretion of PAA at the expense of ATP (Douma et al., 2011; Weber et al., 2012a). Furthermore, it was calculated that the amount of PAA, which enters the cell by passive diffusion, is two orders of magnitude higher than

the maximum biomass specific consumption rate required for penicillin G production. This implicated that passive import of PAA is not a limiting factor for penicillin G production. These results support a PAA transport model, where PAA import occurs by passive diffusion and export is an active process (Douma et al., 2011) consistent with earlier studies on PAA transport (Hillenga et al., 1995).

Toxicity is a phenomenon that has also been associated to higher concentrations of PAA, hence the less toxic POA is often used for fermentation. In *S. cerevisiae*, the ABC transporter PDR12 is involved in a defense mechanism against toxic carboxylic acids by removal of water-soluble carboxylate anions (Piper et al., 1998). In this respect, various gene clusters in *P. chrysogenum* which are involved in transport and detoxification processes are strongly upregulated in the presence of PAA (van den Berg et al., 2008). Recently, it was demonstrated that the ABC transporter ABC40 of *P. chrysogenum* is responsible for PAA extrusion from the cell to prevent toxicity for *P. chrysogenum* when the β-lactam production capacity is low (Weber et al., 2012a). Interesting, in high yielding strains the PAA consumption is relatively rapid resulting in low extracellular PAA concentrations. Since PAA induces the expression of ABC40, in high yielding strains ABC40 is not highly expressed and instead β-lactam synthesis acts a mechanism of PAA detoxification.

Intracellular transport

As discussed in a previous section, microbodies play a crucial role in penicillin biosynthesis. This raises questions with respect to the transport of the intermediates across the microbody membrane. The membrane of microbody is considered to be impermeable to solutes. Molecules like NAD(H), NADP(H), acetyl CoA, ATP and protons cannot freely diffuse through the membrane (Rottensteiner and Theodoulou, 2006). Recently it was proposed that the microbody membrane of *S. cerevisiae* harbors a porin-like structure, which allows for free metabolite flow between the microbody matrix and the cytosol for solutes with a molecular mass up to 400 Da (Antonenkov et al., 2009).

Up to now, very few microbody membrane localized transporters have been identified and characterized. In *S. cerevisiae*, an adenine nucleotide transporter (Ant1p) has been identified. The adenine nucleotide transporters belong to the ADP/ATP Mitochondrial Carrier Family. It was suggested that Ant1p of *S. cerevisiae* represents a novel type of MCF, since it is able to transport AMP, ADP and ATP (Palmieri et al., 2001). ATP is used in the microbody matrix for the activation of fatty acids, including adipic acid (precursor of cephalosporins) but also the activation of the side chain precursors POA and PAA used in the penicillin biosynthesis. Homologs of Ant1p have also been identified and localized to the microbody membrane of *P. chrysogenum*. Deletion of one of the Ant1p homologs resulted in a major reduction in β-lactam biosynthesis (Woszczyńska et al., unpublished) consist with the notion that ATP is needed for precursor activation.

Recently, a gene *paaT* (*P. chrysogenum)* was identified (Fernandez-Aguado et al., 2012) which encodes a homologue of *A. chrysogenum* MFS protein CefT. The *cefT* gene is located in the cephalosporin gene cluster and encodes a cephalosporin transporter (Martín et al., 2005; Ullán et al., 2002). Expression of *paaT* is stimulated by PAA (van den Berg et al., 2008), and a GFP fusion of PaaT localizes to the peroxisomal membrane. Silencing of *paaT* in *P. chrysogenum* led to the reduction of penicillin G production, while levels of isopenicillin N and intracellular penicillin G remained comparable to parental strain (*P. chrysogenum* Wisconsin 54-1255). Silencing as well as overexpression of *paaT* led to changes in the sensitivity to PAA (phenyl acetic acid). Cells with overexpressed *paaT* showed a higher radial growth compared to the deletion mutants. Based on these finding it was suggested, that PaaT is a transporter for PAA (Fernandez-Aguado et al., 2012). This mechanism of PAA transport (Fernandez-Aguado et al., 2012) contradicts a well-defined model for PAA transport (Douma et al., 2011). Possibly, this controversy might relate to differences in substrate concentration and/or be strain dependent.

Taking a similar approach as for PaaT (Fernandez-Aguado et al., 2012) a protein was identified that was suggested to be involved in isopenicillin N transport which is a homologue of the *A. chrysogenum* CefM protein, i.e., PenM (Fernández-Aguado et al., 2014). Based on the in-silico analysis PenM is described as MFS protein, which possess pex19 binding site, enabling insertion into microbody membrane. PenM is a membrane protein containing 12 transmembrane spanning domains that appears to localize to the microbody membrane (Fernández-Aguado et al., 2014) although a DSRed-PenM fusion protein showed only luminal fluorescence instead of membrane staining of the microbody membrane. The role of PenM in the β-lactam biosynthesis was studied by overexpression and silencing studies. Overexpression of *penM* led to substantial increase of isopenicillin N and penicillin G levels. In the cultures of *penM* silenced mutants very low levels of β-lactams where detected, additionally results of bioassays show a substantial reduction of penicillin G biosynthesis. In contrast to isopenicillin N, no intracellular penicillin G accumulation was observed, and the author suggested that in these strains the penicillin G biosynthesis is impaired. It was proposed that PenM transports isopenicillin N across the microbody membrane (García-Estrada et al., 2007; Ullán et al., 2010). CefM of *A. chrysogenum* has been proposed to be involved in the transport of penicillin N, being the D enantiomer of isopenicillin N (Fernández-Aguado et al., 2014; Teijeira et al., 2009).

Excretion of β-lactams into the medium

The mechanism of β-lactam secretion across the plasma membrane has remained elusive. In the industrial production of β-lactams the extracellular concentration of penicillin G is 10-fold higher than inside the cell, which suggests that active transport takes place during the secretion of penicillin G (van den Berg et al., 2008). Based on the outcome of the growth experiment performed with penicillin G in the chemostat *P. chrysogenum*

culture, a model for transport of penicillin G through the cell membrane was proposed, anticipating reversible transport (Douma et al., 2011). After addition of penicillin G to the culture, intracellular levels directly increased. Authors indicates that maintenance of the equilibrium ratio of extracellular to intracellular penicillin G concentration pinpoints that secretion of penicillin G occurs via facilitated diffusion (Douma et al., 2011). Possibly, the export of penicillin G in *P. chrysogenum* DS17690 strain is a limiting factor for β-lactam biosynthesis, as the export capacity is comparable to the maximum biomass specific penicillin G production rate (Douma et al., 2011; van Gulik et al., 2000).

Moreover, intracellular accumulation of penicillin G was observed when verapamil, an inhibitor of ABC transporters, is added to growing cells (Van den Berg et al., 2001). The penicillin biosynthetic cluster of *P. chrysogenum* does not contain any transporter specific open reading frame (van den Berg et al., 2008), in contrast to some other β-lactam producing organisms. In *A. nidulans,* the deletion of the *atrD* gene, encoding a multidrug resistance type of ABC transporter, resulted in a 2-fold decrease in penicillin production (Andrade et al., 2000). In the cephalosporin producing bacteria *Streptomyces clavuligerus and Nocardia lactamdurans,* the *cmcT* gene in the cephalosporin biosynthesis cluster encodes a multidrug efflux transporter, belonging to the major facilitator superfamily (MFS). Although in *S. clavuligerus cmcT* is expressed only under cephamycin producing conditions, its involvement in the secretion of cephamycin remains to be shown (Martín et al., 2005). In *Acremonium chrysogenum*, CefT is involved in the secretion of cephalosporin C. The *cefT* gene is part of the cephalosporin C biosynthesis cluster. Overexpression of CefT resulted in a 2-fold increase of cephalosporin C production, whereas deletion of *cefT* had no effect on production (Martín et al., 2005; Ullán et al., 2002). Moreover, the overexpression of CefT in an engineered strain of *P. chrysogenum* resulted in the improved production of specific cephalosporins (Nijland et al., 2008). As mentioned in the previous section of this chapter, PaaT, a homologue of CefT in *Penicillium chrysogenum* was recently described as a transporter of penicillin G precursor, PAA (Fernandez-Aguado et al., 2012).

Multidrug resistance (MDR) transporters secrete a variety of unrelated, usually hydrophobic compounds across the membrane. Since, penicillins are amphipathic, moderately hydrophobic molecules, MDR transporters are likely candidates for secretion of β-lactams (Evers et al., 2004). In fungi, MDR transporters either belong to the ATP-binding cassettes (ABC) transporter superfamily or the major facilitator superfamily (MFS) transporters. The ABC transporters form a large family of proteins, possessing a broad spectrum of substrate specificity. They can translocate both, small and large molecules, across the membrane. ABC transporters are comprised of two cytosolic nucleotide binding domains (NBDs), each of them containing of highly conserved Walker A and B motifs that specify the nucleotide binding site and the ABC signature motif, located between Walker A and B. The nucleotide binding domains interact with two transmembrane domains (TMDs), which consist of six transmembrane spanning segments. In fungi, the NBDs and TMDs may have different arrangements,

and either are fused into a single large polypeptide (full transporter) or exist as fused NBD/TMDs that arrange as homo- or heterodimers (half-transporters). MFS transporters are either characterized as uniporters, symporters or antiporters and cover a broad range of substrate specificities. MFS proteins are secondary transporters, which transport a solute in a process that is driven by electrochemical gradient of solute and by the proton motive force (Del Sorbo et al., 2000). The genome of *P. chrysogenum* contains 830 transporter protein encoding genes. Of this group, 688 are secondary transporters, with majority being member of the MFS (416 genes). There are 51 ABC transporters. DNA microarray studies with high and low producing strains (DS17690, Wisconsin54-1255) grown under penicillin producing conditions (i.e., with PAA versus without PAA) showed that genes encoding proteins involved in the metabolism, transport and detoxification of the cell are significantly overrepresented in the genes up regulated. This result suggests that MDR-like transporters might be involved in excretion of penicillin. However, because of the abundance of MDR like transporters, penicillin secretion may result from the simultaneous activity of multiple transporters (van den Berg et al., 2008) which makes their identification difficult.

CONCLUDING REMARKS

In the past decade major insights have been obtained in the enzymology and genetics of the penicillin biosynthesis pathway in the filamentous fungus *P. chrysogenum*. After years of classical strain improvement, *P. chrysogenum* has been developed into an excellent cell factory for β-lactam antibiotics and the current strains harbor superb fermentation characteristics. Compartmentalization of β-lactams biosynthesis and localization of the last two enzymatic steps within the microbody matrix, provides an optimal flux of the pathway but also still many elements of this compartmentalization are poorly understood. Although the genome sequencing and microarray analysis have been a major step forward in understanding the molecular basis of the improvement of β-lactam biosynthesis in industrial *Penicillium* stains, still more insight analysis is needed about enzyme specificity, the critical transport steps and possible limiting factors in biosynthesis. This necessitates a more detailed analysis for instance, using proteomics to identify the transporters involved in β-lactam transport across the plasma membrane and the microbody membrane.

During the rounds of classical strain improvement program, the penicillin biosynthesis cluster was intensively amplified between tandem repeats. In may also be a source of genetic instability. Recently, the correlation between the number of penicillin biosynthesis clusters, gene and protein expression and β-lactam biosynthesis has been studied in a series of isogenic strains that only differ in the number of biosynthesis gene clusters (Nijland et al., 2010). This revealed that the functional expression of *pcbAB* gene encoding ACVS lags behind relative to the other structural genes. While the protein levels of ACVS and IPNS correlate well with the transcripts levels, this is

not the case for IAT. Strikingly, the amount of IAT produced by an eight copy-cluster strain is only 2-fold higher than that of the single-cluster-copy-number strain, while the number of microbodies did not change significantly. Additionally, at high cluster copy number, intracellular accumulation of isopenicillin N occurs, which indicates that the IAT activity might be a limiting step for penicillin production in high yielding strain (Nijland et al., 2010). The molecular basis for this inefficient IAT production is unknown but could relate to the processing and/or targeting to the microbodies. On the other hand, activation of the side chain could be a limiting factor as well. Introduction of phl (PCL) and pcbDE (IAT) to single copy penicillin cluster strain *Penicillium chrosygenum* DS47274 had a minor impact on the increase of penicillin biosynthesis. However, in case of high copy number strains the overproduction of IAT led to the decrease of penicillin V and increase of 6-APA concentration (Alvarez et al., 1993; Weber et al., 2012b). Due to imbalance in availability of ACVS, IPNS and excess of IAT, IAT functions as an hydrolase and converts isopenicillin N (or Penicillin V) to 6-APA. This result indicates that in general homeostasis in the availability of penicillin biosynthesis enzymes is critical for synthesis of penicillin. Whereas in high yielding penicillin production strains the amount of available IAT, as well as transport of isopenicillin N or penicillin V out of the microbody and the cell is the limiting factor (Weber et al., 2012b).

Finally, a thorough understanding of the β-lactam biosynthetic pathway and the nature of the classical strain improvement program can help to further develop *P. chrysogenum* into a production strain for secondary metabolites other than antibiotics. This will be a main challenge for the future.

SCOPE OF THE THESIS

The aim of the work described in this thesis was to identify microbody-localized membrane transporters involved in the biosynthesis of β-lactams in *P. chrysogenum* using proteomic and genomic approaches. The focus is on the identity and functionality of the microbody membrane transporters.

Chapter 1 describes a general introduction of β-lactam biosynthesis in *P. chrysogenum* with an emphasis on the compartmentalization of the pathway and the role of transport proteins in the various (sub)cellular steps.

In **Chapter 2**, a proteomic approach employing mass spectrometry is described to map the microbody membrane proteins. First organelles were purified by differential fractionation; membrane proteins where separated by SDS-PAGE, digested with trypsin and analyzed using tandem mass spectrometry. This chapter summarizes the results and discusses the methods used in the isolation and analysis of the membrane proteins.

Chapter 3 describes a study performed on the four *Penicillium chrysogenum* homologs of the yeast adenine nucleotide transporter Ant1. Activation of side chain precursors into CoA derivatives occurs in the microbodies and requires ATP. This chapter examined and discusses the involvement and role of the adenine nucleotide transporters, in particularly Pc13g03380 in β-lactam production.

Chapter 4 describes the cellular localization and the role of the two *P. chrysogenum* peroxisomal ABC-D transporters, namely Pc13g11640 and Pc16g09390. In yeast, these transporters are involved the transport of CoA-derivatized long chain fatty acids, and could potentially be involved in the transport of CoA required for activation of the side chain precursors during in β-lactam biosynthesis. The studies shown that in the filamentous fungus *P. chrysogenum*, these transporters may have a similar role as in yeast but also shows that they are not essential for β-oxidation nor for CoA supply of microbodies. Hence other routes for CoA entry must exist.

Industrial penicillin production levels by the filamentous fungus *P. chrysogenum* have increased dramatically during the years because of an intense classical strain improvement.

Chapter 5 describes the analysis of the gene cluster dose effect on penicillin production using the high yielding *P. chrysogenum* strain DS17690 that was cured from its native clusters. Results of these studies suggest that at high penicillin gene cluster numbers, productivity is limited by the functional levels of acyltransferase and/or the availability of CoA-activated side chains.

In the final chapter of this thesis, the work is summarized and possible future directions of the work are discussed.

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chapter 2

PROTEOMICS OF THE MICROBODY MEMBRANE OF *PENICILLIUM CHRYSOGENUM*

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ABSTRACT

Since the discovery of the important role of microbodies in penicillin biosynthesis by the filamentous fungus *Penicillium chrysogenum*, the mechanisms of intracellular transport of penicillins of its precursors has remained obscure. Here we have used proteomics of isolated microbodies to identify membrane proteins associated with these organelles. Herein, an engineered industrial *Penicillium chrysogenum* strain was used that expressed a variant of Pex11 fused at its C-terminus to Protein A to allow for immunopurification following differential centrifugation. The microbody membrane protein fraction was analyzed by SDS-PAGE followed by tryptic digestion and LC-MS/MS (MALDI) analysis. We identified about 70 proteins, among which enzymes involved in the β-lactam biosynthesis as well as two microbody membrane proteins. The majority of the proteins appear contaminants. These data show that the identification of low abundant membrane proteins remains challenging because of the difficulties to isolate pure organelles.

1. INTRODUCTION

β-lactam antibiotics belong to the oldest but also the most widely used group of anti-microbial medicines. From the event of penicillin discovery, the production of β-lactams by *Penicillium chrysogenum* strain has increased 1,000 fold (Elander, 2003). The increase in the efficiency of β-lactam production was achieved by continues strain improvement program (CSI) as well as by media optimization (Newbert et al., 1997). The β-lactam biosynthesis pathway in *Penicillium chrysogenum* is localized in cytoplasm and lumen of microbodies of the fungal cell. Formation of the tri-peptide δ(L-α- aminoadipyl)- L-cysteinyl-D-valine (LLD-ACV), and cyclization into isopenicillin N (IPN) take place in the cytoplasm. These two reaction are catalyzed by *ACV synthase* (ACVS) and *isopenicillin N synthase* (IPNS), respectively.

The activation of the side chain precursors phenoxyacetic acid (POA) or phenyl acetic acid (PAA) to the CoA esters and the substitution of L-α-aminoadipate group for phenylacetyl- or phenoxyacetyl moiety in IPN, are localized in microbody. Both enzymes involved in this reactions, namely PCL (*phenylacetyl CoA ligase)* and IAT (*acyl-coenzyme A:isopenicillin N acyltransferase)* are active within microbody matrix (Hillenga et al., 1995; Müller et al., 1991; van de Kamp et al., 1999; van den Berg et al., 2008). Microbodies (or peroxisomes) are small (200-800 nm), single membrane-bound organelles. They are present in the cell of each living organism, and contain with a protein rich matrix. They are involved in many versatile biochemical pathways (Antonenkov and Hiltunen, 2006), but generally associated with the oxidation of fatty acids and utilization of hydrogen peroxide. Other microbody functions are more organism specific: glyoxylate cycle, photorespiration, biosynthesis of the plant hormones and penicillin biosynthesis in fungi (van den Bosch et al., 1992). In filamentous fungi, microbodies are involved in: the β-oxidation of fatty acids in *Neurospora crassa, Aspergillus nidulans, Candida albicans* (Kionka and Kunau, 1985; Valenciano et al., 1998; Valenciano et al., 1996), karyogamy in *Podospora anserine* (Berteaux-Lecellier et al., 1995) and penicillin biosynthesis in *Penicillium chrysogenum* (Meijer et al., 2010; Müller et al., 1992).

The penicillin biosynthesis pathway is localized in the cytosol and in microbodies. The presence of functional microbodies is critical for efficient β-lactam biosynthesis. High producing industrial strains of *P. chrysogenum* have a larger membrane surface area over volume of their microbodies as compared to low producing strains such as Wisconsin (Meijer et al., 2010; van den Berg et al., 2008). Moreover, the overexpression of Pex11 induced the proliferation of microbodies concomitantly with an increased penicillin production whereas the level of microbody matrix enzymes remained unaffected (Kiel et al., 2005; Opaliński et al., 2012). Pex14/17 is a novel protein involved in the recognition and targeting of PTS1 and PTS2 signal bearing microbody proteins. IAT and PCL, enzymes that are critical for the last steps in penicillin biosynthesis both contain a PTS1 targeting sequence. However, in a Pex14/17 deletion mutant of *P. chrysogenum*, PCL and IAT were still targeted to microbody matrix, although the level

of penicillin biosynthesis decreased 50-fold. On the other hand, overexpression of Pex14/17 led to the increase of the microbodies numbers which was paralleled by an increase in penicillin production (Opaliński et al., 2010). Interestingly, also the deletion of Atg1, a protein involved in the autophagy of microbodies, resulted in increased penicillin production (Bartoszewska et al., 2011a).

Although the importance of microbodies in β-lactam production is well established, only few attempts have been made to identify membrane proteins, in particular transporters that localized to the microbody membrane and that possibly contribute to penicillin production. The question as to whether transport across the microbody membrane is a specific process or mediated via pores or passive diffusion has been an issue of debate during the last 40 years. Studies on mammalian microbodies suggested that the microbody membrane is freely permeable for small molecules because of presence of pore-forming channels. However specific transporters are needed to transfer compounds as cofactors, ATP and long chain fatty acids (van den Berg et al., 2008). During penicillin biosynthesis, isopenicillin N that is a relatively hydrophilic compound needs to be transported into the microbody lumen. On the other hand, 6-aminopenicillanic acid (6-APA) seems to diffuse rapidly across the microbody and plasma membrane possibly by passive diffusion (Garcia-Estrada et al., 2007; Hillenga et al., 1995). Because of the hydrophobic nature of penicillins, one may argue that these molecules cross the microbody membrane through passive diffusion. However, since during fermentation, the extracellular levels of penicillin can be much higher than the intracellular concentration, it is generally assumed that penicillins are actively secreted across the plasma membrane (Hillenga et al., 1995; van den Berg et al., 2008). So far the transporters involved have not been identified in *P. chrysogenum*. In *Aspergillus nidulans* the ABC transporter AtrD is involved in penicillin production (Andrade et al., 2000). Moreover, penicillin production is sensitive to verapamil, which is an antagonist of the multidrug transporters, suggesting that penicillin secretion is an active process involving MDR-like ABC type transporters. A genetic analysis of a series of expressed ABC transporters by gene inactivation did not reveal an obvious candidate for a penicillin secretion system. In another approach, transporter genes upregulated by the presence of phenylacetic acid were analyzed. This revealed transporters involved in phenylacetate secretion, but none involved in secretion of penicillin (van den Berg et al., 2008).

The goal of this study was to develop a method for membrane isolation of microbodies from *P. chrysogenum* and identify membrane proteins by means of liquid chromatography and mass spectrometry. Herein standard organelle fractionation methods were combined with immunomagnetic isolation to enrich the microbody membranes (Kiel et al., 2009; Kikuchi et al., 2004). The organellar fraction was subjected to SDS-PAGE, and after trypsin digestion, peptides were analyzed by LC-MS/MS (MALDI-TOF) yielding only two microbody membrane proteins.

2. MATERIALS AND METHODS

2.1. Strains and media

Strains used in this study were derived from *P. chrysogenum* DS17690, and kindly supplied by DSM Biotechnology Center, Delft. For microbody isolation, a *P. chrysogenum* DS17690 strain expressing a Pex11-TEV-Protein A fusion protein was used, which was kindly provided by Jan Kiel, MCB, University of Groningen. Cells were grown on penicillin production medium (Nijland et al., 2010) for up to 64 hours, in the rotary shaker at 220 rpm and 25° C (Figure 1).

2.2. Microbody isolation

Organelles were isolated following the protocol described previously (Kiel et al., 2009) and as outlined in Figure 1. Cells of 2 litres of a culture of *P. chrysogenum* Pex11-TEV-Protein A grown for 68 hrs were collected on a paper filter and washed with KC buffer (0.8 M KCl, 10 mM citrate pH 6.2). Protoplasts were generated by incubation of the hyphae at 25°C for about 3 hrs with Glucanex® used at concentration of 25mg/mL (lysing enzymes from *Trichoderma harzianum*, L1412, Sigma). Protoplasts were recovered by filtration on the glass wool and directly cooled down to 4°C. Next, the protoplasts were washed with KC buffer supplemented with 1 mM PMFS (phenylmethyl sulphonyl fluoride), followed by a washing step using KC buffer mixed an equal volume of sorbitol buffer B (1.2M sorbitol in 5 mM MES pH5.5, 1 mM $MgCl_2$ and 1 mM EDTA) and supplemented with 1 mM PMSF.

Finally, the protoplasts were washed with sorbitol buffer B supplemented with 1 mM PMSF, and taken up in cold sorbitol buffer, containing the Complete protease inhibitors cocktail

(Complete, Roche) and 1 mM PMSF. Protoplasts were lysed using a potter homogenizer, and cell debri and non-lysed cells were removed using differential centrifugation: i.e., 4000 x g for 10 min., 6000 x g for 10 min., and 30000 x g for 20 min at 4°C. Finally, an organellar fraction (i.e., a mixture of mitochondria and microbodies) was obtained at 30000 x g (about 300-500 µg of protein) that was subsequently gently taken up in ice-cold 40 % (w/v) sucrose prepared in buffer B, loaded on the discontinuous sucrose gradient (35 – 52 % w/v) (Douma et al., 1985) and centrifuged for 2.5 hrs at 4°C using an SV28 Sorval rotor in a Sorval RC-5 high speed centrifuge. Gradients were collected in 1.5 ml fractions starting from the bottom of the gradient. The sucrose density and protein concentration was measured in each collected fraction. Fractions were precipitated with 25 % (w/v) trichloroacetic acid (TCA) and analyzed by SDS-PAGE and Western-Blot. Microbodies were recovered from the fractions corresponding to about 52 % sucrose (protein concentration about 0.6 mg/ml), lysed with ice-cold 0.1 M Tris-HCl pH 8.0 and centrifuged for 15 mins at 200.000 x g at 4 ° C to remove the matrix proteins. This step was repeated twice yielding a crude microbody fraction.

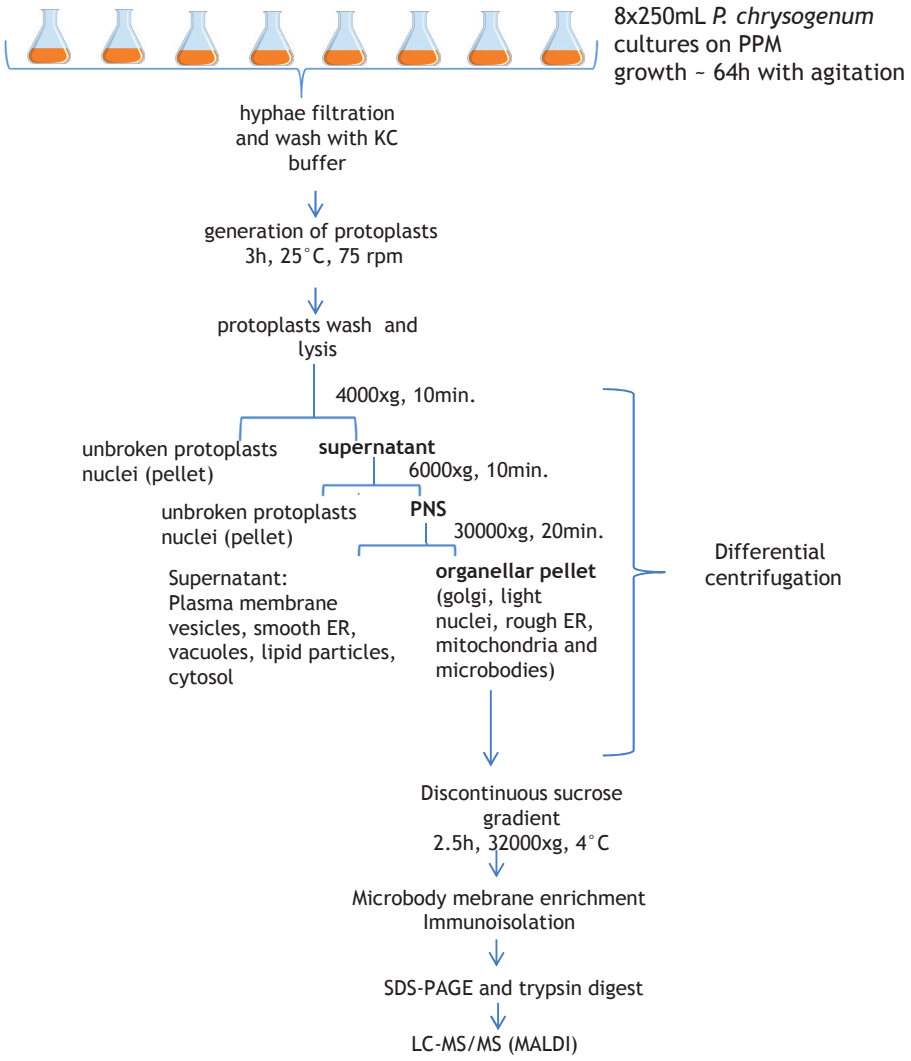

Figure 1. Schematic representation of the subsequent steps in the isolation of organelles.

2.3. Microbody purification and characterization

The crude microbody fraction was further characterized and purified using various methods:

1. Membranes were concentrated 5-fold by centrifugation (15 min at 200.000 x g at 4 °C, resuspended in ice-cold 50 mM Tris-HCl, pH 8.0 and subjected to SDS-PAGE and mass spectrometry.
2. Membranes were further purified by Immunoisolation using two types of magnetic Dynabeads (Life Technology, previous Dynal), i.e., Dynabeads M-500 and M-280

SAR (See Figure 1A). Dynabeads M-500 Subcellular are polystyrene beads (5 µm), which are surface activated with p-toluene-sulphonyl chloride, providing active groups for covalent attachment to antibodies. The surface of M-500 bead is of low hydrophobicity, which minimizes non-specific binding. By the use of microbody-specific antibodies, the use of these beads should ensure a successful separation of the microbody membranes from the protein rich contaminating mitochondrial membranes. The Dynabeads M-280 SAR was first conjugated with anti-sheep Rabbit IgG before binding the microbody specific antibody. Bead activation and antibody coating were performed according to the manufacturer instructions. Beads were vortexed, and the required amount was washed with PBS (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0, 24g KH_2PO_4), pH 7.4. Per sample ~50 µl of beads were used. Beads were resuspended in 0.1 M sodiumborate buffer, pH 9.5 to which the rabbit IgG (ICN) was added that can bind to Protein A. Mixtures were incubated overnight at 37 °C with minimal agitation and subsequently washed twice with PBS at 4°C, once with 0.2 M Tris-HCl, pH 8.5 supplemented with 0.1 % (w/v) bovine serum albumin at 37°C and finally washed with PBS, pH 7.4 for 5 min at 4 ^{0}C to remove unbound rabbit IgG.

Ready to use beads were mixed with the crude membranes (400 µl of beads per 400 µg of protein) and incubated for 4 hrs at 4 °C with gentle shaking. Next, bead complexes were collected by placing the reagent tube in the magnetic stand, and washed three times with PBS buffer, pH 7.4 and 2 mM EDTA. Microbodies were eluted by boiling the sample in 50 µl of 2x SDS sample buffer and analyzed by SDS-PAGE and mass spectrometry. When applicable, the immunoisolation was followed by TEV protease cleavage. The microbody-magnetic beads mixture was incubated for 2 hrs at 25 °C with the TEV cleavage mixture (20x rTEV buffer [#Y02233, Life Technologies, Gibco], 0.1 M DTT and TEV Protease [10127-017, Life Technologies, Gibco]). The unbound fraction was collected and precipitated with 25 % (w/v) TCA, while the bound fraction was recovered by boiling with 2xSDS sample buffer. Samples were analyzed by SDS-PAGE and mass spectrometry. When applicable, the microbody membranes were eluted from the beads by incubation with 0.1 M Na_2CO_3 for 30 min at 4°C according to (Kikuchi et al., 2004). Next, the mixture was washed with 0.25 M (w/v) sucrose, 1 % (v/v) ethanol and 2 mM EDTA and bound protein was recovered with 2xSDS sample buffer.

2.4. Protein identification by mass spectrometry

Samples containing the microbody membranes were separated on 7 and 12 % SDS-PAGE and stained with colloidal CBB (Bio-Rad Bio-Safe Coomassie). Gels were thoroughly washed and a single lane was cut into slices and transferred into clean vials. Gel pieces were washed with water subsequently de-stained with 30 % (v/v) acetonitrile with 25 mM NH_4HCO_3 and in 50 % (v/v) acetonitrile with 25 mM NH_4HCO_3 and finally dried in vacuum. Next, the samples were reduced and alkylated with 10

mM DTT dissolved in 100 mM NH_4HCO_3 and incubated for 30 min at 56 °C followed by the addition of 55 mM iodoacetamide dissolved in 100 mM NH_4HCO_3. Following incubation for 30 min in the dark at room temperature, 50 % (v/v) acetonitrile in 25 mM NH_4HCO_3 was added and after 30 min of incubation with shaking, the solution was removed. The gel pieces were vacuum dried and aliquots of 20 µl of a trypsin solution (10 ng/µl in 25 mM NH_4HCO_3, MS-grade, Promega, Leiden, The Netherlands) was added. Reaction tubes were incubated overnight at 37 °C, whereupon the residual liquid was removed and combined with tryptic peptides extracted from the gel pieces. The material was solubilized in 10 µl of 75 % (v/v) acetonitrile and 1.25 % (v/v) of aqueous formic acid, vacuum dried and subsequently reconstituted with 5 µl of 5 % (v/v) acetonitrile in 0.1 % (w/v) of trifluoroacetic acid (TFA). Peptides were mixed in a 1:1 ration with a solution containing 5 mg/ml α-cyano-4-hydroxycinnamic acid and 1 pmol of the calibration peptide ACTH 18-39 (Sigma Aldrich, mass of 2,464.1989 Da) in 70 % (v/v) acetonitrile and 0.03 % (w/v) TFA and transferred to a stainless steel target plate and analyzed in positive ion mode by MS/MS using a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). Spectra were internally calibrated. A 841.5021 Da trypsin autolytic product and the added calibration peptides were used as references.

For Liquid Chromatography-Mass Spectrometry (LC-MS), tryptic peptides were resuspended in 0.1 % (w/v) TFA and separated on a C18 capillary column (75 µm × 150 mm, 3 µm particle size Zorbax 300SB-C18 HPLC column, Agilent Technologies, Amstelveen) mounted on an MDLC nano liquid chromatography system (GE-Healthcare) prior to trapping on a pre-column (300 µm x 5 mm, C18 PepMap300). Aqueous solutions of 0.05% TFA (A) and 80% acetonitrile, 0.05% TFA (B) were used for elution. A two-step gradient from 4-40% B in 50 minutes was performed at a flow rate of 250 nL·min 1. The column eluent was mixed 1:4 (v/v) with a solution of 2.3 mg/m; α-cyano-4-hydroxycinnamic acid (LaserBio Labs) in 60 % (v/v) acetonitrile and 0.07 % (w/v) TFA. Fractions of 12 seconds width were spotted on a blank MALDI target with a Probot MALDI spotter system (Dionex). Mass Spectrometric analysis was carried out with a MALDI-TOF/TOF 4700 (Applied Biosystems) in the m/z range of 900-4000. Data acquisition was performed in the positive ion mode. Peptides with a signal-to-noise level above 100 were selected for MS/MS fragmentation. Peak-lists of the acquired MS/MS spectra were generated, using default settings and a S/N threshold of 20.

All MS/MS spectra were analyzed with Mascot (Matrix Science, London, UK; version Mascot), and X!Tandem (The GPM, the gpm version 2007.01.01.1) against the *P. chrysogenum* protein sequence database (24). In order to exclude false positives, the same search was performed also against the reversed version of *P. chrysogenum* protein sequence database. Variable modifications like oxidation of methionine and iodoacetamide derivative were specified. Protein identification and verification of MS/MS peptides was performed using Scaffold (version Scaffold 1.9.05, Proteome Software Inc., Portland, OR). Peptide identifications were accepted with probability

greater than 90 % as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identification was accepted if the probability was higher than 95 % and contained at least 1 peptide. This reduced stringency was used to increase the sensitivity of detection as discussed in the results section. Protein probabilities were assigned by the Protein Prophet algorithm (Keller et al., 2002).

2.5. Miscellaneous methods

P. chrysogenum cell culture was mixed in 1:1 ratio with 25 % (w/v) TCA and incubated overnight in -20 °C. Cell samples were thawed on ice, pelleted by centrifugation (10 min, 14.000 rpm, 4 °C, eppendorf centrifuge), washed two times with ice-cold 80 % (v/v) acetone (-20 °C) and air dried. Pellets were suspended in 250 µl of solubilization buffer (1 % [w/v] SDS, 0.1 M NaOH). Following the addition of 65 µl of 5x SDS loading buffer, sample were incubated for 5 minutes at 100 °C and analyzed by SDS-PAGE.

Protein concentrations were determined using Bradford Protein Assay or DC Protein Assay (Bio-Rad) using BSA as standard. SDS-PAGE and Western blotting were performed according to the standard procedure. Western blot was performed using PVDF membranes. Polyclonal antibodies against selected *P. chrysogenum* proteins were used. Secondary anti-rabbit antibodies conjugated with alkaline phosphatase (Sigma) were used and blots were developed by chemiluminescence using CDP-star (Roche Applied Science) and visualized with LumiImager (Roche Applied Science).

3. RESULTS

To gather information about the membrane protein composition of microbodies of β-lactam producing cells of *P. chrysogenum*, cells were grown under antibiotic producing conditions (Nijland et al., 2008). For this purpose a *P. chrysogenum* strain was used that expressed the Pex11-TEV-Protein A at the microbody membrane, see a schematic representation of the protein fusion Figure 2A. Pex11 is a microbody membrane protein (Opaliński et al., 2012), and the presence of the Protein A tag allows for ease of detection of microbodies and eventually immunopurification of the microbody membranes (Figure 2B). The TEV protease cleavage site was introduced to allow for the TEV protease based recovery of the microbodies during the anticipated immunopurification. Protoplasts were generated from the hyphen upon Glucanex treatment, and following several washing steps, the protoplasts were lysed to recover a microbody-containing fraction by differential centrifugation and discontinuous sucrose gradient centrifugation using the method described by Kiel et al. (2009). Microbodies were found in the fractions containing about 52 % (w/v) sucrose (Figure 3). Isolated fractions were evaluated for mitochondrial impurities and the presence of the microbody membranes (α-Pex11 antibody) using SDS-PAGE and immunoblotting (Figure3 and 4). Fractions enriched in microbody membranes were

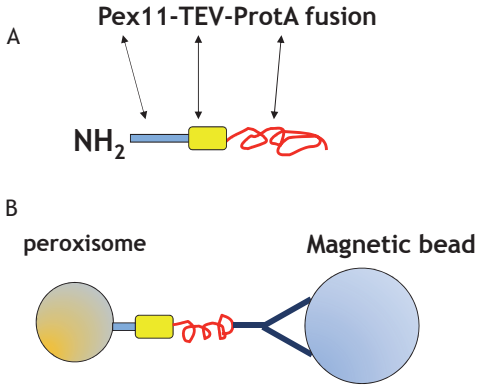

Figure 2. Schematic representation of the immunoisolation approach.

combined and lysed in hypotonic buffer in order to remove the microbody matrix proteins. The crude microbody membrane fraction was subsequently subjected to immunopurification using magnetic beads coupled with rabbit IgG. Material eluted from the magnetic beads was analyzed by SDS-PAGE and by mass spectrometry using a gel that was cut into approximately 25 slices. Upon in gel trypsin digestion extracted tryptic peptides were analyzed on LC-MS/MS. Resulted spectra were analyzed with Mascot, spectra verification and protein identification was performed with Scaffold against *P. chrysogenum* protein database.

A major issue with the bead-based immune-isolation was the degree of non-specific binding to the magnetic beads. There was no major difference in the sample purity obtained from M-500 and M-280 SAR, still a substantial amount of microbody membrane was detected in the unbound fraction examined with SDS-PAGE (data not shown). In case of the M-280 SAR magnetic beads, the preparation procedure was shorter and therefore this method was used for further analysis. Another problem encountered with the immune-isolation was a low efficiency of binding to the resin, as shown by the presence of Pex11 in the unbound fraction (Figure 4).

The P. chrysogenum strain applied in this study was engineered to facilitate a Protein A fused to the membrane protein Pex11. The introduced TEV cleavage site was used to release the microbody membranes from the magnetic beads using TEV protease. However, this method did not work as the amount of Pex11 recovered from the beads was identical irrespective the use of TEV protease. Also the treatment of the microbody membranes with 0.1 M Na_2CO_3 was unsuccessful, as with these membranes no protein could be recovered from the beads (data not shown).

Western blot analysis as well as mass spectrometry indicated that the obtained microbody sample after immune-isolation was contaminated with mitochondria, endoplasmic reticulum, ribosomes, and cytoplasmic proteins. On the SDS-PAGE gel input, washes and eluted fractions were compared, however no distinct enrichment

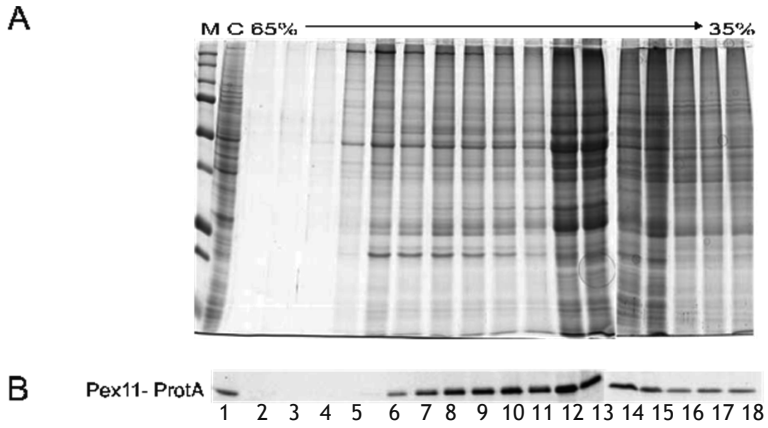

Figure 3. A: SDS-PAGE of organelles separated on a sucrose density gradient, M- Marker, C- crude cell extract. B: Western blot analysis of organellar fractions using the α-Pex11 antibody.

of the low abundant proteins of the microbody fraction was observed in the fraction obtained after immune-isolation (Figure 4). The analysis resulted in the identification of approximately 67 proteins including two known microbody membrane proteins: Pc12g09400 (Pex11p) and Pc21g19300 (similarity to the microbody membrane protein PMP22 of *Arabidopsis thaliana*). Further, proteins were identified that are involved in penicillin biosynthesis, i.e., Pc21g21370 (AT), Pc21g21390 (ACVS) and Pc21g21380 IPNS (Table 2). Only one of these proteins, i.e., AT is microbody localized suggesting that IPNS and ACVS are contaminants of the microbody fraction. The analysis further indicated the presence of eight other membrane proteins that are putative transporters, whereas the remaining proteins are from mitochondrial, endoplasmatic reticulum or cytosolic origin (Table 2), likely to be impurities of the microbody fraction as often only a single peptide fragment was found.

4. CONCLUSIONS AND DISCUSSION

Organellar proteomics is a very challenging subject since the quality of analysis is mostly determined by the methods of sample preparation and the ability to clearly separate various subcellular organelles. Advances in cell imaging indicate that various organelles within the cell are intimately associated through complex cellular networks hence the difficulty in obtaining these organelles in a pure state. While the sensitivity of mass spectrometry has improved, a further issue remains the efficiency of the organelle isolation and its biological reproducibility. In case of membrane proteomics, also the enrichment of the required membrane fraction can be difficult.

Here we have used proteomics in an attempt to map the microbody membrane protein proteome in the filamentous fungus *Penicillium chrysogenum* that is used in the

Table 1. Overview of the protein and peptides identified in the sample of the proteins eluted after immunoisolation.

Protein name (similarity to)	Protein accession numbers	MW (kDa)	Protein coverage (%)	# peptides	Peptide coverage	Peptide sequence
β-lactam biosynthesis						
acyl-coenzyme A:isopenicillin N acyltransferase (acyltransferase) – *P. chrysogenum*	Pc21g21370	39.9	99.3	1	2.8	SIDFAVDLIR
isopenicilline N synthase – *P. chrysogenum*	Pc21g21380	38	99.8	2	7.9	AYNKEHQDQIR GYALALGKEEDFFSR
α-aminoadipyl-cysteinyl-valine synthetase – *P. chrysogenum*	Pc21g21390	425	100	30	11.2	AIIASNQHVER DGWSVAQAVESIEAGR EQNLQNSSPSWVVSPTIVTHENR FIPNPFQSEEDKR FLVGYYVADAALPSAAIR FLYFTDVAAGAVEDR FPDVPQPAETYTPYFEYLEPPR GYLNQPLLTDQR GYLNRPELTPHR IAVVYEETSLTYR IILVGENLTEAR LDDELPWEAR LDQEIASLSQHVFR LIVPPAEFVADDEFYR LNYPLAVIAR LYPFPER NLSDFWLR QQQPQLEYLGR RLPEIINDSAQSSYSPPR SEDEGSLAFNQYRPVQPVDSVAK SGAAYVPIDPTYPDER SGGAYVPIDPGYPNDR STDLAYIIYTSGTTGRPK VQLADYDQVQEQR WIPGSSGEVEYLGR WLPNGEVEYLGR WSVSNPSPLSR YDADTLLPFIR YNTTLSPDLFQR YQLASTGFGDASAAYQER

Table 1. Continued

Protein name (similarity to)	Protein accession numbers	MW (kDa)	Protein coverage (%)	# peptides	Peptide coverage	Peptide sequence
Microbody proteins						
Microbody membrane protein PMP22 - *Arabidopsis thaliana*	Pc21g19300	25.8	98.8	1	6.5	VSWVTSPLCLAFAQK
Protein CAC21806 from Patent WO0071579 – *P. chrysogenum*	Pc12g09400	26.5	99.2	1	3.4	FYAWYLYR
Energy transduction						
Mitochondrial ADP/ATP carrier BAC82547 – *P. chrysogenum*	Pc18g02420	34	100	3	11.7	GFGPSVLGIVVYR TAAAEGVVSLWR YFPTQALNFAFR
Mitochondrial F1-ATPase α-subunit Atp1 – *S. cerevisiae*	Pc12g03370	59.6	100	4	9.9	AAPTEVSSILEQR EAYPGDVFYLHSR GVSEEAGLAETGR TGEIVEVPVGPELLGR
Mitochondrial F1-ATPase β subunit Atp2 – *N. crassa*	Pc21g10070	55	100	5	13.9	AHGGYSVFCGVGER GVAAEDTGAPITIPVGPSTLGR IGLFGGAGVGK VALVFGQMNEPPGAR VVDLLAPYAR
Mitochondrial F1-ATPase δ-subunit Atp16 – *S. cerevisiae*	Pc22g25650	16.6	99.2	1	7.7	LSLTLPHQTIYR
Mitochondrial F1-ATPase subunit 7 Atp7 – *Kluyveromyces lactis*	Pc20g13280	19.3	88.6	1	9.8	VQVLSEQPQTVDFSHYR
Mitochondrial F1-ATPase subunit f Atp17 – *S. cerevisiae*	Pc22g09100	12	100	2	22.9	GPAAPVKPTGLIGR VVTFYAGLPR
Mitochondrial F1-ATPase subunit g Atp20 – *S. cerevisiae*	Pc21g23180	19	100	2	16.0	TLPSPSSILACIR VSASAGPAISNAAGALR
ATP synthase coupling factor (F0) subunit – *S. cerevisiae*	Pc21g05220	10.4	100	2	19.6	FDLEAFLK RAEIEHEYAR

Table 1. Continued

Protein name (similarity to)	Protein accession numbers	MW (kDa)	Protein coverage (%)	# peptides	Peptide coverage	Peptide sequence
Mitochondrial phosphate transport protein Mir1 – *S. cerevisiae*	Pc06g01110	32	100	3	12.1	EEGIGAFYSGFGPILFK ILKEEGIGAFYSGFGPILFK LVSQPTFASGLLPAMSR
Subunits of NADH:ubiquinone reductase – *N. crassa*	Pc12g12260	53	99.6	1	3.8	HYTVNFGPQHPAAHGVLR
	Pc21g05450	19	100	2	15.6	HLLGSQGPPEAFALR WTPSQEVFEQR
	Pc13g12820	18	99.2	1	10.7	TIPGTPEGQVPVHLRPK
	Pc22g03960	21	100	2	15.7	ATALTGSVGLFAAAVQNTLTK GIYGPGYEER
	Pc21g17300	8.5	99.3	1	27.6	ALGDVDPEPVPLTYPIPQGPR
	Pc22g22950	11	100	1	14.9	FLFCQTSEASAATR
	Pc20g11780	15	100	3	17.9	NFVSGFLEGR NFVSGFLEGRN YFRPEEDPGAR
Cytochrome c1 of ubiquinol-cytochrome c reductase Cyt-1 – *N. crassa*	Pc22g22210	34.8	93.3	1	5.0	LSDYIPAPYPNEEAAR
Subunit II of ubiquinol--cytochrome c reductase CAA42214.1 - *Bos primigenius taurus*	Pc12g05480	48.3	99.6	1	2.9	YQPFPGFSDALDR
14 kD subunit of ubiquinol--cytochrome c reductase Qcr7 – *S. cerevisiae*	Pc22g17890	14	100	2	25.4	FDDLIPEESENVQK SWLTPLSHWYTDAAGYR
Subunit VIII of ubiquinol--cytochrome c reductase – *S. cerevisiae*	Pc13g12390	12.5	100	2	23.1	NEFLNSKPGR VLAGAGHAAIFNVFR
Subunit IV of cytochrome c oxidase Cox4 – *S. cerevisiae*	Pc18g04080	22	99.2	1	6.6	TFADYVKPDYWYR
Subunit V of cytochrome c oxidase Cox5 – *A. niger*	Pc21g10080	20	100	4	20.9	AAYYIAFGAHGPR EWQEASNEYALK GFVQSPPAEKS KAAYYIAFGAHGPR

Table 1. Continued

Protein name (similarity to)	Protein accession numbers	MW (kDa)	Protein coverage (%)	# peptides	Peptide coverage	Peptide sequence
Subunit VI of cytochrome c oxidase Cox6 – *S. cerevisiae*	Pc21g18840	18	100	5	31.6	EFDGVQDVFELQR FEKEFDGVQDVFELQR QYLESLEGLR RVNDFPTAVR SGHEDETYEEFSAR
Subunit VIa of cytochrome c oxidase Cox13 – *S. cerevisiae*	Pc20g07780	16.5	99.2	1	6.4	TEYPYQNIR
Subunit VIb of cytochrome c oxidase Cox12 – *S. cerevisiae*	Pc12g07440	11	99.9	1	19.6	GAIPEADPDEVLETKPFK
Ribosomal proteins						
Cytoplasmic ribosomal protein of the small subunit S12 AS1 - *Podospora anserina*	Pc22g20260	18	99.2	1	10.5	GIDLDQLLDLSSEQLR
Cytoplasmic ribosomal protein of the small subunit S18 - *Homo sapiens*	Pc22g00880	18	99.2	1	5.2	IPSWFLNR
Cytoplasmic ribosomal protein of the small subunit S19 – *S. cerevisiae*	Pc18g02490	16.5	99.2	1	12.1	TSASNELPPQDADWFYVR
Cytoplasmic ribosomal protein of the small subunit S14.e – *N. crassa*	Pc22g20650	16	99.2	1	11.9	IFASFNDTFVHVTDLSGR
Cytoplasmic ribosomal protein of the small subunit Ys24 – *S.s cerevisiae*	Pc16g14740	15	99.3	1	4.6	LLGFFY
Translation initiation factor eIF-4A – *Schizosaccharomyces pombe*	Pc20g06740	44.5	97.7	1	4.0	GVYAYGFERPSAIQQR
Translation elongation factor 1 α – *Podospora anserina*	Pc13g02940	49.8	100	2	7.8	SHLNIVVIGHVDSGK TLLEAIDAIEPPVRPSNKPLR
Mitochondrial elongation factor like protein An08g04470 – *A. niger*	Pc12g15010	48	99.9	1	4.1	GLANFLDYASIDKAPEER
Other proteins						
γ-actin Act – *P. chrysogenum*	Pc20g11630	41.8	100	2	7.2	IWHHTFYNELR SYELPDGQVITIGNER

Table 1. Continued

Protein name (similarity to)	Protein accession numbers	MW (kDa)	Protein coverage (%)	# peptides	Peptide coverage	Peptide sequence
Peptidyl-prolyl isomerase An14g00850 – *A. niger*	Pc13g06110	13	99.3	1	11.5	AILTISPDFGYGDR
Cofilin Cof1 – *S. cerevisiae*	Pc21g10090	16.5	99.2	1	10.8	YAVYDVEYELPGEGKR
Nucleoside-diphosphate kinase NDK-1 – *N. crassa*	Pc21g15880	17	98.9	1	11.1	VLLGATNPLASAPGTIR
Histone H4.1 – *A.s nidulans*	Pc16g12260	15	100	3	22.4	DAVTYTEHAK DNIQGITKPAIR TFLEGVIR
Cyclophilin-like peptidyl prolyl cis-trans isomerase CypA – *A. niger*	Pc18g05190	19	100.0	2	16.7	SIYGEKFPDENFVHK VIPQFMLQGGDFTR
Ribose-5-phosphate isomerase RpiB - *Escherichia coli*	Pc22g21440	17	100	3	20.8	AVTAHDPFSVER LATDWLNYR VQAITDYEVQFR
Human regulatory molecule HRM-7 like protein An02g02190 – *A.s niger*	Pc12g11550	16	99.2	1	6.1	FNYAWGLIK
Novel cell death-regulatory protein GRIM19 - *Mus musculus*	Pc20g03810	13	99.2	1	13.6	FIRPTFAYTPANLTQ
Membrane steroid hormone-binding protein MSBP - *Bos taurus*	Pc13g10390	17	100	2	18.7	GLAFQSFDEEMLTK NFYGPGGPYENFAGR
Calmodulin 6 CaM6 - *Arabidopsis thaliana*	Pc20g01370	16	99.3	1	9.0	SVGGDFDFDSFLK
Ubiquitin ubi1 – *A. nidulans*	Pc13g09510 Pc16g08400 Pc22g12390	18	99.1	1	8.4	IQDKEGIPPDQQR
Hex1 – *A. nidulans*	Pc21g00970	59.8	100	9	24.5	AYQSQTDSYKEDDVYLR GSAGETVPIPCHFIR ISDAFADGR ISVSPQTGQHR VGDILILQGRPCQVIR VLHPFSHSEHEESHAGEVAAPR VPIPFSVFPSSYR YLGVDLFTR YSTPTQVNVPQSTTR

Table 1. Continued

Protein name (similarity to)	Protein accession numbers	MW (kDa)	Protein coverage (%)	# peptides	Peptide coverage	Peptide sequence
Skin cell transmembrane protein like protein An07g06340 – *A. niger*	Pc12g16370	12	98.8	1	18.8	PVVAGPPAQAGPGGAPSTFDK
Hypothetical membrane protein YJR085c – *S. cerevisiae*	Pc12g06680	16	99.3	1	13.8	APVPLALGATGLLASYYYQK
Hypothetical protein Afu3g06460 – *A. fumigatus*	Pc20g07680	16	100	2	11.7	YGKEEWDY YQYHPHGDK
Hypothetical protein YKR065c – *S. cerevisiae*	Pc22g22380	25.5	100	2	12.3	FRVDPSTNSISNPVPDYYGEK LDWDSFFK
Hypothetical protein SPAC922.05c - *Schizosaccharomyces pombe*	Pc12g09320	55	99.1	1	2.2	SEIVGLWDTLR
Hypothetical protein Afu3g06640 – *A. fumigatus*	Pc20g03120	9	98.2	1	19.5	VLAVDLLNPTPQAEAR
Hypothetical protein AN9078_2 – *A. nidulans*	Pc21g11710	17	100	3	34.6	AAEIDDPLQNNNYPNPPAVKR DPHGGWWDAQEKR TYPDGLETELGGPNALPAR
Hypothetical protein AN7629_2 – *A. nidulans*	Pc22g14880	10	99.3	1	12.4	NPTGFDIAQFK
Hypothetical protein SPAPJ691.03 - *Schizosaccharomyces pombe*	Pc13g12160	10	100	3	44.0	ATKPVSEALLNEKWDR AWEEADASFR AWPAWVGLGFGAGR
Hypothetical protein NUO-12.3 – *N. crassa*	Pc12g01050	11.6	99.3	1	22.3	KPTVAPTYDGVDFEDNVAVHNAR
Hypothetical protein AN5969_2 – *A. nidulans*	Pc22g20870	15	99.3	1	8.0	KLPFLDTYYDR
Hypothetical protein Afu4g06910 – *A. fumigatus*	Pc22g17950	36	100	2	8.3	GLKAEILTQYQPAK SAHEGPIAGQLEAK
Hypothetical protein Afu4g09870 – *A. fumigatus*	Pc22g11890	9	99.9	2	24.3	ENRVPYYQR LFQNHDGKR
Hypothetical protein An16g07080 – *A. niger*	Pc12g03140	9	100	1	15.0	LQNDYGADYWVR
Hypothetical protein EAA71297.1 - *Fusarium graminearum*	Pc22g06850	10	98.8	1	22.1	GQSSNHQAPTGFEVSNPWK

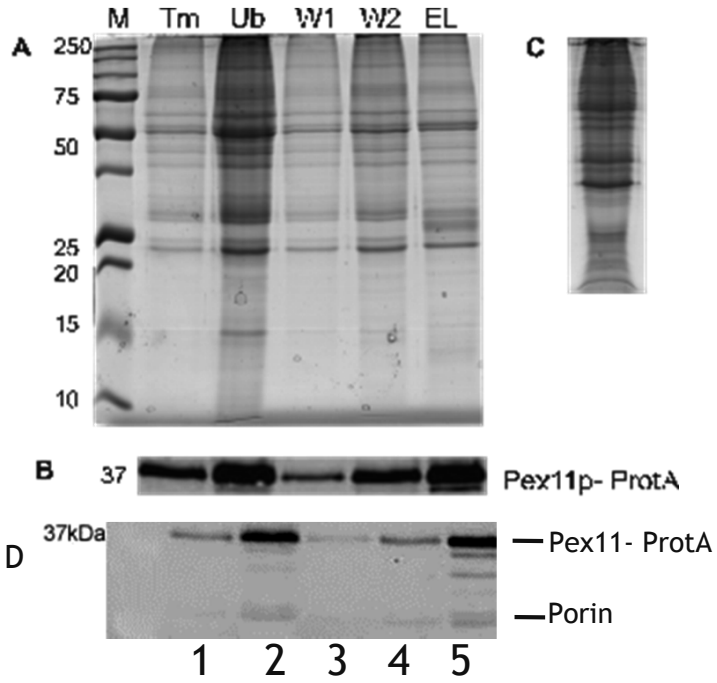

Figure 4. Immuno-isolation of microbody membranes: A: SDS- PAGE of the fractions, where: M- marker, Tm- Tris washed membranes, Ub- unbound fraction, W1 and W2- washes, EL- eluted fraction. B: Western blot analysis of the samples obtained during immune-isolation, decorated with α-Pex11 antibody. C: eluted fraction run on SDS- PAGE gel and used for LC- MS/MS.D: Western blot analysis of the samples obtained during immune-isolation, control of the amount of mitochondrial contamination, decorated with α-Porin antibody.

pharmaceutical industry for antibiotics production. Whereas, 2-D gel electrophoresis has been used to map the proteome of different fungi (Oliveira and Graaff, 2011), organellar proteomics has been relatively scare. Previously, a proteomic analysis of the microbody matrix has been performed (Kiel et al., 2009) which mapped about 80 proteins. Only few studies report on the proteomics of the microbody membrane. Marelli and coworkers (Marelli et al., 2004) isolated microbody membranes from *Saccharomyces cerevisiae* using a novel quantitative ICAT labeling approach, where the initial purification of microbodies was performed by combining subcellular fractionation and immuno-isolation followed by microcapillary LC with electrospray ionization and tandem MS. However, the list of putative microbody proteins still contained numerous contaminants and/or proteins of dual organellar localization. To narrow down the number of identified proteins to the truly microbody membrane proteome, a novel isotope based quantitative ICAT labeling was applied. ICAT labeling utilize heavy ($^{2}H_{8}$, $^{13}C_{9}$) and light isotopes ($^{1}H_{8}$, $^{12}C_{9}$) forming a covalent adduct with the side chains of reduced cysteine amino acyl residues, contain biotin molecule. ICAT labeled samples

prior to μLC/ESI- MS/MS were fractionated by ion exchange and avidin chromatography (Marelli et al., 2004). In that study, microbody and mitochondrial membrane enriched fractions were obtained from subcellular centrifugation of lysed *S. cerevisiae* oleic-grown cells. As an alternative approach, a microbody membrane enriched fraction was obtained from *S. cerevisiae* expressing Protein A fused to the C-terminus of Pex11.The microbody membrane enriched fraction was subjected to IgG affinity chromatography for further purification which resulted in an enrichment of low abundant membrane proteins. In total, Marelli and coworkers (2004) quantified about 306 proteins, with 70 potential microbody proteins including 8 novel proteins (Marelli et al., 2004). In another approach, membranes of rat liver microbodies were isolated using differential centrifugation in sucrose, followed by separation on Nycodenz (Kikuchi et al., 2004). The microbody-containing fraction was subjected to immune-isolation using Dynal magnetic beads coupled with an anti-PMP70 antibody. PMP70 is a microbody membrane protein and the antibody should promote specific binding of the microbody membranes to the resin. Analysis of trypsin peptides was performed using LC-MS/MS QTOF, resulting in the identification of 34 proteins with five of unknown function, whereas the majorities of the identified proteins were either of mitochondrial or ER origin. This analysis did not yield novel membrane proteins, but a homolog of PMP22 and ABC long chain fatty acid transporters was identified (Kikuchi et al., 2004). We have used Protein A fused to the C-terminus of Pex11 for immune-isolation of microbodies of *Penicillium chrysogenum* followed by proteomic analysis. This yielded only two membrane proteins, i.e., Pex11 and a homolog of Pmp22, which is a low number.

One of the critical steps in subcellular proteomics is the isolation of intact organelles. For this purpose, the gentlest method is enzymatic generation of protoplasts. However, this also leads to some disadvantages such as prolonged sample preparation (2-3 hrs). The entire procedure takes about 18 hrs and during that time, sample degradation might be a serious issue despite all kinds of measure to prevent proteolytic degradation and denaturation. Also, the generation of a highly purified organelle sample is not always possible because of the physiological state of cells. In the current study, the isolated microbody membranes also contained mitochondrial and endoplasmic reticulum proteins, while the application of magnetic beads for immuno-isolation of the organelles did not improve the purity. This could be caused by a non-specific binding of the organellar membrane to the magnetic beads, or because these membranes are intimately associated with other organelles in cells, hence a partial co-purification. A further complication is the possibility of dual localization of proteins (Ast et al., 2013) suggested previously (Ast et al., 2013; Girzalsky and Erdmann, 2013; van der Zand and Tabak, 2013). Dual localization may occur for instance for proteins involved in microbody biogenesis. Proteins having a dual localization can be encoded by the same or separate genes. Due to alternative start and stop codons sites or differences in splicing different isoforms may occur that either have different or weak targeting signals. For instance, malate dehydrogenase localizes both to mitochondria,

microbodies and cytoplasm (Ast et al., 2013). Thus, a final conclusion with respect to the subcellular localization is only possible upon experimental validation for instance by using fluorescent fusion proteins. Also, soluble proteins were found to be present in the purified membrane fraction. Again, this could be due to protein degradation during organelle isolation, or because these proteins bind specifically or nonspecifically to the membranes. Interestingly, Muller et al. (1992) showed that ACVS and IPNS most likely localize in close proximity/of bind to the microbody membrane, and these proteins were also found in the microbody membrane fraction in this study. A possible improvement would be to optimize the washing step and remove the absorbed soluble proteins by alkaline membrane extraction. Unfortunately, in the current study this resulted in a major loss of protein, a corresponding low membrane recovery yield and protein amounts to small for reliable MS detection.

Various studies suggest that the number of integral membrane proteins in the microbody membrane is very low. Freeze-facture imaging of the microbody membranes shows a smooth surface, with only very few dimples consistent with a low membrane protein density in the membranes (Bartoszewska et al., 2011b). This might mean that these membranes are equipped with a limited arsenal of transporters. A long-standing question, therefore, is how molecules are transported across the microbody membrane. So far three possible mechanisms are proposed: a) membranes are impermeable to solutes and contain a set of 'specific' transporter proteins, b) membranes contain a porin that mediates the flux of a large number of unrelated compounds (Hettema and Tabak, 2000; van Roermund et al., 2003), or c) a combination of both, a and b (Antonenkov and Hiltunen, 2006). Up to now only transporters involved in the fatty acid metabolism are known in *S. cerevisiae* and mammalian microbodies, which are the adenine nucleotide transporter (PMP34 *H. sapiens* and Ant1p *S. cerevisiae*) and long chain fatty acids transport (ALDP/PMP70 and Pxa1/Pxa2) (Liu et al., 1999; Shani and Valle, 1996). These systems have also been identified in microbodies of *P. chrysogenum* (See chapter 3 and 4). Recently, a transporter of phenylacetic acid in *P. chrysogenum* Wisconsin 54-1255 strain was identified, PaaT, which is a homologue of *Acremonium chrysogenum* CefT, a plasma membrane localized transporter. Both proteins belong to the family of MFS proteins (Major Facilitator Family) (Fernandez-Aguado et al., 2013), and it was suggested that PaaT localizes to the microbody membrane. However, the cell localization study performed was not conclusive. A major obstacle encountered in GFP based localization studies is that overexpression may result in targeting of a fraction of the protein to vacuoles that might be confused with microbodies. Since PaaT did not localize to the membrane of the observed organelle, but rather fluorescence was found to be distributed in the lumen of the organelle, vacuoles may indeed have been confused with microbodies.

Summarizing, the physiology of fungi makes isolation of intact organelles from *P. chrysogenum* difficult but not impossible. Filamentous fungi secrete proteases, which enhance the issue of protein degradation during the lengthy isolation protocol. Fungi

also contain a dense network of microtubules, which may cause unwanted clustering of organelles in the partially purified samples. Therefore, to perform proteomic studies on microbodies, a fast and efficient method is needed that reduces harvesting time thus decreasing the risk of proteolysis. Further methods for the enrichment of microbody membranes might be considered: a) development and application of antibodies against organism specific integral microbody membrane proteins which could facilitate specific binding to magnetic beads (Kikuchi et al., 2004); b) binding of known antibodies to microbody membrane proteins in an organellar mixture, resulting in a pI shift of a particular membrane protein and thus the possibility to separate different (populations and subpopulations) organelles by immuno free flow electrophoresis, (Völkl et al., 1997); c) ICAT labeling followed by peptide separation on a cation exchange and/or reverse phase column (Marelli et al., 2004; Völkl et al., 1997). This may decrease the experimental handeling time, decrease sample loss and possibly reduce the amount of impurities. Once this challenging task is fulfilled, candidate proteins identified by mass spectrometry needs to be carefully selected and subjected to cellular localization studies.

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chapter 3

NUCLEOTIDE TRANSPORT ACROSS THE MICROBODY MEMBRANE IS ESSENTIAL FOR EFFICIENT β-LACTAM PRODUCTION BY *PENICILLIUM CHRYSOGENUM*

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ABSTRACT

One of the key steps in the biosynthesis of β-lactam antibiotics by the filamentous fungus *Penicillium chrysogenum* is the Coenzyme-A activation of side chain precursors such as phenylacetic acid. This process, which occurs in microbodies, requires the hydrolysis of ATP and thus the uptake of ATP into these organelles. In *Saccharomyces cerevisiae,* Ant1p functions as an adenine nucleotide uptake system in the microbody membrane. The genome of *P. chrysogenum* encodes four Ant1p homologs (Pc13g03380, Pc21g09430, Pc12g12500 and Pc22g11190). All four genes are expressed in *P. chrysogenum*, but only Pc13g03380 is upregulated when the fungus is grown in the presence of phenylacetic acid or oleic acid. By the use of C-terminal fusions with the green fluorescent protein, Pc13g03380, Pc12g12500, Pc21g09430 could be localized to the microbody membrane. Gene inactivation revealed that deletion of Pc13g03380 resulted in a marked decrease in β-lactam production. These data suggest that Pc13g03380 is involved in uptake of ATP into microbodies, which is needed for the activation of side chain precursors during β–lactam biosynthesis.

1. INTRODUCTION

Since the discovery of penicillin in 1928 by Alexander Fleming, it has remained one of the most popular and well-studied antibiotics. Penicillins are β-lactam inhibitors of peptidoglycan biosynthesis in bacteria and used clinically worldwide. β-lactam antibiotics were for a long time known as the "miracle drug" and are still among the best-sold drug classes worldwide. Therefore, a detailed knowledge about the biosynthesis pathway including the transport steps is crucial for further development and pharmaceutical exploitation (Weber et al., 2012a).

Penicillins are synthesized only by a few *Penicillium* and *Aspergillus* species (Aharonowitz et al., 1992). During the last decades, industrial strains of *Penicillium chrysogenum* are used for penicillin production and these went through an extensive classical strain improvement program, which resulted in a more than 1000-fold higher production that wild-type like strains. It was only by 1986, that the genetic basis of the penicillin biosynthesis pathway was resolved with the discovery of the genes of the three key enzymes of β-lactam production namely *pcbC* (isopenicillin N synthase, IPNS) (Carr et al., 1986), *pcbAB* (δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase, ACVS) and *penDE* (acyl-coenzyme A:isopenicillin N acyltransferase, AT) (Barredo et al., 1989; Crawford et al., 1995). However, the β-lactam biosynthesis pathway is highly compartmentalized, with different steps occurring in the cytosol and in a specific organelle termed peroxisome or microbody. Compartmentalization provides enzymes with an optimal environment for activity, allows for the regulation or separation of metabolic processes, and even allows for multiple pathways that share metabolites to occur simultaneously. The first two steps of penicillin biosynthesis take place in the cytoplasm, i.e., the condensation of the three precursor amino acids 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 the nonribosomal peptide synthetase ACVS encoded by the *pcbAB* gene (Fierro et al., 1995; van de Kamp et al., 1999). The next step also occurs in the cytosol, which is the cyclization of LLD-ACV into isopenicillin N (IPN) by IPNS encoded by the *pcbC* gene. In this reaction, the penam nucleus is formed, which consists of β-lactam and thiazoline ring. The final steps of β-lactam biosynthesis occur in microbodies wherein the L-α-aminoadipate group is substituted for a phenylacetyl- or phenoxyacetyl moiety in a reaction catalyzed by the acyl-CoA:isopenicillin N N-acyltransferase (AT) encoded by the *penDE* gene. Phenylacetic acid (PAA) or phenoxyacetic acid (POA) serve as precursors but are first activated to their corresponding CoA thioesters in a reaction catalyzed by phenylacetyl coenzyme A ligase (PCL). This enzyme is also localized in the microbody. In this respect, *P. chrysogenum* contains various other CoA ligases that may contribute to side chain activation (Koetsier et al., 2009a; Koetsier et al., 2009b; Wang et al., 2007). The AT mediated reaction with PAA-CoA or POA-CoA finally results in the formation of penicillin G or V, respectively.

The genes *pcbC*, *pcbAB* and *penDE* are organized in a single cluster that is also termed the penicillin biosynthesis gene cluster. Transformation of a wild type strain

with this cluster results in an increased level of penicillin production (Fierro et al., 1995; Newbert et al., 1997; Weber et al., 2012a). The industrial production strains obtained by classical strain improvement show a massive amplification of the penicillin gene cluster with up to 8-10 copies. On the basis of a comparison of strains in a lineage that was optimized by classical strain improvement, it was reported that the relation between the number of gene clusters and amount of penicillin produced is nonlinear (Newbert et al., 1997). Recently, this phenomenon was examined with a series of isogenic *P. chrysogenum* strains in which the penicillin biosynthetic gene cluster was varied between 0 and 8 copies. The data showed that the AT activity is limiting at high gene cluster numbers (Nijland et al., 2010). Despite a massive increase in transcript levels with increasing cluster copy number, the AT protein levels in the microbodies only marginally increased while intracellular IPN accumulated in the cytosol. Although this observation suggested that the AT activity is limiting, other factors may also contribute such as a limited CoA ligase activity, and/or limiting concentrations of co-factors like CoA, ATP/ADP and NADP(H). These processes will influence and maintain the homeostasis of the entire biosynthetic pathway, the transport processes occurring in the cell of *Penicillium chrysogenum* are not very well known yet.

Activation of the side chain could possibly be a limiting factor, however insertion of *phl* (PCL) and *pcbDE* (IAT) to single penicillin cluster strain of *Penicillium chrysogenum* DS47274 had a minor impact on the increase of penicillin biosynthesis. In case of high copy number strains the overproduction of IAT led to the decrease of penicillin V and increase of 6-APA concentration (Alvarez et al., 1993; Weber et al., 2012b). Possibly, due to excess of IAT over availability of ACVS and IPNS, IAT function as hydrolase and converts IPN (or Penicillin V) to 6-APA. This indicates, that homeostasis of penicillin biosynthesis enzymes is critical for synthesis of penicillin, as well as export of the final product out of the microbody and the cell (Weber et al., 2012b).

Microbodies are small organelles surrounded by a single membrane. They are 200-800 nm in diameter and present in the cells of all eukaryotic organisms (Müller et al., 1992). Microbodies are involved in the β-oxidation of medium and long chain fatty acids and the detoxification of hydrogen peroxide, hence the alternative name peroxisomes. Other metabolic pathways may also occur in microbodies such as the glyoxylate cycle and hormone biosynthesis (Rottensteiner and Theodoulou, 2006; Schrader and Fahimi, 2008). In fungi, microbodies play a key role the β-oxidation of fatty acids (Kiel et al., 2009; Kionka and Kunau, 1985; Valenciano et al., 1998; Valenciano et al., 1996; Veiga et al., 2012), karyogamy (Berteaux-Lecellier et al., 1995) and penicillin biosynthesis (Müller et al., 1992). In industrial *P. chrysogenum* strains, the amount of β-lactam produced increases with the number of peroxisomes (Meijer et al., 2010; Müller et al., 1992; Schrader and Fahimi, 2008). Similar observations were made in *Aspergillus nidulans*, where the volume fraction of the microbodies is increased under penicillin producing conditions (Valenciano et al., 1998). The overexpression of the peroxisomal proliferation factor Pex11 in *P. chrysogenum* resulted in increased

numbers of peroxisomes and also in elevated levels of β-lactam production. However, the level of the AT enzyme in the microbody matrix remained unchanged under these conditions. Therefore, it has been suggested that the stimulatory effect of microbody proliferation is related to an increased microbody membrane surface that may allow for an enhanced transport or flux of precursors across the membrane (Kiel et al., 2005). Such a profitable effect in metabolite flux should, however, relate to the last two steps of penicillin biosynthesis that are located in the matrix of peroxisomes (Müller et al., 1992). Therefore, it is of special interest to examine the transport steps required for β-lactam biosynthesis required at the microbody membrane.

Next to the few microbody transporters identified and characterized so far in yeast and in mammals, recently two transporters were suggested to be peroxisomal localized and to be involved in β-lactam biosynthesis in *P. chrysogenum* (Fernández-Aguado et al., 2014; Fernandez-Aguado et al., 2012). The MFS protein, PaaT of *P. chrysogenum* is a homologue of CefT from *A. chrysogenum* (Fernandez-Aguado et al., 2012) has been implicated in penicillin biosynthesis. CefT of *A. chrysogenum* is a transporter involved in secretion of cephalosporin. The expression of *paaT* is stimulated by phenyl acetic acid (PAA) (van den Berg et al., 2008). Fluorescent microscopy suggests an organellar, possibly peroxisomal localization of PaaT (Fernandez-Aguado et al., 2012). Upon mutation of *paaT* a small reduction of extracellular Penicillin G was observed, whereas the intracellular concentration of IPN and Penicillin G remained comparable to the parental strain. Silencing as well as overexpression of *paaT* caused changed the sensitivity of *P. chrysogenum* to phenylacetic acid, and it was suggested that PaaT is a peroxisomal transporter involved in PAA import (Fernandez-Aguado et al., 2012). The peroxisomal localization of this transport is ambiguous the GFP fluorescence occurred in the lumen of the organelles while no clear membrane localized fluorescence could be discerned. Another protein termed PenM, is a homologue of CefM of *A. chrysogenum*, which has been proposed to function as IPN transporter (Fernández-Aguado et al., 2014). PenM contains 12 transmembarne spanning domains, and possess a putative pex19 binding site (Fernández-Aguado et al., 2014). Fluorescent microscopy suggests a peroxisomal localization, although no membrane localization was shown. The overexpression of *penM* caused an increase in the extracellular isopenicillin N and penicillin G levels. Silenced mutants produced reduced amounts of Penicillin G, while the intracellular concentrations of IPN were increased (Fernandez-Aguado et al., 2012). It was proposed that PenM transports IPN through the microbody membrane, as IPN is a hydrophilic molecule, which requires active transport across the membrane (García-Estrada et al., 2007; Ullán et al., 2010).

Of special interest are the microbody adenine nucleotide transporters that belong to the Mitochondrial Carrier Family and that have been implicated as AMP/ATP antiporters and uniporters (Lasorsa et al., 2004). This contrast with the mitochondrial adenine nucleotide transporters, which are strictly ADP/ATP antiporters (Klingenberg, 1989). The *Saccharomyces cerevisiae* Ant1p is the best characterized adenine

nucleotide transporter and is involved in the activation of medium chain fatty acids (MCFA) to fatty acyl-CoA. ATP is required for the activation of fatty acids and in this reaction AMP is generated. The AMP is excreted into the cytosol as microbodies lack an adenylate kinase or an ATP regeneration system (Palmieri et al., 2001). β-lactam biosynthesis depends on the CoA activation of PAA or POA, a step that requires ATP yielding AMP. Here, we have examined the role of *P. chrysogenum* homologs of the yeast Ant1p in β-lactam antibiotic biosynthesis. Our data suggest that one of these homologs fulfills a critical role in β-lactam biosynthesis.

2. MATERIALS AND METHODS

2.1. Fungal and bacterial strains

P. chrysogenum strains used in this study are described and indicated in Table 1. *Escherichia coli* DH5α strain was used as a high frequency recombination strain for the Multisite Gateway® Three-Fragment Vector Construction Kit (Invitrogen) (Table 2). Plasmids DNA was amplified and isolated as described (Sambrook et al., 1989). The four Ant1p homologs Pc12g12500, Pc13g03380, Pc21g09430 and Pc22g11190 were amplified from genomic DNA derived from the DS17690 strain by PCR. Herein, genes were amplified without a STOP codon and flanked with att recombination sites of the Gateway System. Primers used are listed in the Table 3. Amplified genes were cloned separately into the pDONR201 vector using the BP Clonase II enzyme mix yielding plasmids pDONR201-Pc12g12500, pDONR201-Pc13g03380, pDONR201-Pc21g09430 and pDONR201-Pc22g11190 which were combined with the pDONRP2-P3R-GFP-Tat and pENTR4-1-PgpdA vectors, respectively. Next, these were cloned into the pDEST R4-R3/AMDS destination vector in the LR reaction Multisite Gateway® Three-Fragment Vector Construction Kit using the LR Clonase II enzyme mix. These cloning reactions resulted in plasmids expressing carboxyl-terminal fusion of selected genes with GFP (Table 2).

2.3. Generation of gene inactivation mutants of P. chrysogenum

To generate gene deletions, the flanking regions of Pc12g12500, Pc13g03380, Pc21g09430 and Pc22g11190 were amplified from the genomic DNA of *P. chrysogenum* DS17690. The region upstream (5′ flanking region) of the specific gene was amplified with the AttB4-FR5′ and AttB1-FR5′ primers. The downstream (3′ flanking region) region was amplified with AttB2-FR3′ and AttB3-FR3′ (Table 3). The 5′ flanking regions were cloned into pDONRP4-P1R, and the 3′ flanking regions were cloned into pDONRP2R-P3. As a selection markers phleomycin resistance gene or the *amdS* gene were used, which were cloned into the pDONR221 vector resulting in pENTR221-Phleo and pENTR221-AMDS, respectively. Cloning was done using the BP Clonase II enzyme mix. Obtained plasmids were combined and cloned into pDESTR4-R3 destination vector of the Multisite

Table 1. *P. chrysogenum* strains used in this study

Strain	Characteristic	Reference
DS17690	High penicillin yielding strain	(van den Berg et al., 2008)
DS54465	DS17690 Δ*hdfA* with increased homologous recombination	(Snoek et al., 2009)
DsRed.SKL (AmdS$^+$)	DS17690 with integrated P_{pcbC}-*DsRed*-T1.SKL-T_{penDE} cassette; AmdS$^+$	
Δ13g03380::*phleo*^{R}	DS54465 Δ13g03380::*phleomycine*	This study
Δ21g09430::*amdS*	DS54465 Δ21g09430::*amdS*	This study
Δ22g11190::*amdS*	DS54465 Δ22g11190::*amdS*	This study
Δ12g12500::*amdS*	DS54465 Δ12g12500::*amdS*	This study
DsRed.SKL Pc13g03380.GFP	DS17690 DsRed.SKL expressing the Pc13g03380.GFP fusion protein	This study
DsRed.SKL Pc12g12500.GFP	DS17690DsRed.SKL expressing the Pc12g12500.GFP fusion protein	This study
DsRed.SKL Pc21g09430.GFP	DS17690DsRed.SKL expressing the Pc21g09430.GFP fusion protein	This study
DsRed.SKL Pc22g11190.GFP	DS17690DsRed.SKL expressing the Pc22g11190.GFP fusion protein	This study
DS47274	DS17690 with single penicillin biosynthesis gene cluster	(Nijland et al., 2010)

Table 2. Plasmids used in Gateway® cloning system

Plasmid	Characteristic	Reference
pDONR P4-P1R	Multisite Gateway vector Kan^R Cm^R	Invitrogen
pDONR221	Multisite Gateway vector Kan^R Cm^R	Invitrogen
pENTR221- Phleo	pDONR221 with phleomycine expression cassette Kan^R	J.G.Nijland,unpublished
pENTR221- AMDS	pDONR221 with *amdS* expression cassette Kan^R	J.G.Nijland,unpublished
pDONRP2R-P3	Multisite Gateway vector Kan^R Cm^R	Invitrogen
pDONRP2-P3R-GFP-Tat	pDONRP2-P3R with GFP and terminator of *penDE* gene	(Nijland et al., 2008)
pENTR4-1-PgpdA	pDONR P4-P1R with *A. nidulans* gpdA promoter, Kan^R	J.G. Nijland,unpublished
pDEST R4-R3	Multisite Gateway vector Amp^R Cm^R	Invitrogen
pDEST R4-R3/AMDS	pDEST R4-R3 with *A. nidulans amdS* expression cassette	(Meijer et al., 2010)
pEXP 13g03380.GFP	,, ,, with Pc13g03380 C-terminal GFP fusion	This study
pEXP 21g09430.GFP	,, ,, with Pc21g09430 C-terminal GFP fusion	This study
pEXP 22g11190.GFP	,, ,, with Pc22g11190 C-terminal GFP fusion	This study
pEXP 12g12500.GFP	,, ,, with Pc12g12500 C-terminal GFP fusion	This study

Table 3. Primers used in this study

Primes	Sequence (5′→ 3′)
Primers used for cellular localization	
Pc13g03380 AttB1- Fw	GGGGCAAGTTTGTACAAAAAAGCAGGCTCTATGGCCGGTCAATCCAAACCT
Pc13g03380 AttB2- Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTCAGGGTAGCAGGCATGCTGGT
Pc21g09430 AttB1- Fw	GGGGCAAGTTTGTACAAAAAAGCAGGCTCCATGGATGGAAAACAGCTTCCAC
Pc21g09430 AttB2- Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCCGAGGAAAGCAGGAACGTG
Pc12g12500 AttB1- Fw	GGGGCAAGTTTGTACAAAAAAGCAGGCTCTATGGCTGAATACAAGT
Pc12g12500 AttB2 - Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCATCTCGATGCGGCCCAC
Pc22g11190 AttB1- Fw	GGGGCAAGTTTGTACAAAAAAGCAGGCTCCATGTCTCAACCAGGAGCCG
Pc22g11190 AttB2- Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTAGCGATGCGGCCCTTTC
Primers used in construction of the gene inactivation strains	
Pc13g03380 AttB4-FR5′, Fw	GGGGACAACTTTGTATAGAAAAGTTGAATTCTGAAGAACTTGTTCAC
Pc13g03380 AttB1-FR5′, Rv	GGGGACTGCTTTTTTGTACAAACTTGCGATGTACGTTAACGGTATTCG
Pc13g03380 AttB2-FR 3′, Fw	GGGGACAGCTTTCTTGTACAAAGTGGTCTTTTCTTATAGTCAGACGGAGGG
Pc13g03380 AttB3-FR 3′, Rv	GGGGACAACTTTGTATAATAAAGTTGAATTCATAAAGAAAAGAAAAAAGC
Pc22g11190 AttB4-FR 5′, Fw	GGGGACAACTTTGTATAGAAAAGTTGAAGCTTAAATGCCCTTCTATGCGCC
Pc22g11190 AttB2-FR 3′, Fw	GGGGACAGCTTTCTTGTACAAAGTGGTCACAGGAAATGATCCCCAGTCTGC
Pc22g11190 AttB3-FR 3′, Rv	GGGGACAACTTTGTATAATAAAGTTGAAGCTTCAAGCCAGAACAAGCAGATC
Pc21g09430 AttB4- FR5′, Fw	GGGGACAACTTTGTATAGAAAAGTTGAATAACGAGGTTCCCCTGTTTCCG
Pc21g09430 AttB1- FR5′, Rv	GGGGACTGCTTTTTTGTACAAACTTGCAGCGTGCAATAGGTTGCC
Pc21g09430 AttB2- FR3′, Fw	GGGGACAGCTTTCTTGTACAAAGTGGTCAAAACTAACTTGACGGATAGG
Pc21g09430 AttB3- FR3′, Rv	GGGGACAACTTTGTATAATAAAGTTGCACGACCATTCTTGGAGTACCCAAG
Pc12g12500 AttB4- FR5′, Fw	GGGGACAACTTTGTATAGAAAAGTTGAACAGGTGATTGGGTGGACGCGTA
Pc12g12500 AttB1- FR5′, Rv	GGGGACTGCTTTTTTGTACAAACTTGCGGCTCTGCTTCTTCTCAAGTTCG
Pc12g12500 AttB2- FR3′, Fw	GGGGACAGCTTTCTTGTACAAAGTGGTCTATGAGGGCTAAATATACTGT
Pc12g12500 AttB3- FR3′, Rv	GGGGACAACTTTGTATAATAAAGTTGCTCCACCGCGTCCACCGCGTCC
Primers used in the real time qPCR	
Pc13g03380 rtFw	CTTCTATTGGTACAGCGTCG
Pc13g03380 rtRv	TGCGATCGTGGCCAAAGCCT
Pc12g12500 rtFw	CTGAATACAAGTTATCCT
Pc12g12500 rtRv	AAGCCGGCGATACCTTGTT
Pc21g09430 rtFw	CTGCCGCTATTCTGGCCA
Pc21g09430 rtRv	GGGTTGGTGCACAATTGG
Pc22g11190 rtFw	GTTCTGTCGATGGTTTTG
Pc22g11190 rtRv	CAGATTTGCGAGCAGTCA

Gateway® Three-Fragment Vector Construction Kit using the LR Clonase II enzyme mix. These vectors were used to generate the gene inactivation mutants as described below.

2.4. Transformation of P. chrysogenum

For transformation, protoplasts were isolated as described (Carr et al., 1986). The expression vectors containing the GFP C-terminal fusion proteins or the gene deletion constructs were transformed into the *P. chrysogenum* protoplast. Protoplasts were plated on acetamide or phleomycin selection medium. Transformation and selection procedure was performed as described in (Cantoral et al., 1987; Kovalchuk et al., 2012). Single colonies were isolated, sporulated and streaked out to single spores. Ten mutants of each transformation were selected for further analysis.

2.5. Media and growth conditions

For the isolation of genomic DNA and RNA, strains were grown in YGG medium: 0.8 % KCl, 1.6% glucose, 0.66 % Yeast Nitrogen Base, 0.15 % citric acid, 0.6 % K_2HPO_4, 0.2 % yeast extract, pH 6.2, and 100 UI/ml penicillin and 100 µg/l streptomycin (Roche) for 48 h in a rotary shaker at 220 rpm and 25 °C. Expression experiments were performed with cells grown for 48 h on YGG medium. When indicated, 0.1 % oleic acid, 0.05 % Tween 80 and 30 mM potassium phosphate buffer pH 6.8 were added. Alternatively cells were grown on penicillin producing medium with and without POA or PAA for 3 and 5 days.

Strains were cultured on penicillin producing medium with and without side chain precursor, prepared as described earlier (Nijland et al., 2010). As indicated, phenylacetic acid (2.5 g/L) or phenoxyacetic acid (2.5 g/L) was added, and cells were grown for up to 7 days in a rotary shaker at 220 rpm and 25 ˚C. For the formation of adipoyl-6-aminoadipic acid, the penicillin production medium was supplemented with 20 g/L adipic acid, according to (Nijland et al., 2008).

2.6. Southern blotting and hybridization

Southern blotting and hybridization were performed as described in (Nijland et al., 2008). The protocol used for genomic DNA isolation was adapted from a yeast genomic DNA isolation protocol (Harju et al., 2004). Genomic DNA was isolated from mycelium grown for 48 h on YGG medium. Cells were harvested by centrifugation, washed, suspended in lysis buffer (2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris HCl pH 8.0 and 1 mM EDTA pH 8.0) and broken using FastPrep (Qbiogene). Obtained DNA was measured with NanoDrop ND-1000 (Thermo Scientific). Clean genomic DNA (2.5 µg for Δ13g03380::*phleo*^{R} and *5* µg for Δ21g09430::*amdS and* Δ22g11190::*amdS*) was digested with suitable restriction enzymes, separated on 0.8 % agarose gels and transferred onto Zeta-Probe membranes (Biorad). The PCR DIG Probe Synthesis Kit (Roche) was used for labeling of probes. Hybridization was performed overnight at 42 °C in hybridization

buffer: 50 % formamide, 5x SSC (saline- sodium citrate), 2 % blocking reagent (Roche), 0.1 % Na- lauroylsarcosin, and 0.02 % SDS. Next, membranes were washed twice with buffer containing 2x SSC and 0.1 % SDS for 15 min at room temperature and twice with buffer containing 0.2x SSC and 0.1% SDS. Probes labeled with digoxigenin were detected by chemiluminescence using CDP-Star (Roche) and imaging by a Roche LumniImager.

2.7. β-lactam analysis

The concentration of adipoyl-6-aminoadipic acid was determined by isocratic HPLC analysis with Platinum EPS C18 column (Alltech, Deerfield, USA) at 30 °C. The mobile phase consisted of 5 M acetonitrile with 5 mM KH_2PO_4 and H_3PO_4 (Harris et al., 2007). Peaks were identified with reference compounds and integrated to calculate the respective concentration and amount.

To determine the intra- and extracellular concentrations of metabolites, quantitative ^1H NMR experiments were performed at 600 MHz on a Bruker Avance 600 Spectrometer. The residue was dissolved in D_2O and measured at 300 K, and compared to standards to provide an exact measure for the quantity of the β-lactams.

6-APA levels were determined using an Accella1250™ HPLC system coupled to a benchtop ES-MS Orbitrap Exactive™ (Thermo Fisher Scientific, San Jose, CA). A sample of 5 µL of medium fraction was applied on Shim-pack XR-ODS™ C18 column (3.0 x 75 mm, 2.2 µm) (Shimadzu, Japan). Runs were performed at a column temperature of 40 °C and flow 300 µL/min. After 5 min of isocratic flow, a linear gradient was started with 90 % of solvent A (100 % water) and 5 % of solvent C (100% acetonitrile). The first linear gradient at 30 min reached 60 % of C, the second - at 35 min reached 95 % of C. Next, a 10 min washing step at 90 % of solvent C was performed, followed by the column equilibration for 15 min at initial isocratic conditions. Solvent D (2% formic acid) was continuously used in the concentration of 5 % to maintain the final 0.1 % of formic acid in the system. The column fluent was directed to the Exactive™ ES-MS Orbitrap operating at the scan range (m/z 80 – 1600 Da) and switching positive/ negative modes. Voltage parameters used for positive mode were: 4.2 kV spray, 87.5 V capillary and 120 V of tube lens. Voltage parameters used for negative mode were: 3 kV spray, -50 V capillary, -150 V tube lens. The capillary temperature was 325 °C, sheath gas flow 60 arbitrary units auxiliary gas was off to sustain higher detection sensitivity for both positive and negative modes during analysis. The Thermo Excalibur™ processing software was used for quantification of the secondary metabolites. The expected masses and retention time of 6-APA (217.064 $[H]^+$, 2.2 min), PenV (351.10 $[H]^+$, 21.7 min) and IPN (360.12 $[H]^+$, 10.2 min) were determined and used to set up the processing method for automated peak integration and quantification. The ICIS algorithm for component peak detection was applied in this analysis (Ali et al., 2013)

2.8. Confocal fluorescence microscopy

P. chrysogenum strains expressing the GFP fusion proteins of the Ant1p homologs were grown for 24 to 48 h in penicillin production medium supplemented with PAA. For confocal laser microscopy, a Zeiss LSM510 confocal laser microscope was used (CLSM: Zeiss Netherlands, Weesp) equipped with Zeiss Plan-Apochromatic 63x NA 1.4 objective. GFP fluorescence was analyzed by excitation of the mycelium with a 488nm Ar/Kr laser and emission of fluorescence was detect with a band pass filter of 500-530 nm in front of the Photo Multiplier Tube (PMT). DsRed fluorescence was analyzed by excitation at 540nm using a He/Ne laser and emission of the fluorescence was detected with a 565-650 nm filter. Images were taken with AxioCam Camera (Zeiss).

2.9. qPCR expression analysis and gene copy number determination

Expression studies were performed using a Mini Opticon Real-Time quantitative PCR instrument (Bio-Rad Laboratories BV). *P. chrysogenum* strains were cultured as described and samples were taken en filtered. RNA was isolated as described in (Nijland et al., 2010). Mycelium (50 mg) was broken with glass beads in 900 µl of Trizol and 125 µl of chloroform using a Fast Prep shaker (include supplier). Broken cells were centrifuged for 10 min at 13,000 rpm at 4 °C. The liquid phase was transferred to a vial and mixed with 1 volume of chloroform. The sample was centrifuged for 5 min at 13,000 rpm. The liquid phase was transferred to a new vial, mixed with 1 volume of isopropanol and centrifuged for 5 min at 13,000 rpm. The obtained RNA pellet was gently washed with ice-cold 70% ethanol, which was subsequently removed and the pellet was air-dried. Finally, the pellet was dissolved in 200 µl of MilliQ H_2O and incubated in 75 °C for 10 min. The RNA was additionally purified with TURBO DNA-free KIT (Ambion) according the manufacture's recommendations. The RNA was measured with a Nano-Drop ND-1000 system (Thermo Scientific). iScript cDNA synthesis kit (Bio-Rad Laboratories BV) was used for cDNA synthesis, were 500 ng of RNA is utilized in the end volume of 10 µl. The obtained cDNA reactions were diluted into an final volume of 400 µl. Polymerase Chain Reactions were performed with a reaction mix containing: 12.5 µl 5x iQ SYBR Green Sensimix, 0.5 µl (100 mM stock) each of a forward and reverse primer, 7.5 µl MQ H_2O, and 4 µl cDNA. In all qPCR experiments actin was used us reference. Melting curve analysis was included to assure formation of the single DNA product. Primers used in this experiment are listed in Table 3.

2.10. Miscellaneous methods

For dry weight determinations, samples of 10 ml were taken from the cultures and filtered on the pre-weighed paper filter. Filters were washed with demineralized water, dried overnight in the oven (60 °C) and subsequently the weight was determined.

3. RESULTS

3.1. Homologs of peroxisomal adenine nucleotide transporters in P. chrysogenum

The *S. cerevisiae* Ant1p is the best-characterized microbody adenine nucleotide transporter. Homologs of *S. cerevisiae* Ant1p include the *Candida boidinii* Pmp47 and mammalian Pmp34 proteins. These proteins mediate the exchange of ATP and AMP across the microbody membrane (McCammon et al., 1990; van Roermund et al., 2001; Visser et al., 2002). The genome of *P. chrysogenum* contains four potential homologs of peroxisomal adenine nucleotide transporters: Pc13g03380, Pc21g09430, Pc12g12500 and Pc22g11190. Figure 1 shows a phylogenetic tree of these *P. chrysogenum* Ant1p homologs and their relationship with *S. cerevisiae* Ant1p.

To validate the cellular localization of the selected transporters (Pc13g03380, Pc12g12500, Pc21g09430, Pc22g11190), C-terminal GFP fusions were constructed. These proteins are predicted to contain four transmembrane segments with the N- and C-termini exposed to the cytosol. The resultant fusion proteins were expressed in a *P. chrysogenum* strain that also produces a red fluorescent protein targeted to the microbody lumen (DsRed.SKL). Transformants were selected on acetamide as a sole nitrogen source, grown for 20 h on penicillin production medium, and analyzed by confocal laser microscopy. In the case of Pc13g03380, Pc12g12500, Pc21g09430,

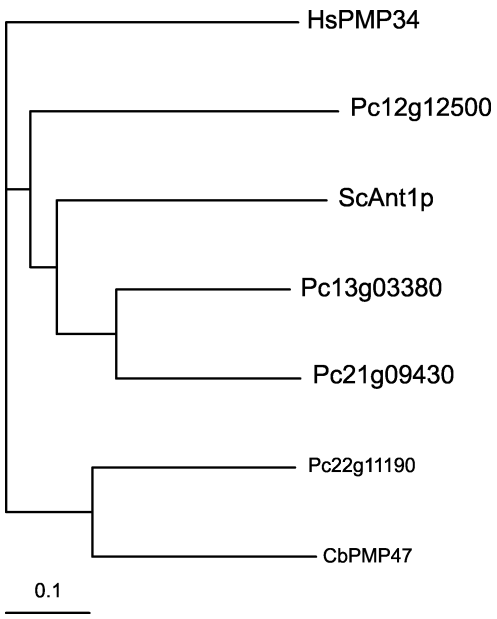

Figure 1. Phylogenetic tree of the putative microbody adenine nucleotide transporters in *P. chrysogenum* with the *S. cerevisiae* Ant1p, human PMP34 and *Candida boidinii* PMP47.

green fluorescence rings were visible around the DsRed.SKL fluorescence that marks the matrix of peroxisomes (Figure 2). This showed that these proteins were localized at the peroxisomal membrane. For Pc22g11190, no reliable localization could be established, suggesting that the fusion protein was unstable.

3.2. Transcription of Ant1p encoding genes of P. chrysogenum

In yeast, Ant1p fulfills a role in β-oxidation (van Roermund et al., 2001) and its expression increases when cells are grown in the presence of oleic acid (Rottensteiner et al., 2002). To examine the expression of the various Ant1p homologs of *P. chrysogenum*, cells were grown on YGG medium with or without oleic acid. Moreover, to evaluate their potential role in β-lactam biosynthesis, cells were also grown on penicillin producing medium with or without the side chain precursor phenylacetic acid (PAA). The expression levels of the Pc13g03380, Pc12g12500, Pc21g09430 and Pc22g11190 genes were examined by qPCR. From cells grown on penicillin producing medium, the RNA was isolated after 3 and 5 days of fermentation. On YGG medium, cells were first grown for 40 h before oleic acid was added and the total RNA was isolated from samples taken 0, 20 and 40 min after addition of oleic acid. The actin gene was used as a reference. All four genes were found to be expressed under the conditions tested (Figure 3). PAA and oleic acid had little effect on the expression levels of Pc22g11190, Pc21g09430 and Pc12g12500, but the expression level of Pc13g03380 increased by 2- and 4-fold in the presence of PAA and oleic acid, respectively. These data suggest that only Pc13g03380 shows a transcriptional response to oleic acid and penicillin producing conditions.

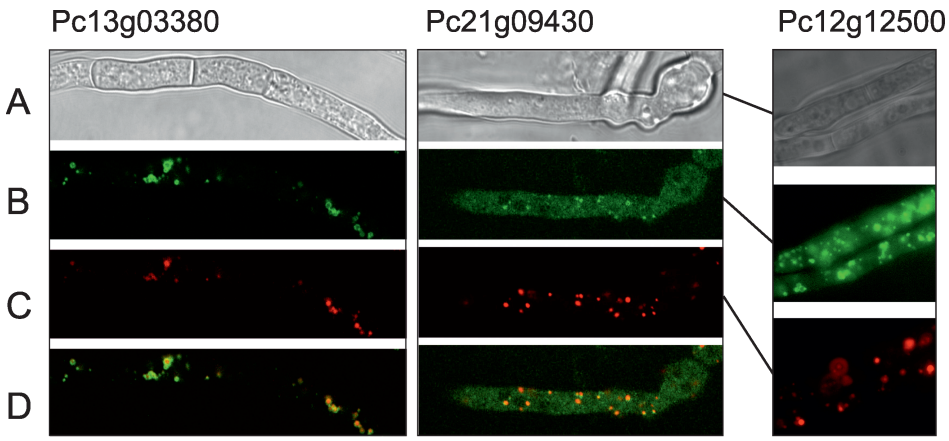

Figure 2. Cellular localization of C-terminally GFP tagged Pc13g03380, Pc12g12500 and Pc21g09430 in the membrane of *P. chrysogenum* microbodies. Images were taken after 20 h of cultivation on penicillin production medium. A, bright field; B, green fluorescence showing the transporter localization in the microbody membrane; C, red fluorescence showing DsRed.SKL in the microbody matrix; and D, merged picture.

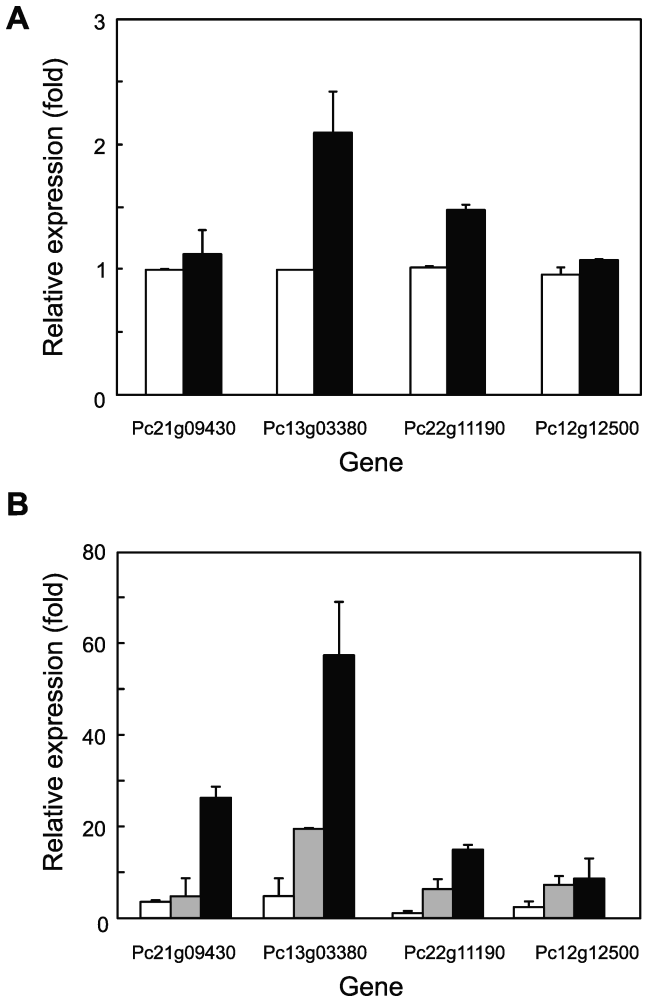

Figure 3. Expression of Pc13g03380, Pc21g09430, Pc12g12500, Pc22g11190 after 3 (white bars) and 5 (black bars) days of growth in the presence of 18 mM phenylacetic acid (A) or 0, 20 and 40 min following the addition of 0.12 % oleic acid (B). Expression was determined by quantitative RT-PCR using the actin gene as a reference. The data presented are averages and mean deviation of X independent replicates.

3.3. Gene inactivation of Ant1p homologs in P. chrysogenum

The expression data suggested that, of the four Ant1p homologs, Pc13g03380 might be involved in penicillin biosynthesis. To further examine the role of these transporters, the genes of the Ant1p homologs were targeted for deletion using the *P. chrysogenum* DS54465 strain, in which the Ku70 (*hdfA*) gene was deleted and which allows for efficient gene inactivation by homologous recombination (Snoek et al., 2009). The flanking regions of all four genes were amplified from genomic DNA of *P. chrysogenum*

DS17690 and used to construct the gene inactivation vector pDESTR4-R3 containing the 5′ and 3′ flanking region with the phleomycine resistance (Pc13g03380) or *amdS* (Pc12g12500, Pc21g09430 and Pc22g11190) as a marker using the Multisite Gateway® Three-Fragment Vector Construction Kit. The destination vectors were transformed into the *P. chrysogenum* Δ*hdfA* strain and mutants were selected by growth on plates with acetamide as a sole nitrogen source or with phleomycin. The obtained gene inactivation mutants were analyzed by PCR (data not shown) and Southern blotting (Figure 4, shown for Pc13g03380). By this method, Pc13g03380, Pc21g09430 and Pc22g11190 were successfully deleted. Despite several attempts we were unable to obtain a deletion strain of Pc12g12500. The obtained deletion strains were used for further analysis.

The gene inactivation mutants were generated in the *P. chrysogenum* DS54465 Δ*hdfA* strain that contains eight copies of the penicillin biosynthesis gene cluster (Nijland et al., 2010). We noted previously that this cluster is highly unstable in this strain, and that multiple copies of the cluster are readily lost when the fungus is subjected to a mock transformation protocol. Therefore, we determined, for five individual clones of each of the gene deletion mutants, the number of penicillin biosynthesis gene clusters using a qPCR-based method on genomic DNA with primers for the genes encoding ACVS and IPNS as described previously (Nijland et al., 2010). Actin was used as a reference. The analysis of the five individual ΔPc13g03380 strains versus the single copy strain DS47274 and the eight-copy strain DS17690 showed that

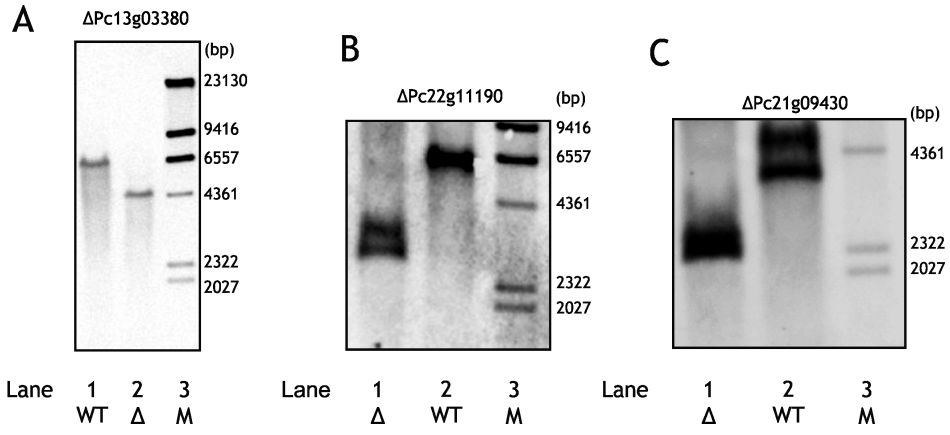

Figure 4. Southern analysis of (A) DS54465 ΔPc13g03380. Genomic DNA was digested with XhoI and hybridized with a probe corresponding to the 3′ Pc13g03380 flanking region yielding a 7418bp band in the parental strain (WT), and a 5119 bp band in the 13g03380::*phleo*^{R} strain (Δ). (B) DS54465 ΔPc22g11190. Genomic DNA was digested with PstI and hybridized with a probe corresponding to the 3′ flanking region of Pc22g11190. Bands of 5832 bp and 3378 bp were found in the parental (WT) and Δ22g11190::*amdS* (Δ) strain, respectively; and (C) DS54465 ΔPc21g09430. Genomic DNA was digested with XbaI and hybridized with a probe corresponding to the 5′ flanking region of Pc21g09430. Bands of 3659 bp and 2102 bp are found in the parental (WT) and Δ21g09430::*amdS* (Δ) strain, respectively.

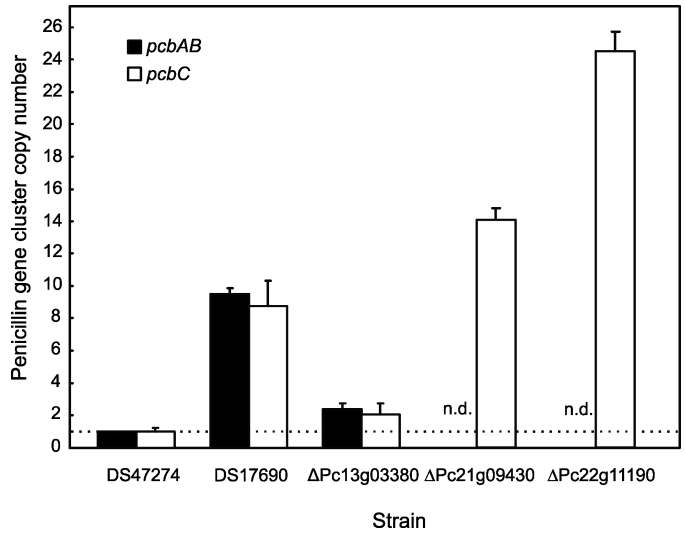

Figure 5. Copy number of penicillin biosynthesis gene clusters in DS47274 (single copy reference strain), DS54465 and ΔPc13g03380, ΔPc21g09430, and ΔPc22g11190. The gene copy numbers of pcbAB and pcbC were determined by qPCR as described in the materials and methods section, and averaged for 5 independent mutants. n.d., not determined. The dashed line indicates the level of the single biosynthetic penicillin cluster copy strain DS47274.

the deletion strains all possessed only two penicillin biosynthesis clusters (Figure 5). A similar analysis revealed that the ΔPc21g09430 and ΔPc22g11190 strains did not lose any copies during the transformation protocol, but instead gained copies (Figure 5).

3.4. Effect of Ant1p gene deletion on β-lactam production

The various gene deletion mutants of the Ant1p homologs of *P. chrysogenum* were tested for their β-lactam production capacity using HPLC, NMR and LC-MS. Herein, individual clones of the various gene inactivation mutants were grown in penicillin production medium in the presence of phenoxyacetic acid (POA). Samples were taken after 5 and 7 days of growth. Mycelium and supernatant fractions obtained by filtration were used to determine of intracellular and extracellular metabolite levels, respectively. For comparison, the *P. chrysogenum* DS54465 strain was included and compared with the ΔPc13g03380, ΔPc21g09430 and ΔPc22g11190 deletion strains. With all of the tested individual clones of the ΔPc13g03380 strain, a substantial decrease by at least 5-fold of the final concentration of penicillin V was observed (Table 4). The intracellular levels of the precursor isopenicillin N were also reduced up to 5-fold after 7 days of fermentation and this was correlated with a reduction in the POA consumption. This correlation was expected, as in these strains the POA side chain is stoichometrically introduced into the β-lactam. In contrast, the ΔPc21g09430 and ΔPc22g11190 mutant

Table 4. Intracellular and extracellular levels of phenoxyacetic acid, isopenicillin N and penicillin V

Metabolite:	penicillin V g/l (g/kg)		phenoxyacetic acid g/l (g/kg)		isopenicillin N g/l (g/kg)	
Strain	5 days	7 days	5 days	7 days	5 days	7 days
DS54465	1.31 (3.28)	2.75 (2.04)	2.18 (6.62)	1.11 (3.53)	0.14 (0.60)	0.22 (2.53)
ΔPc13g03380	0.30 (0.73)	0.42 (0.52)	2.72 (8.08)	1.80 (3.46)	0.07 (0.34)	0.06 (0.50)
ΔPc21g09430	1.87 (3.17)	3.36 (2.21)	1.96 (4.79)	0.68 (2.58)	0.22 (1.60)	0.26 (3.03)
ΔPc22g11190	1.50 (3.00)	3.26 (2.02)	1.82 (6.50)	0.66 (3.81)	0.20 (1.50)	0.24 (2.80)

The levels of the indicated metabolites were determined by NMR as described in the materials and methods section using cells and fermentation broth obtained after 5 and 7 days. *Intracellular levels are indicated between parentheses, and expressed in gram of metabolite per kilogram of cell dry weight.

strains showed no significant difference in penicillin V and isopenicillin N production or POA consumption as compared to the parental strain. With all strains, the amount of used sugar remained unchanged (data not shown), indicating that the observed phenomena are not caused by differences in growth. These data suggest that the inactivation of the Ant1p homolog Pc13g03380 from the genome of *P. chrysogenum* has a strong effect on β-lactam biosynthesis. However, in these gene deletion strains also the number of copies of the penicillin biosynthetic gene cluster is reduced from eight to two. Therefore, we also compared the penicillin V production by these mutants with that of the single copy strain DS47274 (Figure 6A). Importantly, the ΔPc13g03380 strains produced less than 50% of the penicillin V observed with the single copy strain DS47274 whereas an about 2-fold higher level would be expected based on

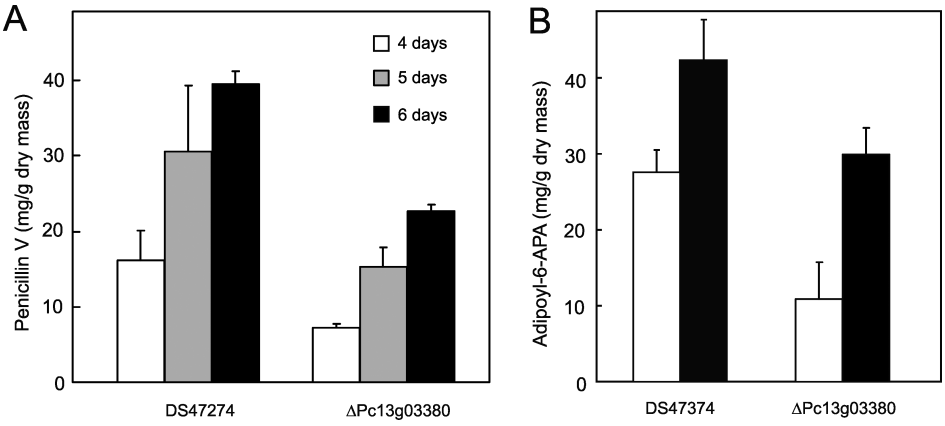

Figure 6. Levels of penicillin V (A) and adipoyl-6-aminopenicillanic acid (B) production by the single penicillin biosynthetic cluster copy strain DS47274 and the ΔPc13g03380 mutant strain containing two copies. Data shown are the averages of two experiments using the 5 independent ΔPc13g03380 strains.

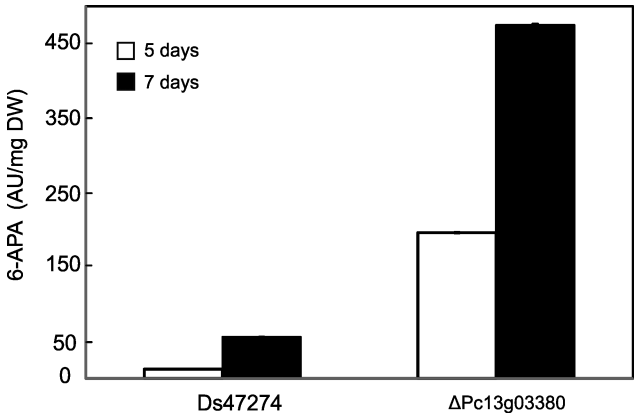

Figure 7. Extracellular levels of 6-aminopenicillanic acid for the single penicillin biosynthetic cluster copy strain DS47274 and the ΔPc13g03380 strain that containing two copies. The data presented are averages and mean deviations of 2 independent replicates.

the copy number (Nijland et al., 2010). This implies that there is a substantial (up to 4-fold) reduction in the amount of penicillin V produced which shows that Pc13g03380 fulfills an important function in penicillin biosynthesis. The lower level of penicillin V production might relate to the lack of CoA activated phenoxyacetic acid caused by a reduced availability of ATP in the microbodies Consequently, one would expect an accumulation of 6-aminopenicillanic acid (6-APA), since acyltransferase (AT) should still be able to hydrolyze the aminoadipate moiety of isopenicillin N.

Therefore, also the extracellular level of 6-APA was determined for the ΔPc13g03380 strain and the DS47274 reference strain. After 5 and 7 days, respectively, a 10-fold and 7-fold increase of the 6-APA concentration in the broth of ΔPc13g03380 was observed as compared to the DS47274 strain (Figure 7). This observation is consistent with the notion that availability of the activated slide chain precursor is reduced in the ΔPc13g03380 strain.

In recent years, recombinant strains of *P. chrysogenum* have been applied for the production of cephalosporins, using adipic acid as a precursor feed. In the absence of the expandase gene, *P. chrysogenum* cells synthesize adipoyl-6-aminopenicillanic acid (adipoyl-6-APA) when fed with adipic acid (Crawford et al., 1995; Nijland et al., 2008). Adipic acid is also metabolized by the cells, likely via a branch of the β-oxidation, which involves an adipic acid CoA ligase (ACS) that is located in the microbody ((Koetsier et al., 2009a); Veiga et al 2012). The latter enzyme is also involved in the formation of adipoyl-6-APA. Therefore, the ΔPc13g03380 strain was grown on mineral medium with adipic acid as a side chain and the medium fraction was analyzed for adipoyl-6-APA production by HPLC. The ΔPc13g03380 strain produced 50% less adipoyl-6-APA as compared to the single copy DS47274 strain (Figure 6B) demonstrating that this Ant1p homolog is also involved in the adipoyl-6-APA production.

4. DISCUSSION

In this report we demonstrated that the *P. chrysogenum* Ant1p homolog Pc13g03380 is required for high-level β-lactam biosynthesis. This protein is homologous to the *S. cerevisiae* Ant1p which functions as a microbody-localized ATP/AMP antiporter. Likely Pc13g03380 fulfills a similar role in *P. chrysogenum*.

P. chrysogenum contains four genes (Pc12g12500, Pc13g03380 Pc21g09430, and Pc22g11190) that encode proteins that are homologous to Ant1p of *S. cerevisiae*. Except for Pc22g11190, the other three proteins could be localized to the microbody membrane by means of a C-terminal fusion with GFP. By a qPCR based expression study, each of these genes was found to be expressed under typical laboratory growth conditions, but only the expression of Pc13g03380 was responsive to the presence of PAA or oleic acid in the medium. This observation suggests that Pc13g03380 is specifically induced under conditions that require a high rate of ATP transport into the microbody, for instance as a result of β-lactam biosynthesis or β-oxidation. To further test this hypothesis, we generated gene inactivation strains of these Ant1p homologs in the *P. chrysogenum* DS54465 strain, which is a *hdfA*Δ derivative of the high penicillin yielding DS17690 strain. We selected five independent deletion strains for each gene: Pc13g03380, Pc21g09430 and Pc22g11190, but were unable to inactivate the Pc12g12500 gene. After transformation of the DS54465 strain with the Pc12g12500 gene inactivation plasmid, cells were not viable which may indicate that this gene is essential in *P. chrysogenum*. However, this would also imply that Pc12g12500 has a different function than Pc13g03380 that likely acts as an ATP/AMP transporter as will be discussed below. When the production of penicillin V was tested for the other gene inactivation strains, only the ΔPc13g03380 strains showed a phenotype with a greatly diminished penicillin V production as compared to the parental strain. However, unlike the other gene deletion strains, the ΔPc13g03380 strains also had lost a substantial number of the penicillin biosynthetic gene clusters during the strain construction protocol. This number had been reduced from 8 to 2, and thus the observed gene cluster instability partially explains the major drop in penicillin V production. When the ΔPc13g03380 strain was compared with the single copy strain DS47274, the level of penicillin V production by the mutants was found to be well below that of the single copy strain which demonstrates that Pc13g03380 indeed plays an important role in β-lactam biosynthesis. A similar result was obtained when POA was replaced by adipic acid, yielding the β-lactam adipoyl-6-aminopenicillanic acid. The reduced pathway activity also resulted in lower levels of the intermediate isopenicillin N. Remarkably, we observed an increase in the level of the extracellular concentration of 6-aminopenicillanic acid (6-APA). In the absence of activated side chain precursors of penicillin, AT can act as a amidohydrolase converting IPN or already formed PenV into 6-APA (Alvarez et al., 1993; Weber et al., 2012b). Thus the increased levels of 6-APA are consistent with the notion that in the ΔPc13g03380 strain, the amount of activated side chain precursor is limiting which can be expected when the supply of

ATP into the microbodies is impaired. Interestingly, the β-lactam production by the ΔPc13g03380 strain was not completely reduced to zero. Therefore, we hypothesize that the activity of one or more of the other Ant1p homologs is responsible for the remaining nucleotide transport activity. It is remarkable that the penicillin biosynthetic gene cluster loss was observed only with the deletion of the Pc13g03380 gene, and not the other three homologs. Perhaps this loss is not accidental but reflects a negative impact of the accumulation of penicillin biosynthesis intermediates in the absence of efficient ATP import into the microbodies.

The exact mechanism by which the deletion of Pc13g03380 results in a reduction of β-lactam production is unknown. However, based on the proposed function of this protein as an ATP/AMP antiporter as shown for the *S. cerevisiae* Ant1p transporter (Palmieri et al., 2001), a possible scenario can be proposed. The removal of Pc13g03380 likely results in a decreased uptake of ATP by the microbodies, and this may result in a reduced β-lactam production as ATP is needed for the CoA activation of side chain precursors such as PAA, POA and adipic acid by microbody CoA ligases (Figure 8) (Alvarez et al., 1993). These aryl-CoA ligases belong to the acyl adenylate protein family (Turgay et al., 1992) and function as activators of the acyl or aryl acids to acyl-AMP or aryl-AMP, at the expense of ATP. In these reactions, AMP is released

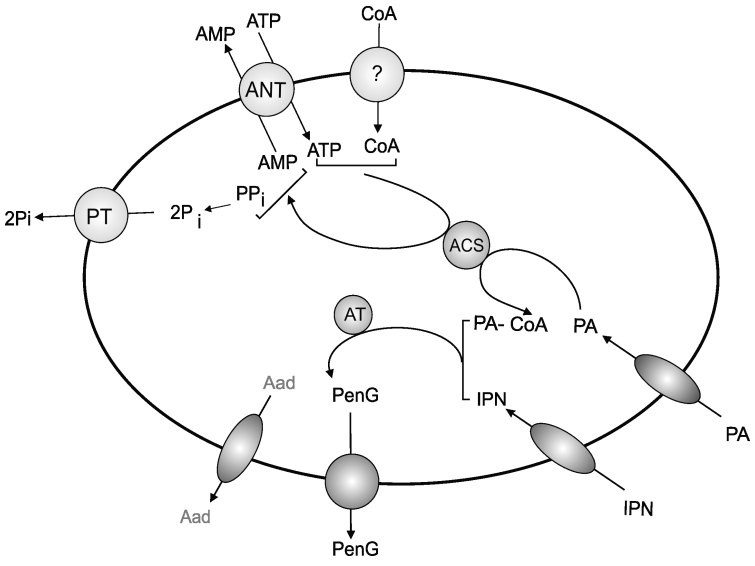

Figure 8. Model for the role of Ant1p in the β-lactam production. We suggest that molecules of ATP used for activation of free fatty acids as well as ones utilized in the reaction of side chain activation during biosynthesis of penicillins might come from the same pool within microbody matrix. PenG: penicillin G; Aad: α-aminoadipic acid; PA: phenylacetic acid; IPN: isopenicillin N; ACS: acetyl CoA-synthetase; AT: acyl-CoA-IPN acyltransferase; ANT: adenine nucleotide transporter; ATP: adenosine-5'-triphosphate; AMP: adenosine monophosphate; CoA: coenzyme A; PP_i: pyrophosphate; PT: hypothetical phosphate transporter.

and the carboxyl group is transferred to the thiol group of CoA forming thioester. The transcription level of Pc13g03380 increased substantially after the addition of oleic acid. This effect might be caused by proliferation of peroxisomes upon the addition of oleic acid (Meijer et al., 2010) suggesting that Pc13g03380 also fulfills a role in β-oxidation by providing ATP for the acyl-CoA ligases present in the microbody matrix. In this respect, Pc13g03380 is also up regulated when cells are grown in the presence of adipic acid (Harris et al., 2009), consistent with the notion that the process of side chain activation during β-lactam biosynthesis has emerged from β-oxidation.

Summarizing, this study indicates that out of the four *P. chrysogenum* Ant1p homologs, only Pc13g03380 seems specifically required for β-lactam production. Pc13g03380 likely fulfills a role as an ATP/AMP antiporter in analogy to the yeast Ant1p, and thus provides the majority of ATP required for side chain activation in the microbody during β-lactam biosynthesis.

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chapter 4

ANALYSIS OF THE ROLE OF MICROBODY LOCALIZED ABC TRANSPORTERS IN β-LACTAM BIOSYNTHESIS BY *PENICILLIUM CHRYSOGENUM*

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ABSTRACT

Penicillium chrysogenum contains two proteins that belong to the subfamily of ABC-D transporters. Pc13g11640 and Pc16g09390 are homologous to the *Saccharomyces cerevisiae* Pxa1 and Pxa2 long chain fatty acid transporters that reside in the microbody membrane. We have studied the cellular localization and role of these two potential transporter proteins in penicillin biosynthesis in *Penicillium chrysogenum*. Pc13g11640 was found to localize at the microbody membrane. Deletion of Pc13g11640 and Pc16g09390 in *P. chrysogenum* had no effect on growth, oleic acid utilization, or production of β-lactam antibiotics. On the other hand, both Pc13g11640 and Pc16g09390 complement the oleic acid utilization defect of the *S. cerevisiae* Pxa1/Pxa2 deletion strain, confirming their role as long chain fatty acid transporters.

1. INTRODUCTION

Penicillum chrysogenum is a filamentous fungus that during the last 60 years is used for the industrial production of β-lactam antibiotics. Current production strains are the result of a classic strain improvement (CSI) program in which a dramatic increase in the penicillin biosynthesis was achieved by chemical and UV-induced mutagenesis of production strains followed by screening of improved producers. One of the main results of the CSI has been the multiplication of the penicillin biosynthesis gene cluster (Elander, 2003). Many of the enzymatic steps in penicillin biosynthesis have been elucidated and studied in detail. The pathway is highly compartmentalized (van de Kamp et al., 1999). The first two steps of penicillin biosynthesis take place in the cytoplasm, where the condensation of the three precursor amino acids: L-α-aminoadipate, L-cysteine and L-valine into the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) is catalyzed by the nonribosomal peptide synthetase *ACV synthetase*. Also the cyclization of LLD-ACV into isopenicillin N (IPN) occurs in the cytosol and is catalyzed by *isopenicillin N synthase (IPNS)*. In this reaction the penam nucleus is formed, which consists of a β-lactam and thiazoline ring. ACVS and IPNS are both localized in the cytosol, and this possibly ensures a fast turnover of the tripeptide ACV into isopenicillin N (Müller et al., 1991; van der Lende et al., 2002b). The final steps in penicillin biosynthesis are localized in the microbody. This concerns the substitution of the L-α-aminoadipate group for a phenylacetyl- or phenoxyacetyl moiety in a reaction catalyzed by *acyl-coenzyme A:isopenicillin N acyltransferase (IAT)* yielding penicillin G or –V, respectively. Another enzyme located in the microbody is the *phenylacetyl CoA ligase (PCL)* that activates phenylacetic acid (PA) or phenoxyacetic acid (POA) into their CoA thioesters that are used as substrate by IAT (Koetsier et al., 2009; Meijer et al., 2010). Several other CoA-ligases were identified, which are localized in the microbody matrix. However, only one of them, namely *acyl CoA-ligase* (ACLA, encoded by Pc22g0270) is upregulated when a fungus is grown on adipic acid (Koetsier et al., 2010). Penicillin titers in ΔaclA, Δpcl and ΔaclA-Δpcl deletion strains, ACLA seems not to be significant for the penicillin biosynthesis. Deletion of phenylacetyl CoA ligase leads to the significant decrease (~ 70%) of the penicillin V production (Koetsier et al., 2010; Lamas-Maceiras et al., 2006). This suggest that one or more of the remaining identified acyl-CoA-ligases might exhibit activity towards precursors of penicillins (Koetsier et al., 2010).

Microbodies are small, single membrane organelles with a diameter of 200-800 nm. They are present in the cells of all eukaryotic organisms (Müller et al., 1992) and involved in various metabolic pathways (Rottensteiner and Theodoulou, 2006; Schrader and Fahimi, 2008; Titorenko and Rachubinski, 2004) such as β-oxidation of the fatty acids and detoxification of the hydrogen peroxide (van den Bosch et al., 1992). In filamentous fungi evidence has been provided that the microbodies are involved in β-oxidation of fatty acids (*Neurospora crassa, Aspergillus nidulans, Candida albicans, Penicillium chrysogenum)* , (Kiel et al., 2009; Kionka and Kunau, 1985; Valenciano et al.,

1998; Valenciano et al., 1996; Veiga et al., 2012) and karyogamy *Podospora anserina*), (Berteaux-Lecellier et al., 1995; Boisnard et al., 2009; Veiga et al., 2012). However, in *P. chrysogenum and Aspergillus nidulans,* the microbodies are also involved in penicillin biosynthesis (Meijer et al., 2010; Sprote et al., 2009). In *P. chrysogenum*, microbodies are essential for penicillin biosynthesis. IAT and PCL both exhibit a pH optimum at slightly alkaline pH, and possibly the luminal pH of microbodies provides an optimal environment for these enzymes (van der Lende et al., 2002a). Also, the co-localization of these two enzymes in a small and closed environment may promote the efficient conversion. Previously, it has been shown that penicillin biosynthesis increases with the volume fraction of microbodies under conditions that the amount of the IAT and PCL enzymes remains the same (Kiel et al., 2005; Meijer et al., 2010). Recently it was observed, that changes of the number and size of peroxisomes (by overproduction or deletion of *pex11* family genes) has no significant influence on the production of antibiotic in the high penicillin producing strain (Opaliński et al., 2012). Interestingly, in the Δpex16 and Δpex3 deletion strains, where most or all of the microbody matrix proteins were mislocalized to cytosol, penicillin titers were significantly reduced (Opaliński et al., 2012). Similar observation was made for *A. nidulans*, where penicillin biosynthesis can also occur in the cytosol and this process involves a cytosolic AT that differs from the microbody enzyme. This cytosolic penicillin biosynthesis seems less efficient, i.e., lower flux, as compared to the microbody localized process (Sprote et al., 2009). Moreover, *Hansenula polymorpha* strain, expressing *pcbAB*, *pcbC*, *penDE* and *phl* from *P. chrysogenum*, although devoid of peroxisomes, is capable to synthesize penicillin G, however at fairly reduced titer comparing to the *H. polymorpha* strain producing microbodies (Gidijala et al., 2009). The latter observation suggests that the transport efficiency in the high producing strains might be less dependent on the size and microbody structure, however, the volume of the microbody fraction is an important element for production of β-lactams.

Little is known about the transport processes that are required in the microbodies for optimal penicillin biosynthesis. A real requirement for transport is already under debate for the last 40 years as only few microbody transporters have been identified so far. Two opposite mechanisms for transport have been suggested: a) metabolite uptake systems that function as antiporters to couple cytosolic processes with those in the microbody (Rottensteiner and Theodoulou, 2006; Visser et al., 2007), or b) free permeation of small solutes through nonselective channels (Antonenkov et al., 2009; Rokka et al., 2009). These channels would be gated to ensure that valuable substrates are not lost from the microbody lumen. A few substrates are known to require the active transport across the microbody membrane. For instance, the uptake of ATP and excretion of AMP is mediated by Ant1p, but the majority of the solutes would permeate the microbody membrane through these gated channels. Also, it is unknown how intermediates and end products of penicillin biosynthesis are transported across the microbody membrane. Since isopenicillin N is relatively polar, it is very likely

that transport of this molecule through the microbody membrane depends on a specific transport protein. Transport of 6-APA (6-aminopenicillanic acid, IAT derived intermediate) occurs relatively efficient by passive diffusion (García-Estrada et al., 2007). However, penicillins vary in polarity, and therefore a mechanism of secretion involving passive diffusion appears unlikely. In this respect, in high yielding strains the extracellular penicillin levels are much higher than intracellular concentration, suggesting that an active transport occurs across the plasma membrane (van den Berg et al., 2008)J, which would also imply an active transport mechanism across the microbody membrane (Douma et al., 2011).

We have recently performed a membrane proteomic approach on isolated microbody membranes using LC-MS/MS. The density of membrane proteins in the microbody membrane is relatively low, and such proteomic approaches are complicated by organellar contaminants as for instance the highly protein dense mitochondrial membranes that make a definite assignment of identified proteins to the microbody membrane difficult. Consequently, the proteomic approach yielded only a few membrane proteins of the microbody membrane for which exclusive microbody localization could be ascertained. On the other hand, in yeast and mammals, ATP-binding cassette transporters (ABC) have been identified in microbodies that are involved in the transport of substrates for β-oxidation. ABC proteins are encoded by the largest gene superfamily in all sequenced genomes and share a wide range of mostly membrane-bound proteins with diverse function. They are characterized by the sequence and organization of an ATP binding domain, which is a nucleotide binding fold (NBF), containing two conserved motifs, Walker A and B with approximately 90-120 amino acids in between (Theodoulou et al., 2006). The functional protein usually consists of two NBFs and two transmembrane domains (TMD), which consists of six membrane-spanning α-helices. In case of eukaryotic ABC transporters these domains can be organized as a full-transporter (build of two NBFs and two TMDs) or two half-transporters (containing one TMD and one NBF). ABC proteins are divided into groups based on the phylogenetic relationship between the nucleotide binding domains. Microbody ABC transporters belong to the ABC-D subfamily. Human and mice ABC-D subfamily possess four members: adrenoleukodystrophy protein (ALDP), adrenoleukodystrophy related protein (ALDRP), PMP70 and PMP69. They are integral membrane proteins (Kamijo et al., 1990; Rottensteiner and Theodoulou, 2006), which are functional as homo- or heterodimers (Liu et al., 1999; van Roermund et al., 2008). In the yeast *Saccharomyces cerevisiae,* two microbody ABC half transporters have been identified, namely Pxa1p and Pxa2p, which function as a heterodimeric ABC transporter (Hettema et al., 1996; Shani and Valle, 1996). In contrast, plant microbodies contain one full size ABC transporter which is known as COMATOSE (CTS) (Zolman et al., 2001). In all organisms mentioned above microbody ABC transporters are involved in the active transport of activated long chain fatty acid (LCFA)-CoA esters across the microbody membrane (van Roermund et al., 2008). CoA is a substrate in various

microbody processes such as β-oxidation but for instance also in β-lactam antibiotics production. The mechanism by which CoA enters this organelle is unknown, but studies on the permeability of microbody membrane for free CoA suggests that it enters via an active transport (Rokka et al., 2009). Interestingly, the uptake of fatty acids could provide a mechanism for the entry of CoA and thus satisfy the need for side chain activation in penicillin biosynthesis. Here we present an analysis of the ALDP homologs of *P. chrysogenum*, and assess their role in β-oxidation and β-lactam antibiotics biosynthesis.

2. MATERIALS AND METHODS

2.1. Fungal strains

P. chrysogenum strains used in this study are described and indicated in the Table 1.

2.2. Bacterial strains used and cloning vectors

To generate expression vectors using Multisite Gateway® Three-Fragment Vector Construction Kit (Invitrogen) *Escherichia coli* DH5α strain was employed. All standard DNA manipulations were performed as described earlier (Sambrook et al., 1989). *P. chrysogenum* DS17690 either DS17690 strain producing DsRed.SKL were utilized for cellular localization of selected proteins (Table 1).

Pc13g11640 and Pc16g09390 were amplified on genomic DNA of DS17690 *P. chrysogenum*. Primers used for this reaction (Table 3) were designed in the way that genes were amplified without STOP codon and were flanked with att recombination sites of the Gateway System. Further, obtained PCR products were cloned separately into pDONR201 using BP Clonase II enzyme mix. Plasmids carrying the genes of interest (pDONR201 - Pc13g11640, pDONR201 - Pc16g09390) were mixed with pDONRP2-P3R-GFP-Tat and pENTR4-1-PgpdA, respectively (J.G. Nijland, unpublished) and cloned into the pDEST R4-R3/AMDS (J.G. Nijland, unpublished) destination vector. This resulted in the generation of plasmids expressing carboxyl-terminal fusion proteins with GFP (Table 2). In these cloning steps LR Clonase II enzyme mix was used from Multisite Gateway® Three-Fragment Vector Construction Kit.

Flanking regions of the Pc13g11640 and Pc16g09390 used to create deletion strains were amplified on genomic DNA of *P. chrysogenum* DS17690. The region upstream (5′ flanking region) of the selected gene was amplified with AttB4-FR5′ and AttB1-FR5′ primers while downstream (3′ flanking region) was amplified with AttB2-FR3′ and AttB3-FR3′ (Table 3). The 5′ flanking regions were cloned into pDONRP4-P1R, while the 3′ flanking regions were cloned into pDONRP2R-P3. During the construction of the Pc16g09390 gene deletion mutant, the phleomycine resistance gene was used as a marker whereas with Pc13g11640, the *amdS* gene was used as a marker. Cloning in pDONR vectors was done using BP Clonase II enzyme mix. Obtained plasmids were

Table 1. *P. chrysogenum* and *S. cerevisiae* used in this study.

Strain	Characteristics	Reference
P. chrysogenum		
DS17690	High penicillin yielding strain	(van den Berg et al., 2008)
DS54465	DS17690 Δ*hdfA* with increased homologous recombination	(Snoek et al., 2009)
DsRed.SKL (AmdS$^+$)	DS17690 with integrated P_{pcbC}-*DsRed*-T1.SKL-T_{penDE} cassette; AmdS$^+$	(Kiel et al., 2009)
Δ13g11640::amdS	DS54465 Δ*13g11640::amdS*	This study
Δ16g09390::amdS	DS54465 Δ*16g09390::amdS*	This study
Δ13g11640/Δ16g09390	DS54465 Δ*13g11640::phleo*/ Δ*16g09390::amdS*	This study
DsRed.SKL Pc13g11640-GFP	DsRed.SKL expressing Pc13g11640.GFP fusion protein	This study
DS47274 (AFF400)	DS17690 containing one penicillin biosynthesis gene cluster	(Nijland et al., 2010)
S. cerevisiae		
BY4741 (Y00000)	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0	(Baker Brachmann et al., 1998)
ΔYKL188c (ΔPxa2) (Y05038)	BY4741; YKL188c::kanMX4	(Baker Brachmann et al., 1998)
ΔYPL147w (ΔPxa1) (Y0210)	BY4741; YPL147w::kanMX4	(Baker Brachmann et al., 1998)

combined appropriately and cloned into pDESTR4-R3 destination of the Multisite Gateway® Three-Fragment Vector Construction Kit with LR Clonase II enzyme mix.

2.3. Media and growth conditions

For the isolation of genomic DNA, RNA as well as for transformation mycelium was grown on YGG medium (48h, 25 °C, 220 rpm) which contain as follows: 0.8 % KCl, 1.6 % glucose, 0.66 % Yeast Nitrogen Base, 0.15 % citric acid, 0.6 % K_2HPO_4, 0.2 % yeast extract pH 6.2, penicillin (100 IU/ml, Roche) and streptomycin (100 µg/l, Roche). If needed 0.12% Oleic Acid, 0.05 % Tween 80 and 30mM potassium phosphate buffer pH 6.8 were added to the culture.

Strains were cultured on penicillin producing medium with and without side chain precursor, prepared as described earlier (Nijland et al., 2010) As indicated phenylacetic acid (PAA) (2.5 g/L) or phenoxyacetic acid (POA) (2.5 g/L) was added, and cells were grown for up to 7 days in a rotary shaker at 220 rpm and 25 °C. For the formation of adipoyl-6-aminoadipic acid, the penicillin production medium was supplemented with 20 g/L adipic acid, according (Nijland et al., 2008). When indicated 0.12 % oleic acid and 0.05 % Tween 80 was added to the medium.

Table 2. Plasmids used

Plasmids	Characteristics	Reference
pDONR P4-P1R	Multisite Gateway vector Kan^R Cm^R	Invitrogen
pDONR201	Multisite Gateway vector Kan^R Cm^R	Invitrogen
pDONR221	Multisite Gateway vector Kan^R Cm^R	Invitrogen
pENTR221-Phleo	pDONR221 with phleomycine expression cassette Kan^R	J.G. Nijland, unpublished
pENTR221-AmdS	pDONR221 with AmdS expression cassette Kan^R	J.G. Nijland, unpublished
pDONRP2R-P3	Multisite Gateway vector Kan^R Cm^R	Invitrogen
pDONRP2-P3R-GFP-T	pDONRP2-P3R with GFP and terminator of *penDE* gene	(Nijland et al., 2008)
pENTR4-1-PgpdA	pDONR P4-P1R with *A. nidulans gpdA* promoter, Kan^R	J.G. Nijland, unpublished
pDEST R4- R3	Multisite Gateway vector Amp^R Cm^R	Invitrogen
pDEST R4-R3/AMDS	pDEST R4- R3 with A. nidulans *amdS* expression cassette, Amp^R Cm^R	J.G. Nijland, unpublished
pEXP 13g11640.GFP	pDEST R4-R3/AMDS with Pc13g03380 C-terminal GFP fusion	This study
pEXP i-16g09390.GFP	pDEST R4-R3/AMDS with Pc16g09390 C-terminal GFP fusion	This study
pSUM1	pDONR P4-P1R with *TDH3* promoter	(Baerends et al., 2008)
pSUM3	pDONR P4-P1R with MET25 promoter	R. Baerends, unpublished
pSUM90	pDONR_P2RP3 with Myc-tag, TAA-stop codon and CYC1-terminator	R. Baerends, unpublished
pSUM2H	pDEST™R4-R3 with 2µ replication origin and HIS4-markermarker	(Baerends et al., 2008)
pSUM2U	pDEST™R4-R3 with 2µ replication origin and URA3-marke	R. Baerends, unpublished

2.4. Transformation of P. chrysogenum

Protoplasts were isolated and transformation of *P. chrysogenum* was performed as described (Cantoral et al., 1987; Kovalchuk et al., 2012) Expression vectors containing the C-terminal fusions of the indicated genes with GFP or deletion constructs were transformed into *P. chrysogenum* protoplasts. Mutants were selected by growth on the plates with acetamide (Kolar et al., 1988) as a sole nitrogen source (Pc13g11640) or on phleomycin regeneration medium (Pc16g09390), respectively. Phleomycin regeneration plates were prepared according to the protocol described by (Kovalchuk et al., 2012). Single colonies were isolated, sporulated and streaked out to single spores. Ten mutants out of each transformation were picked up and selected for further analysis. The double mutant Δ*13g11640/16g09390* of *P. chrysogenum* DS54465 was made by transforming the destination vector carrying flanking regions

Table 3. Primers used.

Primers	Sequence (5′→ 3′)
Primers used for cellular localization	
Pc13g11640 AttB1- Fw	GGGGACAAGTTTGTACAAAAAAGCAG GCTCGATGGCCGCTCAGTCCAAACTAC
Pc13g11640 AttB2- Rv	GGGGACCACTTTGTACAAGAAAGCTGG GTGAGCCGTTAACTCGGCAACTCGGCG
Pc16g09390 AttB1- Fw	GGGGACAAGTTTGTACAAAAAAGCAG GCTCGATGGCTGTCCAGTCAACCCTTC
Pc16g09390 AttB2- Rv	GGGGACCACTTTCTACAAGAAAGCTG GGTGATTCGCAGTTGCTTCAACTAGCG
Primers used in construction of the gene inactivation strains	
Pc13g11640 AttB4 FR5′ Fw	GGGACAACTTTGTATAGAAAAGTTG TTTAAAGCTCTAGGGTCACAGATTG
Pc13g11640 AttB1 FR5′ Rv	GGGGACTGCTTTTTTGTACAAA CTTGCCATGACGGAGGTGAC
Pc13g11640 AttB2 FR3′ Fw	GGGACAGCTTTCTTGTACAAAGTG GTTCGTGAATACTTAGGATCGTG
Pc13g11640 AttB3 FR3′ Rv	GGGACAACTTTGTATAATAAAGTTG ATCGATATACAGCAGTGTACAAACC
Pc16g09390 AttB4 FR5′ Fw	GGGGACAACTTTGTATAGAAAAGTT GTTCGAAGTTGCGTCCTGGATCTC
Pc16g09390 AttB1 FR5′ Rv	GGGACTGCTTTTTTGTACAAACT TGCTCCGACTCACGAGGTAAC
Pc16g09390 AttB2 FR3′ Fw	GGGACAGCTTTCTTGTACAAAGTG gACCATTGCCGAGTTCTAGCTC
Pc16g09390 AttB3 FR3′ Rv	GGGACAACTTTGTATAATAAAGTTG AAGCTTCAATGATATCCACCATATGC
Primers used in the real time qPCR	
Pc13g11640 rtFw	GAGTCTGCTAGTGCAGTT
Pc13g11640 rtRv	CATTCGACGATTTGTGAC
Pc16g09390 rtFw	GCAGTAGAGGGCAAGAAGG
Pc16g09390 rtRv	GGTGTAATACGAATCGGC
Primers used for *S. cerevisiae*	
attB1FPc13g11640	GGGGACAAGTTTGTACAAAAAAGCAGG CTCGGATAATGTCTGCCGCTCAGTCCA
attB2RPc13g11640	GGGGACCACTTTGTACAAGAAAGCT GGGTGAGCCGTTAACTCGGCAACTC
attB1FPc16g09390	GGGGACAAGTTTGTACAAAAAAGCAGG CTCGGATAATGTCTGCTGTCCAGTCAAC
attB2rPc16g09390	GGGGACCACTTTGTACAAGAAAGCT GGGTGATTCGCAGTTGCTTCAACTA
Pc13g11640 rtFw	GAGTCTGCTAGTGCAGTT
Pc13g11640 rtRv	CATTCGACGATTTGTGAC
Pc16g09390 rtFw	GCAGTAGAGGGCAAGAAGG
Pc16g09390 rtRv	GGTGTAATACGAATCGGC

of Pc16g09390 and phleomycin marker gene into the *P. chrysogenum* DS54465 13g11640Δ::*amdS* strain.

2.5. Southern blotting and hybridization

Hybridizations and southern blots were performed as described (Nijland et al., 2008). The method of genomic DNA isolation was adapted from yeast DNA isolation protocol (Harju et al., 2004). Genomic DNA (2.5 µg) was digested with suitable restriction enzymes, separated on 0.8 % agarose gel and transferred on Zeta- Probe membrane (Biorad) (Sambrook et al., 1989). Probes were labeled using the PCR DIG Probe Synthesis Kit (Roche). Hybridization reactions were performed overnight at 42 °C in hybridization buffer (50 % formamide, 5x SSC (saline- sodium citrate), 2 % blocking reagent (Roche), 0.1 % Na-lauroylsarcosin, 0.02 % SDS). Next, the membrane was washed two times with buffer containing 2xSSC and 0.1% SDS and two times with buffer containing 0.2xSSC and 0.1 % SDS each for 15 min. For detection of the digoxigenin labeled probes, chemiluminescent CDP-Star (Roche) was employed.

2.6. Confocal fluorescence microscopy

P. chrysogenum strains expressing C-terminal fusions of the transporter proteins and the marker GFP were grown for up to 48h in penicillin production medium supplemented with PAA. Images were made with a Zeiss LSM510 confocal laser microscope (CLSM: Zeiss Netherlands, Weesp, The Netherlands) equipped with Zeiss Plan-Apochromatic 63xNA 1.4 objective. GFP fluorescence was analyzed by excitation of the cells with a 488nm Ar/Kr laser. The emission of fluorescence was detected with a band pass of 500-530 nm Photo Multiplier Tube (PMT). DsRed fluorescence was analyzed by excitation of the cells with a 540nm He/Ne laser and emission of the fluorescence was detected by a 565- 650 nm Photo Multiplier Tube. Pictures were taken with an AxioCam Camera (Zeiss).

2.7. Expression studies

RNA was isolated from collected mycelium according to the procedure described earlier (Nijland et al., 2010), gently washed with ice-cold 70% ethanol and air dried. The resulted pellet was dissolved in 200 µl of milliQ water and incubated for 10 min at 75 $^{\circ}$ C. The RNA was cleaned with TURBO DNA-free KIT (Ambion), the RNA concentration was estimated with Nanodrop ND-1000 (Thermo Scientific) and diluted to the concentration of 500 ng per cDNA reaction mixture. cDNA synthesis was performed in the end volume of 10 µl with iScript cDNA synthesis kit (Bio-Rad) as described previously (Nijland et al., 2010) using an iCycler Mini Opticon Real-Time PCR machine (Bio- Rad Laboratories BV). Actin was used as reference in all qPCR experiments. Melting curve analysis was included to assure formation of the single DNA product.

2.8. Complementation in yeast

Yeast strains used in this study were purchased from Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html): ΔYKL188c (Δpxa2) (BY4741;Mata;his3Δ1;leu2Δ0;met15Δ0;ura3Δ0; YKL188c::kanMX4); ΔYPL147w(Δpxa1) (BY4741;Mata;his3Δ1;leu2Δ0;met15Δ0;ura3Δ0;YPL147w::kanMX4); BY4741 (wild type strain) (MATa;his3Δ1;leu2Δ0;met15Δ0;ura3Δ0) (Table 1). Deletion strains obtained from Euroscarf were constructed as described (Baker Brachmann et al., 1998). Pc13g11640 and Pc16g09390 of *P. chrysogenum* were amplified from genomic cDNA with primers flanked with an att recombination sites of the Gateway System (Table 3). The PCR products were cloned into pDONR221, yielding the donor vectors pDONR221-13g11640 or pDONR-16g09390. Each donor vector was combined with pSUM1 or pSUM3 and pSUM90 and cloned into pSUM2H and pSUM2U destination vectors respectively (Table 2). This cloning was performed in one recombination step. The *in vitro* recombination during the LR reaction yielded the pSUM2U1-13g11640, pSUM2U3-13g11640, pSUM2H1-Pc13g11640 and pSUM2U3-Pc13g11640 expression vectors. The same procedure was repeated for Pc16g09390, yielding pSUM2U1-16g09390, pSUM2U3-16g09390, pSUM2H1-16g09390, and pSUM2H3-16g09390. Next, the expression vectors were transformed (Gietz and Schiestl, 2007) into the *S. cerevisiae* strains BY4741, ΔYKL188c, and Δ*YPL147w,* respectively. The empty pSUM2U and pSUM2H vectors were included as controls. Yeast transformants were selected and grown on plates with minimal medium containing: 0.67% yeast nitrogen base without amino acids, 0.3% glucose, agar 20g/L and amino acids as needed (20-30 μg/ml) (van Roermund et al., 2001). For DNA isolation, yeasts were grown in 5-10 ml of liquid minimal medium, with agitation at 30 °C to an OD_{600} of 3.0.

To study the growth on fatty acids, cells were first inoculated in liquid minimal medium and grown overnight. Cultures were diluted to an OD_{600} of 0.1 and transferred to medium consisting of: 0.5 % potassium phosphate buffer (pH 6.3), 0.3 % yeast extract, 0.5 % peptone and either 3 % glycerol or 0.12 % oleic acid- 0.05 % Tween 40 (van Roermund et al., 2001). Cultures were grown for 24 hrs at 30 °C and the OD_{600} was measured in time.

2.9. Miscellaneous methods

For dry weight determinations, samples of 10 ml of the cultures were filtered on the pre-weighed paper filter and washed with demineralized water, dried overnight in the oven (60 °C) and subsequently weighed. The extracellular concentrations of PAA, POA, penicillin G and V as well as adipoyl-6-aminoadipic acid were measured by isocratic HPLC analysis with Platinum EPS C18 column (Alltech, Deerfield, USA) at 30 °C. The mobile phase consisted of 5 M acetonitrile with 5 mM KH_2PO_4 and H_3PO_4 (Harris et al., 2007).

3. RESULTS

3.1. Cellular localization of microbody ABC transporters in P. chrysogenum

The *P. chrysogenum* genome contains two homologs of known yeast microbody ABC transporters namely Pc16g09390 and Pc13g11640 (Figure 1). These proteins are homologues to the yeast Pxa1p and Pxa2p (*Saccharomyces cerevisiae*), respectively. The represent so-called ABC half-transporters. In order to form a functional transporter, these two modules are believed to dimerize, but also in a homodimeric form these transporters may harbor some functionality. To validate their cellular localization, the gene of the fluorescent reporter protein GFP was fused to the C-termini of Pc16g09390 and Pc13g11640, respectively. According to the predicted membrane topology of these ABC transporters, the C-termini are exposed to the cytosol. The resultant

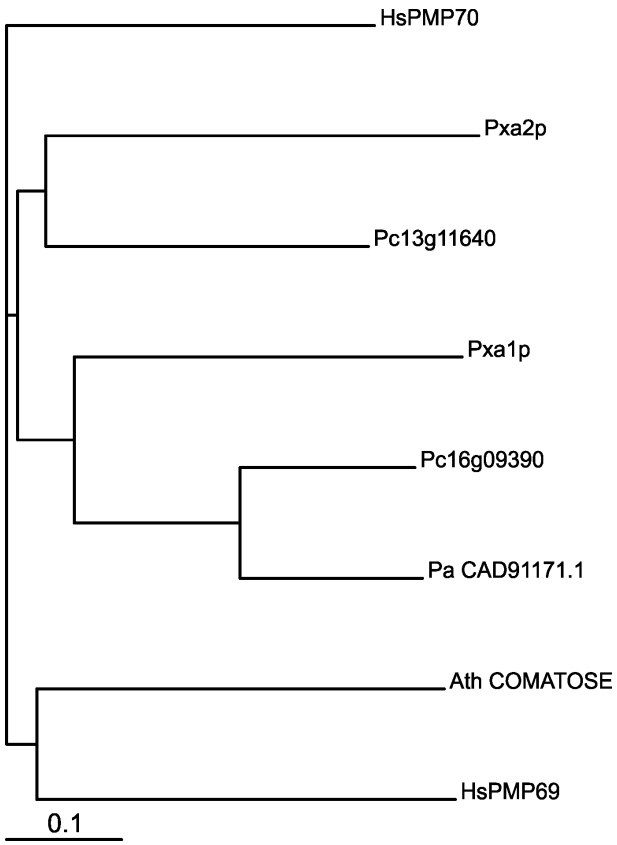

Figure 1. Phylogenetic tree of the relationships of the protein sequences of ABC-D transporters of P. chrysogenum, S. cereviseae (ScPxa1p, ScPxa2p), Podospora anserina (CAD91171.1), Homo sapiens (HsPMP69, HsPMP70) and Arabidopsis thaliana (COMATOSE)

proteins were expressed in a *P. chrysogenum* strain that produces the microbody lumen marker DSRed.SKL. Mutants were selected on acetamide as a sole nitrogen source and cultivated on penicillin production medium. Mycelium was collected after at least 20 h of cultivation, and visualized by confocal laser microscopy. In the case of Pc13g11640, green fluorescence rings were visible around DsRed.SKL fluorescence demonstrating the microbody membrane localization (Figure 2). Unfortunately, with the Pc16g09390 protein neither the C-terminal fusion, nor an additional constructed N-terminal fusion protein, resulted in detectable levels of fluorescence.

3.2. Expression analysis of Pc13g11640 and Pc16g09390 in P. chrysogenum

To examine the expression level of Pc13g11640 and Pc16g09390 *P. chrysogenum* DS54465 was grown for several days in the shaken flasks on penicillin production medium, with and without the side chain precursor phenylacetic acid (PAA). Mycelium was harvested, total RNA was isolated and tested using qPCR to determine the expression level of Pc13g11640 and Pc16g09390. As a reference the actin gene was used. Both Pc13g11640 and Pc16g09390 are transcribed irrespective the presence or

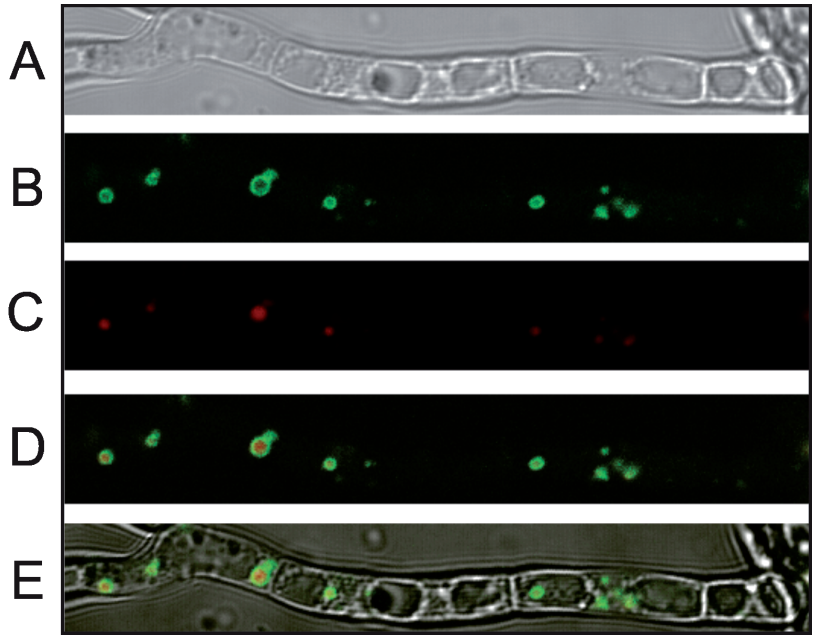

Figure 2. Cellular localization of Pc13g11640-GFP in *P. chrysogenum*: A, bright field image; B, GFP fluorescent image showing microbody membrane localization of Pc13g11640-GFP; C, Fluorescent image showing DSRED-SKL localization in the microbody matrix; D, Merged image of B and C and E, merged image of A, B and C.

absence of the side chain precursor. In case of Pc13g11640, a 2-fold increase in the transcript levels was observed after 5 days of growth in the presence of PAA (Figure 3A).

Since microbody ABC transporters are likely involved in β-oxidation of fatty acids, the expression levels of Pc13g11640 and Pc16g09390 were also determined for cells grown in the presence of oleic acid. For this purpose, *P. chrysogenum* DS54465 was grown for 40 hrs on YGG medium, whereupon oleic acid was added. Samples were collected at various time intervals (with 1 hr) following oleic acid additions, and analyzed by qPCR. Oleic acid did not affect the expression level of Pc13g11640 and Pc16g09390 (Figure 3B). These data suggest that the two ABC transporters are constitutively expressed in *P. chrysogenum*.

3.3. Expression of Pc13g11640 and Pc16g09390 in Saccharomyces cerevisiae

To further investigate the function of Pc13g11640 and Pc16g09390, the genes were cloned and expressed in the ΔPxa1 and ΔPxa2 strains of *S. cerevisiae* (Baker Brachmann et al., 1998). Yeast strains were grown on minimal medium supplemented with oleic acid as a sole carbon source. The growth defect of the *S. cerevisiae* ΔPxa2 strain on oleic acid was complemented by Pc16g09390 but not by Pc13g11640 (Figure 4). Conversantly, growth of the ΔPxa1 strain was complemented by Pc13g11640 (Figure 4)

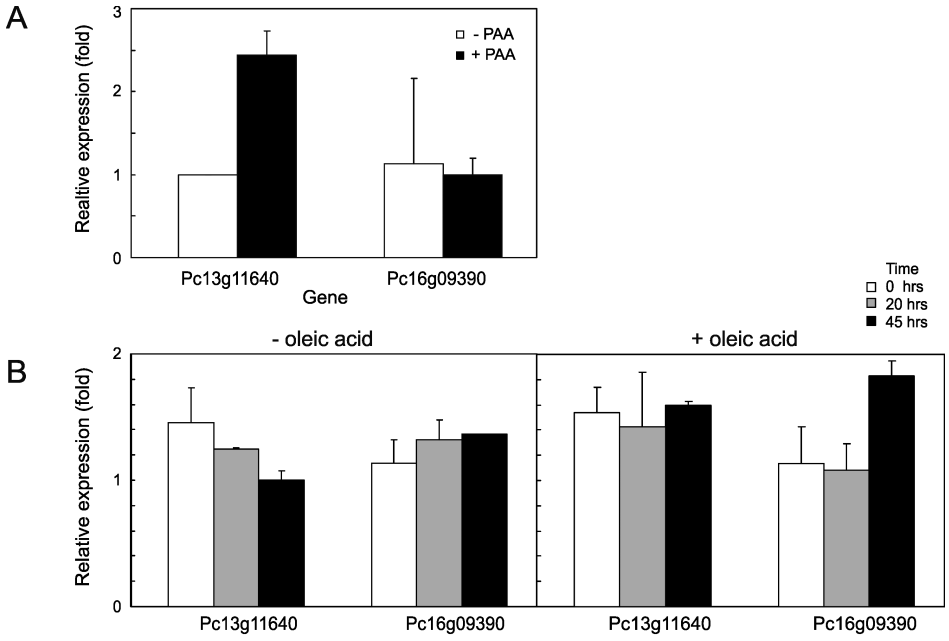

Figure 3. Effect of the side chain precursor phenylacetic acid (A) and oleic acid (B,C) on the expression of Pc13g11640 and Pc16g09390 in cells grown on YGG.

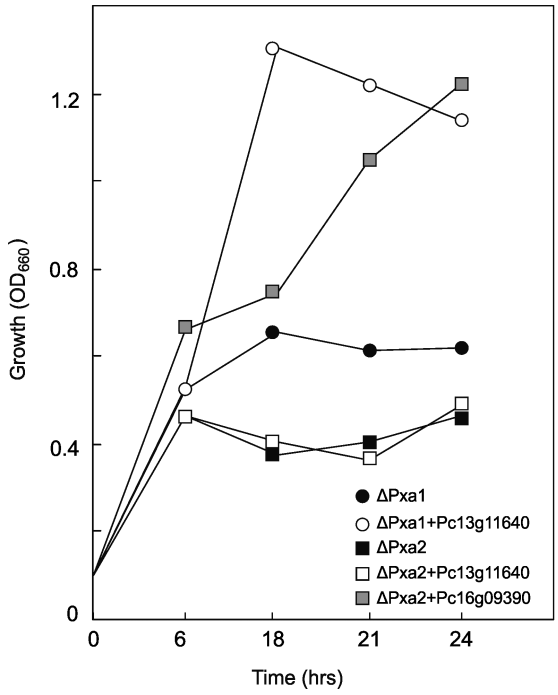

Figure 4. Growth of the*Saccharomyces cerevisiaeΔPxa1* andΔPxa2strains on oleic acidwiththe introduced Pc13g11640 and Pc16g09390 genes.

but not by Pc16g09390 (data not shown). These results suggest that Pc13g11640 and Pc16g09390 are functional homologs of Pxa1 and Pxa2 of *S. cerevisiae*, respectively, and likely involved in the β-oxidation of long chain fatty acids.

3.4. Growth on fatty acids and β-lactam production by microbody ABC transporter gene deletion strains

To examine the function of Pc13g11640 and Pc16g09390 in *P. chrysogenum*, genes were inactivated either individual or together in the high penicillin yielding strain DS54465. Constructed vectors carried the 5′ and 3′ flanking regions of these genes and the appropriate marker (Table 1), and these were transformed to *P. chrysogenum* DS54465. Mutants were selected by growth on plates with acetamide as a sole nitrogen source (ΔPc13g11640) or on phleomycin regeneration medium (ΔPc16g09390 and the double mutant Δ*Pc13g11640/Δ16g09390*), respectively. All transformants were confirmed by PCR and Southern blot analysis (Figure 5), and the correct mutants were used in the further studies.

The *P. chrysogenum* DS54465 strain contains eight copies of the penicillin biosynthetic gene cluster, but this region of the chromosome is highly unstable giving

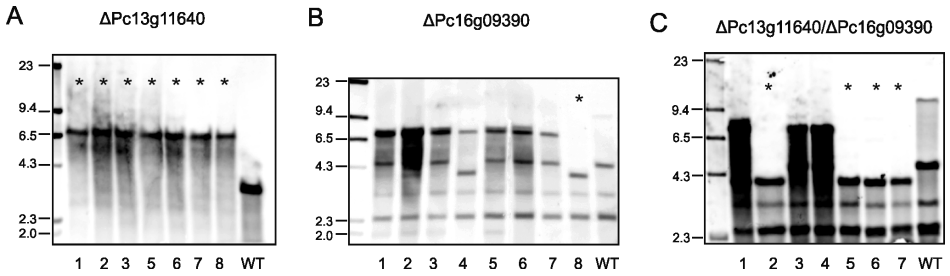

Figure 5. Southern analysis of the ΔPc13g11640 and ΔPc16g09390 strains. (A)Genomic DNA of *P. chrysogenum* DS54465 Δ13g11640strain was digested with HindIIIand probed with a 3′ flanking region of 13g11640 gene. The band of 6257bp corresponds to the*Δ13g11640::amdS* mutant,whereas the 3414 bp band corresponds the same region in the *P. chrysogenum*DS54465 strain (WT). (B) Genomic DNA of *P. chrysogenum* DS54465 *Δ16g09390* was digested with BamHIand probed with a 3′ flanking region of 13g09390 gene. The band of 3320 bp corresponds to the*Δ16g09390::phleo* mutant,whereas the 4487 bp band corresponds to the same region in the *P. chrysogenum*DS54465 strain (WT). (C)Genomic DNA of *P. chrysogenum* DS54465 *Δ13g11640/Δ16g09390* was digested with BamHIand probed with a 3′ flanking region of the 16g09390 gene. The band of 3320 bp corresponds to the*Δ16g09390::phleo* mutant,whereas the 4487 bp band corresponds to the same region in the *P. chrysogenum*DS54465 strain. Correct deletion mutants are indicated by an asterisk.

rise to the spontaneous loss of clusters. Therefore, we used qPCR to determine the number of copies of the IPNS gene in the deletion mutants. Herein, cells were grown for 40 hrs on YGG, mycelium was harvested and the DNA was isolated. The single copy strain DS47274 was included as a reference. In all cases, the transformation protocol and the construction of the gene deletions did not result in a loss of penicillin biosynthesis clusters (Figure 6).

The single and double gene inactivation strains were grown on penicillin production medium in the presence of POA, and analyzed for penicillin V production. The production was compared with that of the parental DS54465 strain. None of the mutants showed a significant growth defect or reduction in the production of penicillin V, although with the ΔPc16g09390 strain production was somewhat enhanced (Figure 7). Also in the presence of oleic acid no significant difference in growth was observed, whereas penicillin V production was somewhat reduced in the deletion strains but not abolished (Figure 7). Finally, the growth experiment was performed with adipic acid in the medium. Typically, adipic acid is metabolized by β-oxidation but it can also be used as side chain in the β-lactam biosynthesis to yield adipoyl-6-APA (ad-6-APA). Again no significant difference in ad-6-APA production was observed, when the single and double mutants were compared with the parental strain (data not shown). These data suggest that Pc13g11640 and Pc16g09390 do not fulfill a direct role in the β-lactam biosynthesis pathway, thus indicating that CoA must enter the microbody by a different mechanism that does not depend on these long chain fatty acid transporters or that levels of CoA in the microbodies is sufficient for both metabolic pathways, as β-oxidation and β-lactam biosynthesis.

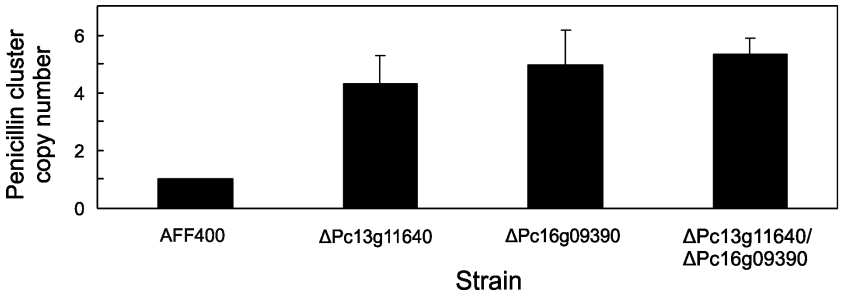

Figure 6. qPCR analysis of the penicillin cluster copy number in *P. chrysogenum*: AFF400 (contains a single penicillin cluster), DS54465 Δ13g11640, DS54465 Δ16g09390 and DS54465 Δ13g11640/ Δ16g09390. For cluster analysis the copy number of the*pcbC*gene was analyzed.

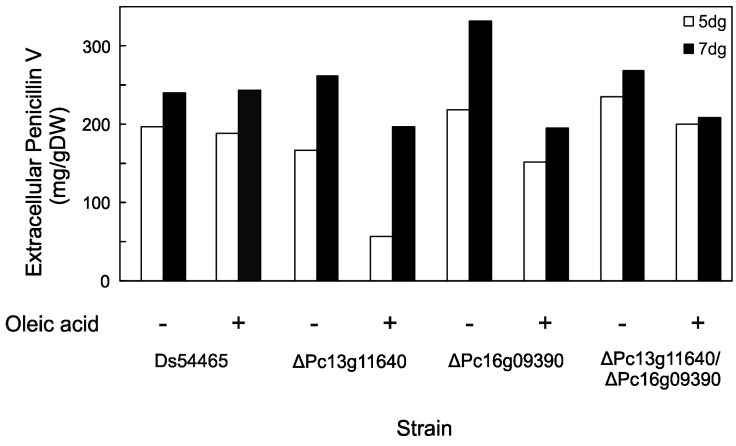

Figure 7. Extracellular Penicillin V production levels measured after 5 and 7 days of cultivation on penicillin production medium for *P. chrysogenum*DS54465 strain, and the Pc13g11640 and Pc16g09390 and Pc13g11640/Pc16g09390 deletion strains. Cells were grown on the production medium without (A) or with (B) 0.12% oleic acid.

4. DISCUSSION

In filamentous fungi, β-oxidation occurs in microbodies where long chain fatty acids are shortened and subsequently directed to the mitochondria. As it have been indicated by (Veiga et al., 2012) the β-oxidation pathway in *P. chrysogenum* is a complex pathway, a numerous set of genes and regulations involved suggests that its physiological role is broader than utilization of linear fatty acids e.g. metabolism of adipic acid. Here we have examined the role of two ABC-D transporters in fatty acid utilization and β-lactam production in *P. chrysogenum*. The two genes Pc13g11640 and Pc16g09390 are homologous to microbody ABC transporters Pxa1 and Pxa2 of *Saccharomyces cerevisiae* that function in long chain fatty acid oxidation (Hettema et al., 1996). The growth defect

of the *S. cerevisiae* Δ*Pxa2* strain on oleic acid was successfully complemented by the Pc16g09390 gene, whereas the growth of ΔPxa1 was restored by Pc13g11640. Since Pxa1 and Pxa2 form a heterodimer in *S. cerevisiae*, the complementation data suggest that the same will hold for the *P. chrysogenum* homologs.

Our results suggest that the *P. chrysogenum* Pxa1 and Pxa2 homologs are actively involved in the β-oxidation of long chain fatty acids. However, inactivation of the Pc13g11640 and Pc16g09390 genes in *P. chrysogenum* had little effect on growth on oleic acid and other fatty acids. In mammalian cells 4 orthologous proteins exist which belong to the ABC-D family (microbody ABC transporters) namely: ALDP, ALDRP, PMP70 and PMP69. The first three are involved in utilization of very long chain fatty acids (VLCFA), while the function of PMP69 is not known. ALDP, ALDRP and PMP70 are able to form homo- or hetero-dimers (Liu et al., 1999). Mutations in ALDP lead to the dysfunction of microbodies and to the disruption of the β-oxidation pathway resulting in the cellular accumulation of VLCFA. Likewise, inactivation of the Pxa1 and Pxa2 genes of *S. cerevisiae* results in the defect of the long chain fatty acids oxidation. However, in the fungus *Podospora anserina* such gene deletions did not affect the vegetative growth on fatty acids. Even when the double ABC transporter mutant was combined with the deletion of the *fox2* gene that encodes the multifunctional β-oxidation microbody protein (Hiltunen et al., 1992), growth on oleic acid was not inhibited (Boisnard et al., 2009) while only minor changes in the germination of ascospores were observed. It has been suggested that in addition to these ABC transporters there might be another, unknown mechanism to import notnot-activated long chain fatty acids (Zolman et al., 2001) into the microbodies where they follow usual oxidation pathway (Boisnard et al., 2009). Alternatively, oxidation of long chain fatty acids by the filamentous fungus occurs elsewhere in the cell, i.e., in the mitochondria (Maggio-Hall and Keller, 2004).

The lack of a clear phenotype of the single as well as double deletion of the ABC-D half-transporters Pc13g11640 and 16g0939 in *P. chrysogenum* suggests that these transporters specify a redundant function. Importantly, it shows that they do not fulfill a critical role in the transport of CoA into the microbody. CoA is a cofactor required for β-lactam production, and used to activate the side chain precursors, a process that occurs in the lumen of the microbodies. CoA is also required for activation of medium chain fatty acids, prior to utilization. The general view is that CoA enters the microbodies as a thioester of activated LCFA (long chain fatty acids) (Figure 8). Our data suggests that there must be an alternative route for CoA to enter the microbody matrix. Taking into account the stoichiometry of the reactions occurring in the lumen of the microbodies, CoA must be provided by other means in order to maintain the pool needed for optimal β-oxidation and β-lactam production (Rokka et al., 2009). Future studies should be directed to reveal the mechanism of CoA import into microbodies.

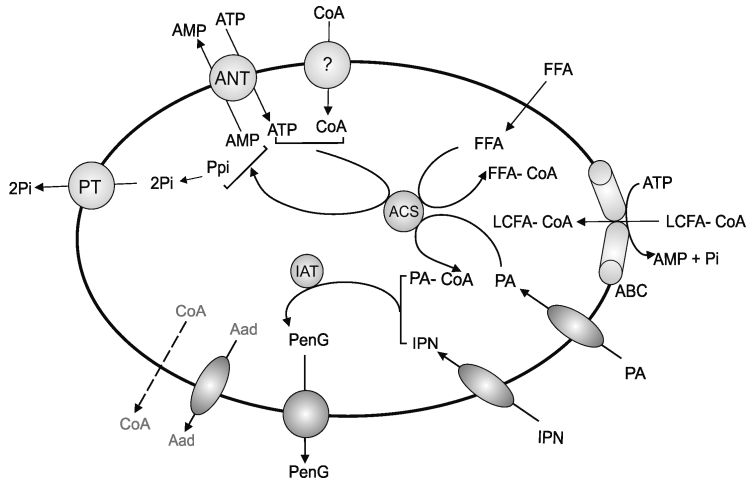

Figure 8. Putative scheme of the pathways involved in fatty acid β-oxidation and β-lactam production in microbodies of *P. chrysogenum* including adenine nucleotide and long chain fatty acid transporters. Abbreviations: PenG- penicillin G, Aad- α- aminoadipic acid, PA- phenyl acetic acid, IPN- isopenicillin N, ACS- acetyl CoA- synthetase, IAT- acyl- CoA: IPN acyltransferase, ANT- adenine nucleotide transporter, ATP- adenosine- 5 '- triphosphate, AMP- adenosine monophosphate. CoA- coenzyme A, PPi- pyrophosphate, PT- putative phosphate transporter, FFA-free fatty acid, LCFA-long chain fatty acid, ABC- ABC transporter which can consist of a Pc13g11640 and Pc16g09390, homo- or hetero-dimer.

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chapter 5

A NONLINEAR BIOSYNTHETIC GENE CLUSTER DOSE EFFECT ON PENICILLIN PRODUCTION BY *PENICILLIUM CHRYSOGENUM*

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ABSTRACT

Industrial penicillin production levels by the filamentous fungus *Penicillium chrysogenum* increased dramatically by classical strain improvement. High yielding strains contain multiple copies of the penicillin biosynthetic gene cluster that encodes the three key enzymes of β-lactam biosynthetic pathway. We have analyzed the gene cluster dose effect on penicillin production using the high yielding *P. chrysogenum* strain DS17690 that was cured from its native clusters. The amount of penicillin V produced increased with the penicillin biosynthetic gene cluster number but saturated at high copy number. Likewise, transcript levels of the biosynthetic genes *pcbAB* [γ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase], *pcbC* (isopenicillin N synthase) and *penDE* (acyltransferase) correlated with the cluster copy number. Remarkably, the protein level of acyltransferase that localizes to peroxisomes saturated already at low cluster numbers. At higher copy numbers, intracellular levels of isopenicillin N increased suggesting that the acyltransferase reaction presents a limiting step at high gene dose. Since the number and appearance of the peroxisomes did not change significantly with the gene cluster copy number, we conclude that the acyltransferase activity is limiting for penicillin biosynthesis at high biosynthetic gene cluster numbers. These results suggest that at high penicillin production level, productivity is limited by the peroxisomal acyltransferase import activity and/or the availability of CoA-activated side chains.

1. INTRODUCTION

During the last 70 years, the increase in penicillin production by industrial strains of the filamentous fungus *Penicillium chrysogenum* has been based mainly on classical strain improvement (Elander, 2003; Penalva et al., 1998). The pathway of the biosynthesis of penicillin and the cellular localization of most of the critical enzymatic steps are known (Muller et al., 1991). Penicillin biosynthesis starts with the formation of the tripeptide γ-(L-α-aminoadipyl)-L-cysteinyl-D-valine by the nonribosomal peptide synthetase ACVS (encoded by *pcbAB*, Pc21g21390), whereupon the β-lactam ring is formed by isopenicillin N synthase (IPNS; encoded by *pcbC,* Pc21g21380) resulting in the formation of isopenicillin N (IPN). These two enzymatic steps occur in the cytosol. Next, the isopencillin N enters the peroxisome, an alkaline compartment that contains acyltransferase (AT; encoded by *penDE,* Pc21g21370), an enzyme that catalyzes side chain exchange using a CoA activated organic acid (or fatty acids) as a substrate. The genes encoding these three key enzymes of the biosynthetic pathway are organized in a gene cluster, also termed the penicillin biosynthetic gene cluster (Figure 1A). Side chain activation is catalyzed by a group of enzymes termed CoA-ligases. One of the enzymes involved in the activation of phenylacetic acid and phenoxyacetic acid in the biosynthesis of penicillin G and V, is phenoxyacetic acid CoA-ligase (PCL) that is encoded by the *phl* gene (Pc22g14900) (Koetsier et al., 2009; Penalva et al., 1998). The *phl* gene is located outside the biosynthetic gene cluster elsewhere in the genome. Together with acyltransferase, PCL is peroxisome-localized (Meijer et al.).

A remarkable phenomena that occurs during classical strain improvement is the amplification of the penicillin biosynthetic gene cluster between tandem repeats ("amplicons") (Fierro et al., 1995; Newbert et al., 1997; Smith et al., 1989) and this has been associated with increased levels of β-lactam production. The introduction of additional copies of the penicillin cluster genes into a single copy strain like Wisconsin54-1255 also leads to increased penicillin titers (Theilgaard et al., 2001). In addition, in a lineage of strains with increasing levels of penicillin production also the number of peroxisomes increases (Meijer et al.; Muller et al., 1992) although this does not seem to be a linear relationship. A recent transcriptomic analysis indicates that the genes encoding the main biosynthetic pathways yielding the three amino acid building blocks are upregulated in high yielding strains relative to earlier production strains (van den Berg et al., 2008).

Recently, the complete genome sequence of *P. chrysogenum* has been elucidated (van den Berg et al., 2008). Also, a genetic toolbox for *P. chrysogenum* has been extended by the inactivation of the *ku70* gene (Snoek et al., 2009). Ku70 is involved in the non-homologous end-joining (NHEJ) pathway that causes random integration, and its inactivation results in an increased gene targeting efficiency by homologous recombination. Therefore, now the phenotype of gene deletions can be analyzed in industrial relevant strains. A disadvantage we noted in the use of these so-called "high

copy-number strains" is the genetic instability of the penicillin biosynthetic gene cluster containing amplicons. During the transformation protocols, copies of the penicillin gene cluster amplicons are readily lost. This phenomenon has been exploited to generate a series of strains derived from a high copy high penicillin yielding strain that differ in the number of penicillin biosynthetic gene clusters containing amplicons (Harris et al., 2009). This now allowed for the first time a quantitative analysis of the dose effect of the penicillin biosynthetic gene cluster in a host that has been optimized for production by classical strain improvement. Thus except for defined penicillin biosynthetic gene cluster amplification, this host bears all necessary modifications and mutations that render it an excellent vehicle for β-lactam production. Here we have analyzed this series of strains at the level of DNA, RNA, and protein, the number of peroxisomes and the main β-lactam biosynthetic pathway intermediates and end products. Our data suggest that the acyltransferase activity becomes limiting at high copy numbers. The implications of these results for high level penicillin production are discussed.

2. MATERIALS AND METHODS

2.1. Fungal stains, media and culture conditions

Penicillium chrysogenum strain DS17690 and its derivatives, and *P. chrysogenum* Wisconsin54-1255, the ancestor of DS17690 were kindly supplied by DSM Anti-infectives. Spores were inoculated in YGG-medium containing (g/l): KCl, 10.0; glucose, 20.0; YeastNitrogenBase (YNB), 6.66; citric acid, 1.5; K_2HPO_4, 6.0; Yeast Extract, 2.0 and grown for 24 hrs in a shaking incubator at 220 rpm and 25°C. On day 0, the mycelium was diluted 7-times in Penicillin V production medium (PPM) containing (g/l): glucose, 5.0; lactose, 75; urea, 4.0; Na_2SO_4, 4.0; CH_3COONH_4, 5.0; K_2HPO_4, 2.12; KH_2PO_4, 5.1 and phenoxy acetic acid, 2.5; supplemented with a trace element solution (pH 6.3 ± 0.1) and grown for 5 days in a shaking incubator at 220 rpm and 25°C.

2.2. Genomic DNA extraction, total RNA extraction and cDNA amplification

Genomic DNA (gDNA) was isolated after 96 hours of inoculation in penicillin producing medium using a modified Yeast genomic DNA isolation protocol (Harju et al., 2004) in which the fungal mycelium is broken in a Fastprep FP120 (Qbiogene). Isolated genomic DNA was measured in a Nanodrop ND-1000 (Thermo Scientific) and set at a concentration of 40 ng per qPCR reaction of 25 µl. Total RNA of transformants was isolated after 5 days of growth in a penicillin producing medium using Trizol® (Invitrogen) with additional DNAse treatment by the Turbo DNA-free kit (Ambion). Total RNA was measured in a Nanodrop ND-1000 and set at a concentration of 500 ng per cDNA reaction. cDNA was synthesized using iScript™ cDNA synthesis kit (Bio Rad) in a 10 µl end volume.

2.3. qPCR analysis

The primers for analysing the expression of the genes in the penicillin biosynthetic gene cluster (*pcbAB, Pc*21g21390; *pcbC,* Pc21g21380, and *penDE,* Pc21g21370) and the phenoxyacetic acid CoA ligase (*phl*, Pc22g14900) are indicated in Table 1. Primers were designed around an intron in the case of γ*-actin* (Pc20g11630), *penDE* and the *phl* gene in order to be able to separate amplification on gDNA and cDNA. For expression analyses γ*-actin* was used as a control for normalisation. A negative reverse transcriptase (RT) control was performed to determine the gDNA contamination in the isolated total RNA. To analyse the number of penicillin clusters in the strains, γ*-actin* and an intergenic target (between Pc20g07090 and Pc20g07100) were used as reference templates. The primers for *pcbAB* and *pcbC* were used to assess the cluster copy number with gDNA. *P. chrysogenum* Wisconsin 54-1255 and strain DS50652 were used as controls containing a single and no penicillin gene clusters, respectively.

The expression levels and gene copy numbers were analysed, in triplo, on a Miniopticon™ system (Bio Rad) using the Bio Rad CFX manager software in which the C(t) values were determined automatically by regression. The SensiMix™ SYBR mix (Bioline) was used as a master mix for qPCR with 0.4 µM primers. The following thermocycler conditions were used: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec and subsequently a melting curve was performed to determine the specificity of the qPCR reaction. The efficiency of the primers used for the copy number determination assessed through the use of four dilutions of gDNA. The reference γ*-actin*, the intergenic region, and the *pcbAB* and *pcbC* genes showed an efficiency of 100.17% (R^2=1.000), 94.34% (R^2=0.997), 96.78% (R^2=0.999) and 102.86% (R^2=0.993), respectively. Likewise, for transcript levels, four dilutions of cDNA were made, and the primer efficiency for the reference gene γ*-actin* and the genes of interest *pcbAB*, *pcbC*, *penDE* and *phl* was 98.19% (R^2=1.000), 96.78% (R^2=0.997), 102.86% (R^2=0.993), 96.38% (R^2=0.999) and 88.57% (R^2=0.981), respectively.

2.4. Biochemical techniques

Cell free extracts of *P. chrysogenum* were isolated as follows: After 5 days of growth in penicillin producing medium, 1 ml of the culture was mixed with 1 ml 25% trichloroacetic acid (TCA) and frozen at – 20 °C. After thawing on ice, cells were pelleted by centrifugation (10.000 x *g* for 10 min) and washed twice with cold (- 20 °C) 80 % acetone. The pellet was air-dried and solubilized in 200 µl solubilization buffer containing 1 % SDS and 0.1 M NaOH and subsequently 50 µl 5x SDS-PAGE loading buffer was added. Samples were boiled for 5 minutes and centrifuged for 10 minutes at 10.000 x *g*, whereupon the supernatant was used for SDS-PAGE analysis. Western blots were stained with polyclonal antibodies raised against the indicated penicillin biosynthetic pathway proteins and transcription elongation factor EF1-α as a reference protein (Kiel et al., 2007).

Table 1. Primers used for qPCR.

Target	Forward Sequence (5'à 3')	Reverse Sequence (5'à 3')
γ-actin cDNA	CTGGCGGTATCCACGTCACC	AGGCCAGAATGGATCCACCG
γ-actin gDNA	TTCTTGGCCTCGAGTCTGGCGG	GTGATCTCCTTCTGCATACGGTCG
IGR Pc20g07090	GTTCCTATAGGACGTAGCTCCGC	AAATCAGCTCTACTAGCGATCCGC
pcbAB	CACTTGACGTTGCGCACCGGTC	CTGGTGGGTGAGAACCTGACAG
penDE	CATCCTCTGTCAAGGCACTCC	CCATCTTTCCTCGATCACGC
pcbC	AGGGTTACCTCGATATCGAGGCG	GTCGCCGTACGAGATTGGCCG
phl	CTGGGTATGGAGACAGCTGCCG	CGTGCCTCGACTCCAGGGAGC

2.5. Transformation of P. chrysogenum with the DS-RED gene

Protoplasts were isolated of *P. chrysogenum* DS17690 and the derivatives DS47274 and DS47276 (Harris et al., 2009) and cells were co-transformed with plasmid pBBK-007 (Meijer et al.) and pBlue-AMDS bearing the acetamidase-gene (*amdS*) gene as selection marker. Plasmid pBBK-007 contains the DS-RED gene fused with the peroxisomal targeting signal SKL under control of the *gpdA* promotor of *Aspergillus nidulans* and the terminator of the *penDE* gene of *P. chrysogenum*. Transformants were selected on plates with acetamide as sole nitrogen source (Snoek et al., 2009). Positive colonies were selected based on the reddish DS-RED color.

2.6. Confocal fluorescence microscopy

Confocal images were made using a Zeiss LSM510 confocal laser scanning microscope (CLSM) (Zeiss Netherlands). DS-RED fluorescence was analyzed by excitation the mycelium with a 543-nm helium-neon ion laser and detection of fluorescence emission using a band pass LP 560nm filter. Positive DS-RED transformants were inoculated in penicillin producing medium and grown for 96 hours before counting the number of peroxisomes.

2.7. Determination of metabolite concentrations

The extracellular titers of phenoxyacetic acid and penicillin V in the culture medium was determined by HPLC using an isocratic flow of acetonitrile, 350 g/l; KH_2PO_4, 640 mg/l; H_3PO_4, 340 mg/l. Peaks were separated on a Platinum™ C18-EPS 5 µm column (Grace) at a flow of 1 ml/min and detected at a wavelength of 254nm (Harris et al., 2006). Intracellular metabolite levels were analysed after 5 days of growth in penicillin producing medium and 5 ml of the broth was washed 3 times with 15 ml of 60% MeOH (-40°C) and frozen in liquid N_2 for storage (Seifar et al., 2008). Freeze dried cell-free-extract (50 mg) was prepared in 1 ml 90 °C MilliQ water to lyse the cells. Samples were firmly shaken for 20 seconds, rapidly cooled in ice-water, and diluted 25 times prior to

analysis. Intracellular metabolites were separated by liquid chromatography on a Waters sunfire column (2.1 x 150mm) at 30 °C at a flow of 0.25 ml/min using a sample injection of 25 µl. Eluates were analysed by MS/MS on a LTQ orbitrap in positive ion mode (*m/z* range of 200-1000) with- and without spiking (100 ng/ml) of the compound of interest.

3. RESULTS

3.1. Variation of the penicillin amplicon number.

In a previous study, the high yielding strain *P. chrysogenum* DS17690 was cured from its penicillin biosynthetic gene cluster containing amplicons by protoplasting and plating without any selection (Harris et al., 2009). This yielded a series of strains (DS47272, DS47273, DS47274, DS47276, DS48082 and DS50652) that varied in their penicillin titre. Finally, a penicillin gene cluster free strain (DS50652) was obtained by a directed deletion of the remaining single cluster in strain DS47274. To determine the penicillin biosynthetic gene cluster copy number in these DS17690 derived strains, gDNA was isolated, and quantitative PCR was performed on the *pcbAB* and *pcbC* genes. Primers amplifying *γ-actin* and an intergenic region on gDNA were used as reference. The sequenced ancestry strain Wisconsin54-1255 (van den Berg et al., 2008) was used as a reference as it is known to contain a single copy of the penicillin gene cluster, while the cluster free strain DS50652 indeed lacked the penicillin gene cluster. The number of penicillin biosynthetic gene cluster in strains DS47272, DS47273, DS47274 and DS47276 was 7, 3, 1 and 4 copies, respectively. The original strain DS17690 contained 8 copies (Figure 1B). Furthermore, the copy number of the *phl* gene that is not part of the penicillin biosynthetic gene cluster amplicon was determined to exclude amplification or reduction events of critical genes in the various strains. All DS17690 derivatives including Wisconsin54-1255 contained only one copy of the *phl* gene in their genome (data not shown). These data demonstrate that in the selected strains, the number of penicillin biosynthetic gene cluster amplicons varies from 0 to 8.

3.2. Expression analysis

The transcript levels were determined of the various penicillin biosynthetic genes (*pcbAB, pcbC,* and *penDE*) and *phl*. Herein, the set of strains that vary in the penicillin gene cluster copy number were grown for 5 days in penicillin producing medium and total RNA was isolated. In the qPCR experiments, *γ-actin* was used as a reference gene. For comparison, the expression of the analysed genes in the strain with a single copy of the penicillin cluster (DS47274) was set at 12.5%, as it reflects 1/8th of the number of clusters present in the DS17690 strain. As expected, the expression levels of the three structural penicillin biosynthetic genes increased nearly linearly with the cluster number, but started to saturate at a very high amplicon cluster numbers (Figure 2). In contrast, the expression of *phl* decreased with the copy number of the penicillin cluster.

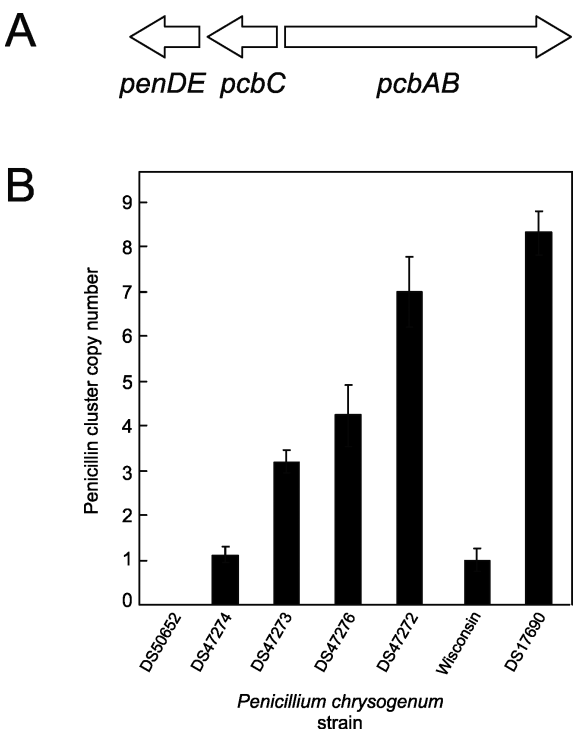

Figure 1. A. Scheme of the penicillin biosynthetic gene cluster. B. qPCR analysis on the penicillin cluster copy number in *P. chrysogenum* DS17690 strain and derivatives. DS50652 is a cluster-free control strain and Wisconsin54-1255, the ancestor of DS17690, contains a single penicillin cluster.

3.3. Protein levels

To correlate the transcriptome with the proteome, the relative protein levels of the three penicillin biosynthetic genes and PCL was analysed by immunoblotting using specific polyclonal antibodies directed against these proteins. Protein levels were determined in mycelia obtained after growth for 5 days in penicillin producing medium but essential similar results were obtained after 7 days of fermentation. Cells were harvested and lysed whereupon proteins were isolated by rapid trichloracetic precipitation. Immunoblots of the cell lysates were developed against the elongation factor EF1-α for normalisation purposes. For comparison, again the protein levels in the single copy strain DS47274 was set at 12.5% (see also above). Protein levels of ACVS (encoded by the *pcbAB* gene) and IPNS (encoded by the *pcbC* gene) increased with gene copy number and saturated at the higher gene cluster numbers (Figure 3). Thus, the transcript and protein levels of these enzymes show a good correlation. Remarkably, the level of AT seemed to saturate already at a copy number above one, and only a small increase was observed at higher gene clusters numbers (Figure 3). This shows that the protein and transcript levels of AT do not correlate. Finally, the

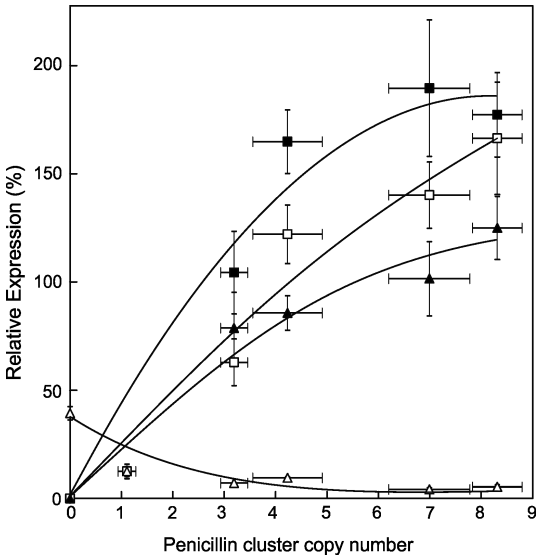

Figure. 2. Relative expression levels of the key genes of penicillin biosynthesis as a function of the genomic copy number of the penicillin cluster. Expression of *pcbAB* (closed squares), *pcbC* (closed triangles), *penDE* (open squares) and *phl* (open triangles). Transcript levels were normalized with *γ-actin* and the expression levels in the single copy strain DS47274 was set at 12.5% to relate to the 8-fold increased copy number in the *P. chrysogenum* DS17690 strain. Because of the experimental error in the determination of the expression level in the single copy strain, the maximum expression levels may scale beyond 100%.

protein levels of PCL decrease in the strains with increasing penicillin biosynthetic cluster copy number, although less dramatically as the transcript levels (Figure 2). These data indicate that the AT levels do not increase proportionally with the transcript level and genomic copy number.

3.4. Quantitation of peroxisomes

Since AT localizes to the peroxisomes, and because penicillin production levels increase with the number of cellular peroxisomes (Meijer et al.), we also determined if the number of these organelles varied in the set of strains. Herein, we selected three different strains, i.e., *P. chrysogenum* DS17690, DS47276 and DS47274 containing 8, 4 and 1 penicillin gene clusters, respectively. The strain were co-transformed with plasmid pBlue-AMDS and pBBK-007 containing the AmdS and DS-RED encoding genes as markers, respectively. The DS-RED protein was fused at its C-terminus to the SKL amino sequence, which targets the protein to the peroxisomes thereby allowing the detection of the organelles by fluorescence microscopy. For the penicillin gene cluster copy number determination, the gDNA was isolated from two transformants each that were selected on the basis of the DS-RED staining of the colony. The

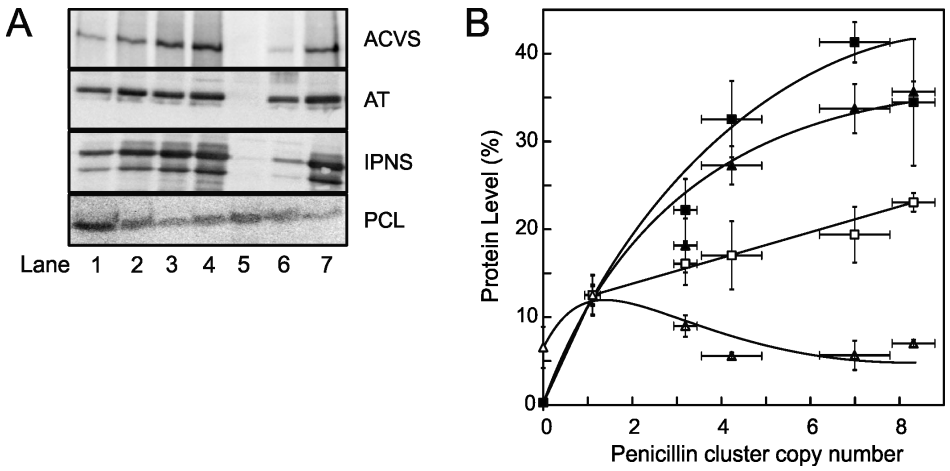

Figure. 3. Protein levels of the key enzymes of penicillin biosynthesis as a function of the genomic copy number of the penicillin gene cluster. A. Relative levels of the enzymes ACVS, AT, IPNS and PCL determined by western blotting and immunostaining using specific polyclonal antibodies. Samples to the western blots were normalized with antibodies directed at elongation factor EF1-α. Strains analysed are: lane 1, DS47274 (1 copy); lane 2, DS47273 (3 copies); lane 3, DS47276 (4 copies); lane 4, DS47272 (7 copies); lane 5, DS50652 (cluster free); lane 6, Wisconsin54-1255; and lane 7, DS17690 (8 copies). B. Quantitation of the protein levels as a function of the biosynthetic gene cluster number. Protein levels of ACVS (closed squares), IPNS (closed triangles), AT (open squares) and PCL (open triangles). The protein levels in the single copy strain DS47274 was set at 12.5% to relate to the 8-fold increased copy number in the *P. chrysogenum* DS17690 strain.

DS-RED strains were selected that showed the same copy number as before the transformation. The strains were grown for 4 days in penicillin producing medium and analysed by confocal laser-scanning microscopy (Figure 4B). The CLSM analysis indeed showed the DS-RED staining of the peroxisomes, while counting revealed that the strains do not differ significantly in the number of peroxisomes (Figure 4D). These data demonstrate that the number of peroxisomes does not change in the set of *P. chrysogenum* strains that vary in penicillin biosynthetic gene cluster number.

3.5. Extra- and intracellular penicillin pathway metabolites and penicillin V production

To relate the biosynthetic gene cluster copy number to β-lactam production, the extracellular level of penicillin V was determined after 5 and 7 days of growth. The production levels were corrected for growth differences by dry weight determination. The penicillin V production increased with the penicillin gene cluster copy number but saturated at higher copy number (Figure 5A) much akin the trend of the protein and transcript levels. In addition, intracellular levels of the key intermediates of biosynthesis were determined by LC-MS/MS. Remarkably, isopenicillin N accumulated

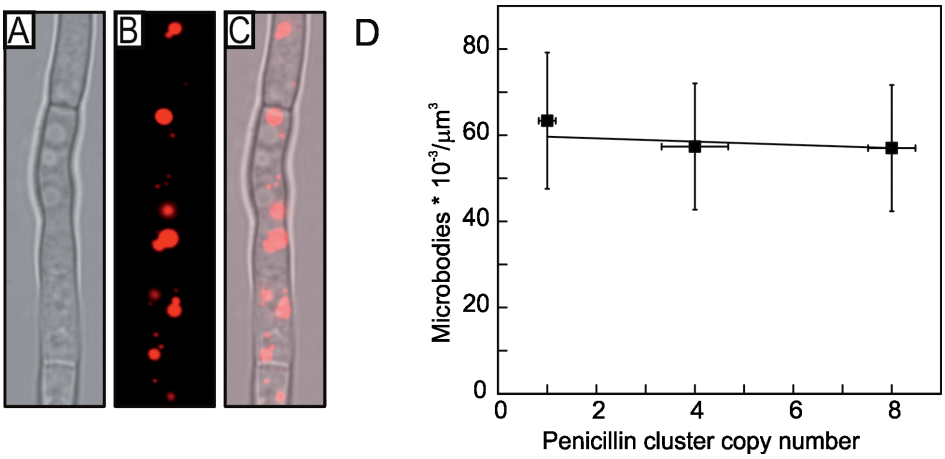

Figure. 4. Quantitation of the peroxisome numbers in *P. chrysogenum* strains that vary in the number of penicillin biosynthetic gene clusters. Visualization of hyphen and peroxisomes through DS-RED fluorescence as recorded by CLSM of strain DS47274, containing a single penicillin gene cluster: A. Bright field image, B. DS-RED fluorescence, and C. merged image. D. The number of peroxisomes per 1000 µm^3 was counted in DS47274, DS47276 and DS17690 strains and plotted against the penicillin cluster copy numbers. The hyphen of the different cells had identical diameters and dimensions.

significantly at higher gene cluster copy number (Figure 5B), i.e., from 0 to up to 5 mg IPN/gDW. There was barely any accumulation of the tripeptide ACV (up to 0.26 mg/gDW), while only smaller amounts of penicillin V stayed within the cells of the higher copy number strains (up to 1.17 mg/gDW). Importantly, the analysis revealed no significant levels of the β-lactam nucleus 6-aminopenicillanic acid (6-APA) (data not shown) or any other β-lactam derived byproducts, neither intra- nor extracellularly. With the DS17690 strain the intracellular and extracellular penicillin V:IPN ratio was 0.23 and 44, respectively. Thus the accumulation of IPN within cells indicates that the conversion of IPN into penicillin V is a rate determining step at high numbers of gene cluster copies. This enzymatic conversion involves AT and PCL.

3.6. Comparison of the single biosynthetic gene copy number strains Wisconsin54-1255 and DS47274

The industrial strain DS17690 was derived from the ancestor Wisconsin54-1255 strain via an extensive classical strain improvement programme that lasted at least 30 years. Wisconsin54-1255 contains only a single penicillin biosynthetic gene cluster, which is the same number as in the DS47274 strain derived directly from strain DS17690 through amplicon curing (see Figure 6A). However, strain DS47274 produces two-times as much penicillin V than Wisconsin54-1255 in the shaken flask fermentations after 5 days (Figure 6D). Remarkably, also the expression of all analysed penicillin biosynthetic pathway

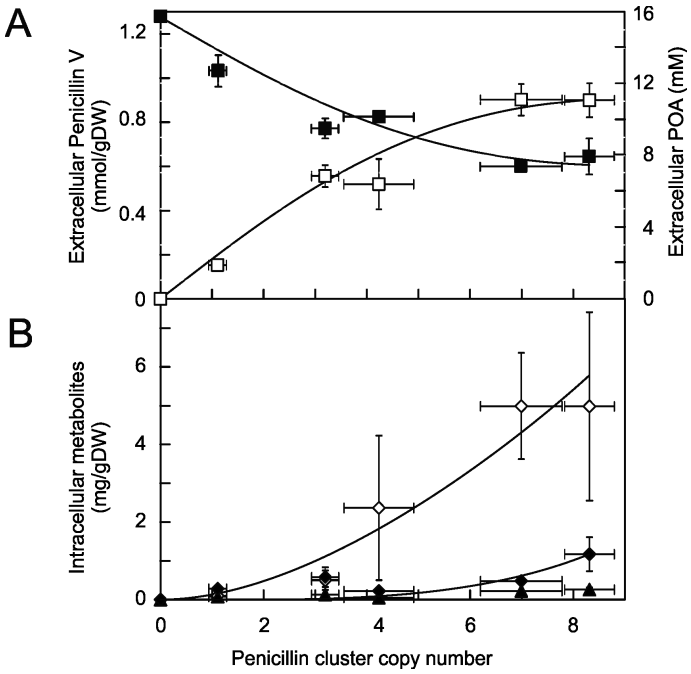

Figure. 5. Production of penicillin V and the intracellular accumulation of β-lactam intermediates in *P. chrysogenum* strains that vary in the number of biosynthetic gene clusters after 5 days of fermentation. A. The extracellular penicillin V production (open squares) and phenylacetic acid levels (closed squares). B. Intracellular levels of ACV (closed triangles), IPN (open diamonds) and penicillin V (closed diamonds).

genes *pcbAB*, *pcbC*, *penDE* and *phl* is increased by more than two-fold in the DS47274 strain as compared to Wisconsin54-1255 (Figure 6B). Likewise, the protein levels of ACVS, IPNS and PCL increased in the DS47274 strain (Figure 6C). However, both strains showed the same levels of the AT enzyme, despite the 2-fold difference in transcript levels. Overall these data suggest that DS47274 is more efficient than Wisconsin54-1255 due to regulatory mutations that cause the up-regulation of the key enzymes in penicillin biosynthesis and likely other mutations that affect the overall performance.

4. DISCUSSION

Industrial β-lactam production processes depend on strains of *Penicillium chrysogenum* that have been subjected to an intense classical strain improvement (CSI) programme involving random mutagenesis, screening and selection. Strain DS17690 used in this study was developed from the Wisconsin54-1255 lineage over a period of many years. One of the most remarkable results of CSI is the amplification of the penicillin biosynthetic gene cluster between tandem repeats. The conserved amplicon flanking

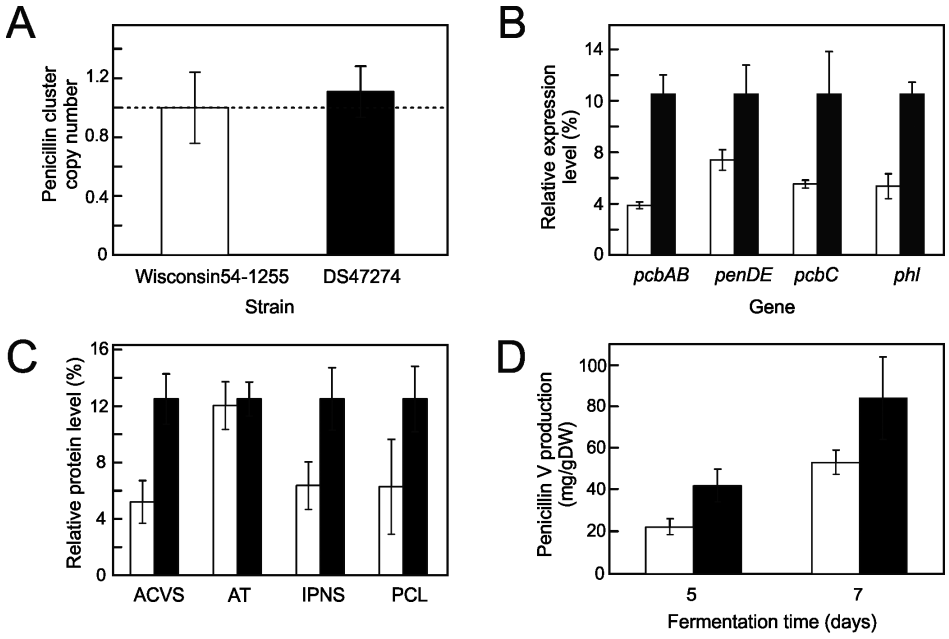

Figure. 6. Comparison of two single penicillin biosynthetic gene cluster strains of *P. chrysogenum* Wisconsin54-1255 (white bars) and DS47274 (black bars). A. Quantification of the penicillin cluster copy number. B. Relative expression levels of *pcbAB*, *penDE*, *pcbC* and *phl*. C. Relative protein levels of ACVS, AT, IPNS and PCL. D. Extracellular levels of penicillin after 5 and 7 days. Samples were taken after 5 days of fermentation unless indicated otherwise. The transcript and protein levels of DS47274 were set at 12.5 % as in Figures 2 and 3.

hexanucleotide might be hot spots for site-specific recombination that results in the amplification of the penicillin cluster (Fierro et al., 1995). However, the region might also be responsible for the genetic instability of the strains with amplified biosynthetic gene clusters. We noted that after protoplasting, gene clusters are readily lost while only the last remaining cluster required a directed deletion approach for removal (Harris et al., 2009). At the same time, this offered us the possibility to 'reverse' study the effect of penicillin gene cluster amplification by obtaining a series of isogenic strains that differ only in the number of biosynthetic gene clusters.

Previously, penicillin production was compared among different strains derived from CSI (Muller et al., 1991; Penalva et al., 1998). However, those reports differs fundamentally from this study as they did not take into account the many other alterations that occurred during the CSI, other than amplicon amplification. In a recent analysis of the genome sequence of *P. chrysogenum* Wisconsin54-1255 and a transcriptomic comparison with strain DS17690 (van den Berg et al., 2008), the presence of multiple mutations and alterations introduced by the CSI programme was readily demonstrated. For instance, CSI resulted in the up-regulation of genes involved in the biosynthesis of

α-aminoadipate, cysteine and valine, and various transporters in addition to the down-regulation of many secondary metabolite biosynthesis gene clusters (van den Berg et al., 2008). Also, the number of peroxisomes varies among strains in a lineage and this may have a direct impact on the productivity (Kiel et al., 2005). Therefore, only through the use of isogenic strains, an exclusive insight in the gene copy dose effect of the penicillin gene cluster on β-lactam production can be obtained.

Quantitation of the genomic copy number of the biosynthesis gene clusters showed that the high-yielding strain DS17690 contains 8 copies, a number that was almost stepwise reduced to 0 in a series of derived strains. Transcription of the biosynthetic genes in these strains appeared very efficient and increased almost linearly with the cluster copy number only saturating at the (very) high copy numbers. Based on our qPCR analysis referenced against γ-actin, it appeared that *pcbAB* is expressed at lower levels than the other two cluster genes *pcbC* and *penDE*. This is in contrast to recent micro-array experiments that showed that *penDE* is expressed at a 2.5 times lower level than *pcbAB* and *pcbC* (van den Berg et al., 2008). The apparent discrepancy is mostly likely caused by differences in growth conditions and/or penicillin production medium. Although the protein levels of ACVS and IPNS correlated well with the transcript levels, this was not the case for AT. While a substantial level of AT protein is produced by the single cluster copy strain, only a marginal 2-fold increase occurred when in the strain with an 8-fold higher gene copy number. Importantly, this phenomenon was accompanied with a substantial increase in the intracellular level of isopenicillin N suggesting that at high penicillin gene cluster numbers the AT activity becomes limiting for production. Why do the AT protein levels not correlate with the *penDE* transcript levels? Possibly, there are limitations at the level of *penDE* translation, import into the peroxisome, maturation of the AT and/or the stability of the protein that may cause this large discrepancy in the protein level. AT contains the PTS1 peroxisomal targeting sequence ARL which is less optimal than the consensus sequence SKL (Kiel et al., 2009). A replacement of the ARL targeting sequence into SKL might boost the production of penicillin.

The various isogenic DS17690 strains did not differ in the number of peroxisomes, but the numbers were almost 3-fold higher as compared to the NRRL1951 strain (Meijer et al.). This has been attributed to the upregulation of *pex11*, a peroxisome proliferation gene (Kiel et al., 2005). Possibly, the AT protein level produced by the single copy strain already challenges the import capacity of the peroxisomes. Another factor that might potentially be limiting is the activity of the phenoxyacetic acid CoA ligase (PCL), one of the side chain activating enzymes that also localizes to the peroxisome (Koetsier et al., 2009). This enzyme is encoded by the single copy *phl* gene that localizes outside of the penicillin biosynthetic gene cluster. In shaken culture, the *phl* transcript levels decreased with increasing cluster copy number and a similar but weaker effect was observed for the PCL protein levels. Although *phl* expression is induced by phenoxyacetic acid and the alternative side chain, phenylacetic acid, these

organic acids seems only weak inducers as substantial amounts of phenoxyacetic acid remained in the medium even at high gene copy number (Figure 5A) (van den Berg et al., 2008). It however seems less likely that the activity of PCL and related enzymes is limiting at high penicillin gene copy numbers as we did not notice any intra- or extracellular accumulation of 6-APA or of 8-hydroxy penicillanic acid that is produced from 6-APA upon reacting with CO_2.

Summarizing, we conclude that in a strain optimized by classical strain improvement, the production of penicillin saturates at high copy numbers of the biosynthetic genes and that the major limitation resides in the production of sufficient AT protein. Therefore, further "overexpression" by increasing the penicillin cluster copy number will likely not result in improved levels of penicillin and this necessitates more unconventional approaches in future metabolic engineering programmes, for instance, the improvement of AT targeting to the peroxisomes, the stabilisation and maturation of the enzyme or possibly an increased number of peroxisomes. Reconstruction of the β-lactam biosynthetic pathway through synthetic biology employing refactoring tools aimed at the optimisation of the expression and targeting of the biosynthetic enzymes may result in genetically more stable strains and a better balance between the various enzymes involved in β-lactam biosynthesis with a potential for enhanced levels of production.

ACKNOWLEDGEMENTS

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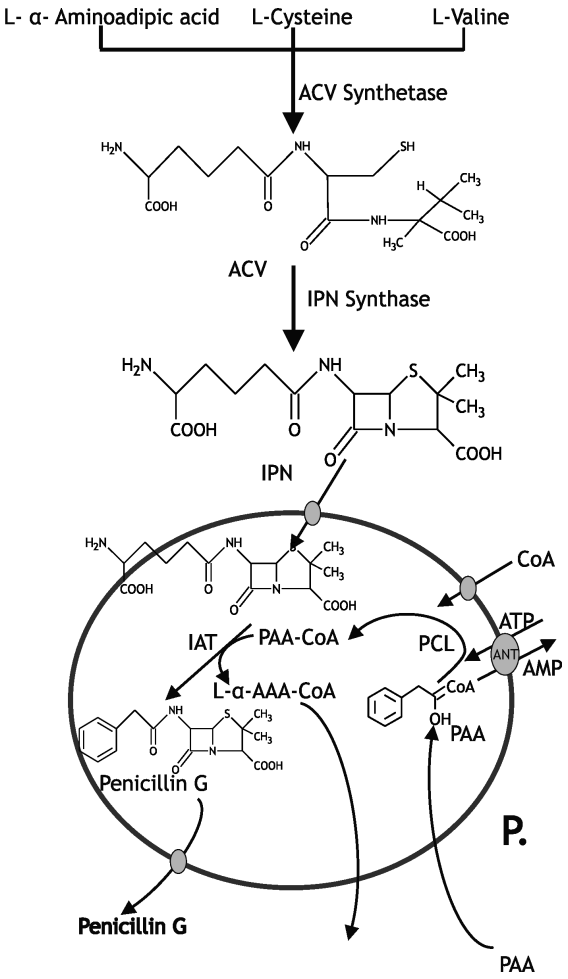
chapter 6

SUMMARY AND OUTLOOK

The discovery of Penicillin took place in 1928 by the British scientist Sir Alexander Fleming. *Staphylococcus spp.* plates were accidently infected with mould spores through an open laboratory window and on these plates a halo was observed around a green mould that inhibited bacterial growth. This was an indication that the mould released a substance, which actively inhibited bacterial growth. Later on it was shown that this substance, penicillin, is effective against gram-positive bacteria, while being safe for animals and humans. This accidental discovery, followed by thorough studies, has had a substantial impact on the quality of human lives. *Penicillium* is able to produce ß-lactam antibiotics, i.e. antimicrobial substances that possess a ß-lactam ring, which is the active entity of the molecule. However, a range of molecules can be made with different side chains such as the penicillins G, L, K and V that differ in antibiotics spectrum, stability and potency (Evers et al., 2004). In the last decade the β–lactam antibiotics cover 65% of the antibiotic market consisting mainly of penicillins and cephalosporins. Currently, these molecules are made at an industrial scale through fermentation and/or semi-synthesis where the fermentation product is further modified through chemical synthesis or enzyme-catalysis. Years of process optimization led to an increased productivity, where costs are substantially reduced, with 90% of total active substance recovery (Elander, 2003). After half a century of work performed jointly by academia and industry, the enzymatic basis of the penicillin biosynthesis pathway is now very well characterized (Figure 1).

Chapter 1 summarizes the current knowledge with regard to the β-lactam production process in *Penicillium chrysogenum* with a focus on the compartmentalization of the cellular pathway, and the transport processes between various (sub)cellular compartments. In this pathway, microbodies fulfill an important role as they harbour the last two enzymes of the pathway. Consequently, also their biogenesis is discussed. The biosynthesis of β-lactams occurs in the subapical part of the hyphae in two different compartments of the cell, namely the cytosol and microbodies. Biosynthesis of β-lactam antibiotics starts in the cytosol with the condensation of three amino acids L-α-aminoadipic acid, L-cysteine and L-valine into the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, in a reaction catalyzed by δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS). In the following step, the β-lactam ring is formed in a reaction catalyzed by isopenicillin N synthase (IPNS). The final steps of the penicillin biosynthesis take place in microbodies. This concerns the CoA activation of a carboxyl acid precursor for β-lactam production, for instance phenylacetic acid (PAA) or phenoxyacetic acid (POA) for penicillin G and V production, respectively. For PAA, the CoA activation is mediated by a phenylacetyl CoA ligase (PCL), but depending on the type of carboxylic acid, also other CoA ligases may contribute to the process. In the last step, a side chain exchange reaction occurs, where the aminoadipate moiety of isopenicillin N is replaced by the side chain precursor. This step is catalyzed by isopenicillin N-acyl transferase (IAT) that also localizes to the microbody. Microbodies are small organelles that are surrounded by a single membrane. They provide the



enzymes with a specific environment, namely a slightly alkaline pH, allowing an optimal activity. Since microbodies are crucial for an efficient β-lactam biosynthesis process, also their biogenesis mechanism is of interest. A range of Pex proteins have been identified in *P. chrysogenum* and their role in microbody biogenesis and β-lactam biosynthesis has been studied (Bartoszewska et al., 2011a; Bartoszewska et al., 2011b; Meijer et al., 2010). Interestingly, overproduction of Pex11 results in an increased proliferation of the microbodies and concomitantly, improved β-lactam biosynthesis (Kiel et al., 2005; Meijer et al., 2010). Remarkably, despite the increased numbers of mostly smaller microbodies, the levels of the biosynthetic enzymes were unaffected by the overproduction of Pex11. Therefore, the improved biosynthesis has

been attributed to an increase in the membrane surface to volume ratio, which may allow higher fluxes of substrates and products.

The enzymes involved in the biosynthesis of penicillin are largely specified by a single gene cluster that in high producing strains is amplified in multiple copies. In contrast to some other β-lactam producing organisms, the *P. chrysogenum* penicillin biosynthesis gene cluster does not contain any transporter specific open reading frame (van den Berg et al., 2008. The molecular mechanism of transport of the biosynthetic intermediates as well as the final product through microbody membrane remains an unresolved issue. The aim of my thesis was to examine the microbody membrane of *P. chrysogenum* with the goal to identify transporters and possible other factors involved in β-lactam biosynthesis.

Chapter 2 described a proteomics approach to identify microbody membrane proteins using cellular fractionation, immuno-magnetic separation and mass spectrometry. A *P. chrysogenum* strain was used that expressed the microbody localized Pex11 protein fused to Protein A. The Protein A moiety would facilitate specific microbody purification using an immune-isolation method. A method for microbody isolation was used that was adjusted for specific membrane isolation (Kiel et al., 2009; Kikuchi et al., 2004). First, protoplasts were made that subsequently were broken to recover a mixture of cellular organelles. Next, the (largely) intact organelles were purified by means of differential centrifugation followed by sucrose gradient centrifugation. In the following steps, the microbodies containing fractions were pooled, lysed in high alkaline Tris buffer and subjected to immune-isolation using magnetic beads conjugated with IgG. The immune-purified membrane fraction was further analyzed by SDS-PAGE and mass spectrometry. This work identified several microbody membrane proteins, i.e., a protein similar to peroxisomal membrane protein PMP22 from *Arabidopsis thaliana* and Pex11. In addition, many mitochondrial, ribosomal, as well as soluble cytosol and microbody matrix proteins were found. At the time when these studies were performed, the fungal organelle proteomics was in it's infancy and a large number of technical difficulties were encountered that needed to be solved. For instance, it was challenging to purify intact microbodies and recover the membrane proteins in reasonable quantities. To generate about 0.5 mg of total organelle mixture, 2L of *P. chrysogenum* culture needed to be grown for 64-72 hrs. In this time period, also already considerable microbody breakdown can occur. Therefore, a balance between growth yield and degradation needs to be determined with the caveat that β-lactam production takes place during the late growth phases close to the stationary or death stage. In the subsequent steps of isolation, i.e., the purification of the microbody membranes, a major share of the protein is lost and membranes are obtained with a very low protein content. Indeed, a frozen specimen of microbody membranes shows a smooth structure, indicating that the number of integral membrane proteins is relatively low (Bartoszewska et al., 2011b). Fungi possess a thick cell wall and therefore enzymatic cell wall degradation is required with the risk of unwanted protein degradation. In addition, fungal cells also contain

many endogenous proteases. When the cells are lysed, rapid protein degradation may occur. Therefore, the method of isolation and purification needs to be fast. The current protocol extends over several days and thus the risk of degradation is difficult to battle. Substantial differences exist between cells in a hyphen and this adds to the complexity of organelles isolation. For instance, β-lactam biosynthesis occurs predominantly in apical cells, and it is difficult to enrich a biological sample for only such cells. Because of the presence of microtubules cell growth is polarized, and clusters of microbodies are observed that sometimes are intimately associated with other organelles like mitochondria and the endoplasmic reticulum. Such associations make it difficult to obtain a pure organellar fraction, which is also evident from the proteomic work conducted. Microbodies originate from the endoplasmic reticulum, and thus at a certain point of the organelle lifetime, the dual localization of proteins is expected. Apart from the above-mentioned difficulties, there are general issues with membrane protein proteomics where integral membrane proteins are difficult to identify because of their hydrophobicity and poor accessibility to proteases used to generate peptide fragments that can be detected in the mass spectrometry setup. Thus, microbody membrane proteomics remains a challenge for future work. Nevertheless, it will be important to examine how the protein content of microbodies varies during growth and how this impacts β-lactam production.

Our proteomic analysis did not reveal any new candidates as potential microbody membrane transporters, therefore a genomic approach was subsequently followed. So far, in a variety of organisms ranging from yeast to mammalian cells, only a few transporter proteins have been firmly localized to the microbody membrane. These are the nucleotide exchanger Ant1p, and the long chain fatty acid transporter ALDP. These transporters were the focus for further work. Their distribution in *P. chrysogenum* was examined and their role in β-lactam biosynthesis was analyzed.

Chapter 3 focuses on the requirement of the energy source ATP in the final biosynthetic steps of β-lactam formation. The CoA activation of side chain precursors by CoA ligase is an ATP dependent process, which implies that there must be a mechanism for ATP formation or transport in the microbodies. The *P. chrysogenum* genome encodes four homologs of the *Saccharomyces cerevisiae* Ant1p, namely Pc13g03380, Pc21g09430, Pc12g12500 and Pc22g11190. The yeast Ant1p functions as adenine nucleotide uptake system in the microbody membrane and exchanges ATP for ADP. The genes of all four putative Ant1p homologs are expressed during growth of *P. chrysogenum*, but only *pc13g03380* is upregulated when the fungus is grown in the presence of phenylacetic acid or oleic acid. This suggests a functional link to the degradation pathways for these molecules. Typically, oleic acid is degraded by β-oxidation a process that in fungi can occur in the microbodies and mitochondria. Likewise, fungi can also degrade PAA via the homogentisate pathway in microbodies. However, in *P. chrysogenum* Wisconsin54-1255, this pathway is blocked due to a mutation in the *pahA* gene encoding phenylacetate hydroxylase (Rodríguez-Sáiz et al., 2001). Pc13g03380 was also localized

to the microbody membrane. Deletion of it's gene had a profound effect on β-lactam production reducing it to low levels. Taken together these data suggest that Pc13g03380 is involved in uptake of ATP into microbodies, which is needed for the activation of side chain precursors during β–lactam biosynthesis as outlined in Figure 1. Importantly, the deletion analysis also signified a problem with gene deletions in an industrial strain of *P. chrysogenum*. Such strains contain multiple copies of the penicillin biosynthetic pathway and during the transformation protocol, gene copies of this pathway can splice out yielding colonies with reduced productivity. This intrinsic instability was in particular pronounced with the deletion of the *pc13g03380* gene in which all 5 independently selected clones had lost 6 out of 8 copies of the penicillin biosynthetic pathway. As this is a rare event with the deletion of other genes, one may argue that gene deletion that cause a defect in the formation of penicillin are particular prone to this instability. For instance the deletion of the *pc13g03380* gene results in higher levels of 6-APA and this may cause some toxicity to the cells. A more systematic survey would be needed to establish the validity of this hypothesis.

Chapter 4 describes studies on *P. chrysogenum* homologues of ABC-D transporters subfamily (Kovalchuk and Driessen, 2010), as these are ABC transporters that are typically associated with the microbody membrane. Pc13g11640 and Pc16g09390 are homologous to the *S. cerevisiae* Pxa1 and Pxa2 proteins. These are long chain fatty acid transporters localized in the microbody membrane. Pxa1 and Pxa2 are so-called ABC half-transporters, which function either as hetero- or homodimers and are involved in the transport of coenzyme A (CoA) activated long chain fatty acids. CoA is also required for the activation of the side chain precursor, and the mechanism of CoA entry in the microbody has not been elucidated. To analyze the impact of the Pxa1/2 homologs on β-lactam biosynthesis, single as well as double deletion strains of Pc13g11640 and 16g0939 were generated in *P. chrysogenum*. However, the deletion analysis showed no effect on β-lactam biosynthesis suggesting that these transporters are not critical for CoA supply in the microbody matrix. Also no effect was observed on oleic acid utilization. Based on these data, we conclude that there must be an alternative way to provide the microbody matrix with CoA, in order to carry out processes such as β-oxidation of fatty acids and β-lactam production. Both Pc13g11640 and Pc16g09390 are able to complement the oleic acid utilization defect of the *S. cerevisiae* Pxa1/Pxa2 deletion strain, confirming their role as long chain fatty acid transporters.

Chapter 5 The genes involved in the penicillin biosynthesis in *P. chrysogenum* are organized in the penicillin biosynthetic gene cluster. The classical strain improvement program has led to a significant amplification of the gene cluster that was paralleled by increased penicillin biosynthesis. In this respect, the addition of extra amplicons to multicopy strains as well as to the single copy strain Wisconsin 54-1255 resulted in increase of penicillin biosynthesis (Theilgaard et al., 2001), but no systematic studies have been conducted on the gene dose effect. By the generation of a *P. chrysogenum* strain in which the *ku70* gene was inactivated (Snoek et al., 2009), it became possible

to delete genes in a directed manner. As Ku70 is part of the non-homologues end-joining pathway, its inactivation resulted in an increased gene targeting efficiency by homologous recombination. The Δku70 strain provided an excellent tool to construct and study deletion mutants of industrial strains, and this method was applied to examine the β-lactam gene cluster dose response as presented in **Chapter 5** using an industrial strain that by classical strain improvement was optimized for β-lactam production. The strains used varied in the number of β-lactam gene clusters ranging from 1 up to 8, and the protein and mRNA levels were determined for the key biosynthetic genes. The transcription levels of *pcbAB*, *pcbC* and *penDE* matched with the number of gene cluster copy number. Interestingly, the cellular level of the AT protein readily saturated at low gene cluster copy numbers, while the level of PCL went down with increasing gene cluster copy numbers. No significant variation in the number and morphology of peroxisomes was observed in relation to the number of gene clusters. Importantly, the β-lactam production increased with the gene cluster copy number, but this increase was not linear and rather leveled off at low gene cluster copy numbers. This implied that there is a major limitation at high copy numbers that is not due to transcription issues, but rather relates to translation, maturation and/or assembly. This bottleneck seems to be related to the levels of acyltransferase and possibly its import into microbodies. Likewise, the availability of CoA activated side chains might also pose problems at high copy numbers as the expression of PCL decreased with the copy number, likely because of a more rapid depletion of PAA, whereas this molecule is needed to induce the PCL expression. Importantly, another study has shown that an enforced increase in the *penDE* copy number can elevate the β-lactam production in high copy number strains (Weber et al., 2012). However, this can also lead to an overdoses of acyltransferase activity and this in turn results in the accumulation of 6-APA. Therefore, it was concluded that penicillin production in the industrial strain examined is limited by the acyltransferase activity and that a careful balance of expression is needed among the different enzymes in order to maximize production. Such quantitative studies are essential to understand the molecular mechanisms of high β-lactam yielding strains used in industry and provide detailed information how the classical strain improvement programme has led to a successful lineage of *P. chrysogenum* strains.

NEDERLANDSE SAMENVATTING EN TOEKOMSTPERSPECTIEF

In 1928 werd penicilline ontdekt door Britse wetenschapper Sir Alexander Fleming. Voedingsbodems beent met *Staphylococcus spp.* werden per ongeluk besmet met schimmelsporen, omdat een laboratoriumraam had open gestaan. Op deze voedingsbodems werd rond een groene schimmel verrassend genoeg een halo gezien die de bacteriële groei remde. Dit wees erop dat de schimmel een stof uitscheidde die actief de bacteriële groei tegen ging. Later werd aangetoond dat deze stof, penicilline genaamd, effectief was tegen Gram-positieve bacteriën, maar veilig was voor gebruik in dieren en mens. Deze toevallige ontdekking, gevolgd door grondige studies, heeft een aanzienlijke effect gehad op de kwaliteit van het menselijk leven. *Penicillium* kan ß-lactam antibiotica produceren. Dit zijn antimicrobiële stoffen die een ß-lactam ring bevatten. De ß-lactam ring is het actieve deel van het molecuul. Verscheidene moleculen kunnen worden gevormd met verschillende zijketens zoals penicillines G, L, K en V die anders zijn in hun antibioticum werkingsspectrum, stabiliteit en potentie (Evers et al., 2004). De antibiotica-markt bestaat voornamelijk uit penicillines en cefalosporines. In het afgelopen decennium hadden de β-lactam antibiotica een marktaandeel van 65%. Momenteel worden deze moleculen gemaakt op een industriële schaal middels gisting en/of semi-synthese, waarbij het fermentatieproduct verder wordt gemodificeerd door chemische synthese of enzym-katalyse. Jaren van procesoptimalisatie hebben geleid tot een hogere productiviteit met een opbrengst van 90% van de totale werkzame stof waarbij de productiekosten substantieel werden verlaagd (Elander, 2003). Na een halve eeuw van gezamenlijk werk door de academische wereld en industrie is de enzymatische basis van de penicilline biosynthese route inmiddels zeer goed gekarakteriseerd (Figuur 1).

Hoofdstuk 1 geeft een samenvatting van de huidige kennis van het β-lactam productieproces in *Penicillium chrysogenum* met aandacht voor de enzymatische route en transportprocessen tussen verschillende (sub)cellulaire compartimenten. In dit systeem vervullen "microbodies" een belangrijke rol, omdat ze de laatste twee enzymen van de β-lactam route bevatten. Ook de biogenese van microbodies wordt besproken. De biosynthese van β-lactamen vindt plaats in het subapicale deel van de schimmeldraden (hyfen) in twee verschillende compartimenten van de cel - het cytosol en de microbodies. De biosynthese van β-lactam antibiotica begint in het cytosol met de condensatie van de drie aminozuren L-α-aminoadipinezuur, L-cysteïne en L-valine in het tripeptide δ-(L-α-aminoadipyl)-L-cysteïnyl-D-valine in een reactie gekatalyseerd door het δ-(L-α-aminoadipyl)- L-cysteïnyl-D-valine synthetase (ACVS). In de volgende stap wordt de β-lactam ring gevormd door een reactie die gekatalyseerd wordt door isopenicilline N synthase (IPN). De laatste stappen van de penicilline biosynthese vinden plaats in de microbodies. Dit betreft de CoA activering van een carbonzuur precursor voor β-lactam productie, bijvoorbeeld fenylazijnzuur (PAA) of fenoxyazijnzuur (POA) voor respectievelijk penicilline G en V productie. Voor PAA wordt de CoA activatie gemedieerd door een fenylacetyl CoA ligase (PCL), maar afhankelijk van het type carbonzuur kunnen ook andere CoA ligasen een bijdrage leveren aan dit proces. In de

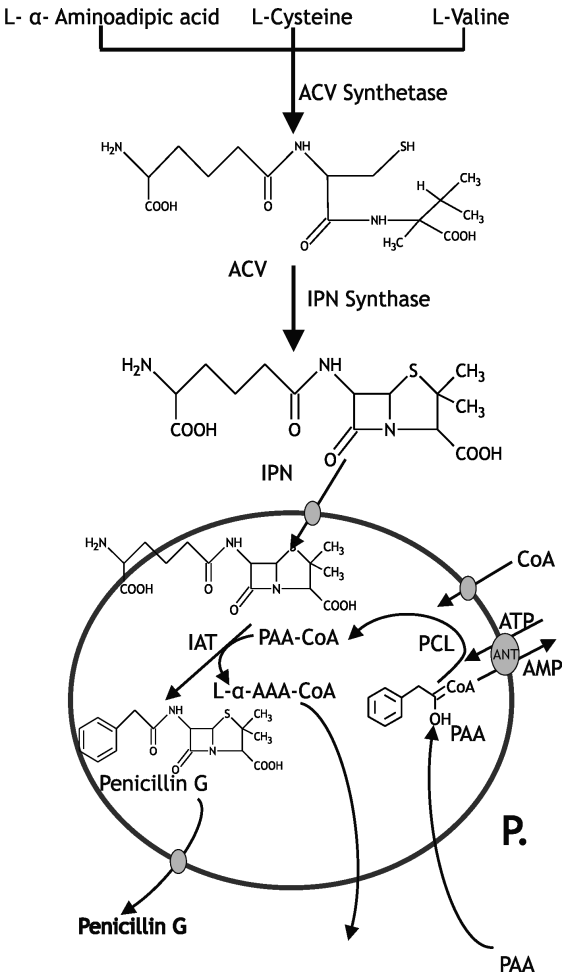


Figure 1. Een vereenvoudigd schema van de penicilline biosynthese in de hyfen van *Penicillium chrysogenum*.

laatste stap vindt een reactie plaats waarbij de aminoadipaat zijketen van isopenicilline N wordt vervangen door de zijketen precursor. Deze stap wordt gekatalyseerd door isopenicilline N-acyl transferase (IAT) dat zich ook in de microbody bevindt. Microbodies zijn kleine organellen die omgeven zijn door een enkelvoudig membraan. Zij bieden de enzymen een omgeving met een licht alkalische pH die voor optimale activiteit zorgt. Het biogenese mechanisme van microbodies is van belang, omdat microbodies essentieel zijn voor een efficiënte β-lactam biosynthese. Er zijn verschillende Pex eiwitten gevonden in *P. chrysogenum*. Hun rol in microbody biogenese en β-lactam biosynthese is onderzocht (Bartoszewska et al., 2011a; Bartoszewska et al., 2011b; Meijer et al., 2010). Interessant is dat overproductie van Pex11 resulteert in een verhoogde

proliferatie van microbodies, en dientengevolge een verbeterde β-lactam biosynthese (Kiel et al., 2005; Meijer et al., 2010). Opvallend genoeg waren de niveaus van de biosynthetische enzymen niet veranderd door de overproductie van Pex11 ondanks de toegenomen hoeveelheid van voornamelijk kleinere microbodies. Daardoor werd de verbeterde biosynthese geweten aan een toename in het membraanoppervlak/volume ratio welke voor hogere fluxen van substraten en producten zou zorgen.

De enzymen betrokken bij de biosynthese van penicilline worden met name gecodeerd door één gencluster. Dit gencluster is in stammen, die veel penicilline produceren, in meerdere kopieën aanwezig. In tegenstelling tot sommige andere β-lactam producerende organismen bevat het *P. chrysogenum* penicilline biosynthese gencluster niet een gen dat codeert voor een transporteiwit (van den Berg et al., 2008). De moleculaire mechanisme van ht transport van de biosynthetische intermediairen en het eindproduct over het membraan van de microbodies is vooralsnog onbekend. Mijn promotieonderzoek richtte zich op het bestuderen van het microbody membraan van *P. chrysogenum* met als doel transporteiwitten en mogelijke andere factoren te identificeren die betrokken waren bij de β-lactam biosynthese.

Hoofdstuk 2 beschrijft een proteomics aanpak voor het identificeren van microbody membraaneiwitten door middel van cellulaire fractionering, immunomagnetische scheiding en massaspectrometrie. Hiervoor werd een *P. chrysogenum* stam gebruikt die het microbody gelokaliseerde Pex11 eiwit tot overexpressie bracht gekoppeld aan Proteïne A. Het Proteïne A domein faciliteert de opzuivering van microbodies met behulp van immuno-isolatie. Een methode voor het isoleren van microbodies werd gebruikt (Kiel et al., 2009; Kikuchi et al., 2004) die verder werd aangepast voor specifieke membraanisolatie Als eerste werden protoplasten gemaakt waar vervolgens een mix van cellulaire organellen uit werd geïsoleerd. Daarna werden de (voornamelijk) intacte organellen opgezuiverd door middel van differentiële centrifugatie gevolgd door sucrose gradiënt centrifugatie. Vervolgens werden de fracties van de microbodies samengevoegd, gelyseerd met hoge alkaline Tris buffer en geïsoleerd met immuno-isolatie gebruikmakende van magnetische beads geconjugeerd met IgG. De membraanfractie werd verder geanalyseerd met SDS-PAGE en massaspectrometrie. Dit leidde tot de identificatie van verschillende microbody membraaneiwitten, namelijk Pex11 en een eiwit dat vergelijkbaar was met het peroxisomale membraaneiwit PMP22 van *Arabidopsis thaliana*, maar ook vele mitochondriale, ribosomale, en oplosbare cytosolische en microbody matrixeiwitten. Ten tijde van deze studies stond de proteomics van schimmelorganellen nog in de kinderschoenen. Er waren een groot aantal technische problemen die opgelost dienden te worden. Het was bijvoorbeeld een grote uitdaging om intacte microbodies op te zuiveren en membraaneiwitten te isoleren in redelijke hoeveelheden. Om 0.5 mg van totale organelmix te verkrijgen was 2 liter van *P. chrysogenum* cultuur nodig die gedurende 64-72 uur diende te groeien. Er vindt echter tijdens het groeiproces ook aanzienlijke afbraak van microbodies plaats. Het is dus belangrijk om een goede balans te vinden tussen groeisnelheid en

afbraak. Met name omdat β-lactam productie plaats vindt gedurende de late groeifase vlak voorafgaand aan de stationaire fase of celdood. In de volgende isolatiestappen, namelijk de opzuivering van de microbody membranen, gaat een groot aandeel van het eiwit verloren en ontstaan membranen met een zeer laag eiwitpercentage. Een bevroren monster van microbody membranen dat bestudeert wordt middels freeze-fracture electronenmicroscopie heeft een gladde structuur wat aangeeft dat het aantal integrale membraaneiwitten relatief laag is (Bartoszewska et al., 2011b). Schimmels hebben een dikke celwand en daardoor is enzymatische celwand afbraak nodig met het risico op ongewenste eiwitafbraak. Daarnaast bevatten schimmelcellen ook veel endogene proteases. Wanneer de cellen worden gelyseerd, kan er snelle eiwitafbraak plaatsvinden. Om die reden dienen de isolatiemethode en opzuivering snel plaats te vinden. Het huidige protocol duurt enkele dagen wat het moeilijk maakt om het risico op afbraak tegen te gaan. De aanzienlijke verschillen tussen de verschillende cellen in een hyfen maken de organelisolatie nog complexer. Bijvoorbeeld, β-lactam biosynthese vindt voornamelijk plaats in apicale cellen, terwijl het moeilijk is om een biologisch monster voor deze cellen te verrijken. Vanwege de aanwezigheid van microtubuli is de celgroei gepolariseerd, en worden clusters van microbodies gezien die soms geassocieerd zijn met andere organellen zoals mitochondriën en het endoplasmatisch reticulum. Zulke associaties maken het moeilijk om een pure organelfractie te krijgen, wat ook duidelijk blijkt uit het uitgevoerde proteomics-werk. Microbodies worden gevormd door het endoplasmatisch reticulum, en daardoor zal gedurende de biogenese cyclus van organellen, gelijktijdige lokalisatie op kunnen treden van eiwitten. Er zijn algemene problemen met het identificeren van integrale membraaneiwitten doormiddel van proteomics. De hydrofobiciteit en moeilijke toegankelijkheid van de proteases die gebruikt worden om peptide fragmenten te genereren en die gedetecteerd kunnen worden door massaspectrometrie, blijft een uitdaging. Oftewel de techniek van microbody membraan proteomics dient zo optimaal mogelijk te zijn voor toekomstige studies. Belangrijk is om onderzoek te doen naar de variatie van het eiwitgehalte van microbodies tijdens de groei, en hoe dit invloed heeft op de β-lactam productie.

Aangezien onze proteoomanalyse niet tot de ontdekking van nieuwe mogelijke microbody membraantransporteiwitten leidde, werd besloten om een genoomaanpak te volgen. Tot nog toe werden in verschillende organismen, variërend van gist tot zoogdiercellen, maar slechts enkele transporteiwitten met zekerheid gelokaliseerd tot het microbody membraan. Dit zijn de nucleotide uitwisselaar Ant1p, en het lange keten transporteiwit ALDP. De focus van verdere studies was om de distributie van deze transporteiwitten in *P. chrysogenum* te bestuderen en hun rol in de β-lactam biosynthese te onderzoeken.

Hoofdstuk 3 richt zich op de behoefte aan de energiebron ATP in de laatste biosynthetische stappen van de β-lactam vorming. De CoA activatie van zijketen precursors door CoA ligase is een proces dat van ATP afhankelijk is. Dit doet vermoeden dat er een mechanisme voor ATP vorming moet zijn, of transport naar de

microbodies. Het *P. chrysogenum* codeert voor vier homologen van de *Saccharomyces cerevisiae* Ant1p; Pc13g03380, Pc21g09430, Pc12g12500 en Pc22g11190. De gist Ant1p fungeert als adenine nucleotide opnamesysteem in het microbody membraan en wisselt ATP uit voor ADP. De genen van alle vier mogelijke Antp1 homologen worden tot expressie gebracht gedurende de groei van *P. chrysogenum*, maar alleen *pc13g03380* wordt opgereguleerd wanneer de schimmel groeit in de aanwezigheid van fenylazijnzuur of oliezuur. Deze gegevens suggereren een functionele koppeling tussen de degradatieroutes van deze moleculen. Over het algemeen wordt oliezuur afgebroken middels β-oxidatie. Dit is een proces dat in schimmels plaats vindt in microbodies en mitochondriën. Daarnaast kunnen schimmels ook fenylazijnzuur afbreken via de homogentisate route in microbodies. In *P. chrysogenum* Wisconsin54-1255 is deze route echter geblokkeerd door een mutatie in het *pahA* gen dat codeert voor fenylacetaat hydroxylase (Rodríguez-Sáiz et al., 2001). De vermoedelijke ATP transporter Pc13g03380 kon worden gelokaliseerd in de microbody membraan. De deletie van het gen resulteerde in een aanzienlijke reductie van de β-lactam productie. Deze gegevens suggereren dat Pc13g03380 betrokken is bij de opname van ATP in microbodies, en vervolgens gebruikt wordt voor de activatie van zijketen precursors tijdens β–lactam biosynthese (Figuur 1). Deze deletie-analyse bracht ook een probleem aan het licht van gendeleties in een industriële stam van *P. chrysogenum*. Zulke stammen bevatten meerdere kopieën van de penicilline biosynthetische route en tijdens het transformatieprotocol kunnen genkopieën van deze route eruit gespliced worden wat resulteert in kolonies met gereduceerde productiviteit. Deze intrinsieke instabiliteit werd met name gezien bij de deletie van het *pc13g03380* gen waarbij alle vijf onafhankelijk geselecteerde kolonies 6 van de 8 kopieën van de penicilline biosynthetische route hadden verloren. Aangezien dit zelden voorkomt bij de deletie van andere genen, mag men veronderstellen dat een gendeletie die een defect veroorzaakt in de penicillinevorming in het bijzonder vatbaar is voor instabiliteit. De deletie van het *pc13g03380* gen bijvoorbeeld resulteert in hoge niveaus van 6-aminopenicillinezuur en dit kan mogelijk leiden tot toxiciteit voor de cellen. Een meer systematische benadering is nodig om de validiteit van deze hypothese te testen.

Hoofdstuk 4 beschrijft studies van *P. chrysogenum* homologen van de ABC-D transporteiwit subfamilie (Kovalchuk and Driessen, 2010). Deze ABC transporteiwitten zijn over het algemeen geassocieerd met het microbody membraan. Pc13g11640 en Pc16g09390 zijn homologen van de S. *cerevisiae* Pxa1 en Pxa2 eiwitten. Deze lange keten vetzuur transporteiwitten bevinden zich in het microbody membraan. Pxa1 en Pxa2 zijn zogenaamde ABC "half-transporters" die functioneren als hetero- of homodimeren en betrokken zijn bij het transport van coenzym A (CoA) geactiveerde lange keten vetzuren. CoA is ook nodig voor de activatie van de zijketen precursor, en hoe CoA de microbody in gaat is nog niet opgehelderd. Om de bijdrage te analyseren van Pxa1/2 homologen op de β-lactam biosynthese werden zowel enkele als dubbele deletie stammen van Pc13g11640 en 16g0939 gemaakt in *P. chrysogenum*. De deletie-

analyse toonde echter geen effect op de β-lactam biosynthese wat suggereert dat deze transporteiwitten niet essentieel zijn voor de levering van CoA aan de microbody matrix. Ook werd er geen effect waargenomen op de oliezuur omzetting. Op basis van deze gegevens concluderen we dat er een alternatieve route moet zijn om CoA aan de microbody matrix te leveren om toch de processen van β-oxidatie van vetzuren en β-lactam productie uit te kunnen voeren. Zowel Pc13g11640 en Pc16g09390 zijn in staat om het defect in oliezuurgebruik te compenseren in de *S. cerevisiae* Pxa1/Pxa2 deletie stam. Dit bevestigt hun rol als lange keten vetzuren transporteiwitten.

De genen die betrokken zijn bij de penicilline biosynthese in *P. chrysogenum* zijn georganiseerd in het penicilline biosynthetische gencluster. De klassieke stamverbetering heeft geleid tot een aanzienlijke uitbreiding van het gencluster wat vervolgens geresulteerd heeft in een toegenomen penicilline biosynthese. Bijvoorbeeld de toevoeging van additionele amplicons aan multie-kopie stammen alsook aan de enkele-kopie stam Wisconsin 54-1255 leidde tot een toegenomen penicilline biosynthese (Theilgaard et al., 2001). Er zijn echter geen systematische studies uitgevoerd ten aanzien van het gen dosiseffect. Door het maken van een *P. chrysogenum* stam waarin het gen *ku70* werd geïnactiveerd (Snoek et al., 2009) werd het mogelijk om gericht genen te verwijderen met een hogere efficiëntie. Aangezien *ku70* een rol speelt bij niet-homologe eind-verbinding, resulteert inactivatie van dit gen in een toegenomen efficiëntie van gene-targeting door homologe recombinatie. De Δku70 stam bood een uitstekend hulpmiddel om industriële deletie mutanten te maken en te bestuderen.

In **Hoofdstuk 5** werd deze methode gebruikt om de dose-response te bestuderen van het β-lactam gencluster met behulp van een industriële stam die door klassieke stamverbetering was geoptimaliseerd voor β-lactam productie. De stammen varieerden in het aantal β-lactam genclusters van 1 tot 8. De mRNA- en eiwitniveaus werden bepaald voor de belangrijkste biosynthetische genen. De transcriptie niveaus van *pcbAB*, *pcbC* en *penDE* kwamen overeen met het aantal kopieën van het gencluster. Interessant was dat het niveau in de cel van het AT eiwit al snel verzadigd was bij een laag aantal kopieën van het gencluster, terwijl het niveau van PCL naar beneden ging met een toenemend aantal kopieën. Er werd geen significante variatie gevonden in het aantal en morfologie van de peroxisomen in relatie tot het aantal genclusters. Belangrijk was verder dat de β-lactam productie toenam met het aantal kopieën van het gencluster, maar dat deze toename niet lineair was, en dat deze stijging al afvlakte bij een laag aantal kopieën. Dit impliceerde dat er een belangrijke beperking is bij het hoge aantal kopieën die niet komt door transcriptie problemen, maar die eerder te maken heeft met translatie, maturatie en/of translatie. Deze 'bottleneck' lijkt te maken te hebben met de niveaus van acyltransferase. Mogelijk speelt ook de invoer van acyltransferase naar de microbodies een rol. Daarnaast kan de aanwezigheid van CoA-geactiveerde zijketens mogelijk problemen geven bij een hoog aantal kopieën, omdat de PCL expressie afneemt met het aantal kopieën. Dit laatste heeft hoogstwaarschijnlijk te maken met een snelle verwijdering van PAA terwijl dit molecuul

nodig is om de PCL expressie te induceren. Belangrijk is dat een studie heeft laten zien dat een verhoging in het aantal kopieën van het *penDE* cluster kan zorgen voor een toegenomen β-lactam productie in stammen met een hoog aantal kopieën (Weber et al., 2012). Dit kan echter ook leiden tot overdosis van acyltransferase activiteit, en dit resulteert vervolgens in accumulatie van 6-APA. Om die reden werd geconcludeerd dat penicilline productie in de industriële stam gelimiteerd is in acyltransferase activiteit, en dat de balans van expressie tussen de verschillende enzymen nauw luistert om productie te maximaliseren. Zulke kwantitatieve studies zijn essentieel om de moleculaire mechanismen te begrijpen van hoge β-lactam producerende stammen gebruikt in de industrie. Verder zullen zulke studies gedetailleerde informatie geven hoe klassieke stamverbetering geleid heeft tot succesvolle *P. chrysogenum* stammen.

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PODSUMOWANIE

Penicylina została odkryta w 1928 roku przez brytyjskiego uczonego Sir Alexandra Fleming'a. Przypadkowo płytki ze szczepem *Staphylococcus*zostały zainfekowane sporami pleśni, która dostała sie do labolatorium przez otwarte okno. Następnego dnia napłytkach zaobserwowano efekt "halo"spowodowany zahamowaniem wzrostu bakterii wokół zielonej pleśniSubstancją aktywną, hamującą wzrost bakterii była penicylina, która hamując wzrost bakterii Gram-dodatnich, równocześnie jest bezpieczna dla ludzi i zwierząt.To przypadkowe odkrycie oraz następujące po nim dokładne badania, w znacznym stopniu poprawiły jakoś ówczesnego życia. Grzyb zwany *Penicillium* jest w stanie produkować antybiotyki ß-laktamowe, czyli substancje przeciwbakteryjne posiadające pierścień ß-laktamowy, stanowiący o specyficznej aktywności chemicznej całej cząsteczki.

Wśród penicylin wyróżniamy penicyline G, K, L i V, które różnią się łańcuchem bocznym, a także spektrum działania bakteriobójczego, stabilnością i aktywnością (Evers et al., 2004).

W ostatniej dekadzie antybiotyki β–laktamowe stanowiły 65% antybiotyków dostępnych na rynku, głównie są to penicyliny i cafalosporyny. Obecnie te związki są produkowane na skalę przemysłową w procesie całkowitej fermentacji i/lub w procesie semi-syntezy, gdzie produkt usyskany w procesie fermentacji podlega dalszym modyfikacjom poprzez syntezę chemiczną lub katalizę enzymatyczną. Lata optymalizacji procesu doprowadziły do wzrostu produktywności, znaczącej redukcji kosztów z jednoczesnym 90% odzyskiem substancji aktywnej (Elander, 2003). Pół wieku pracy wykonanej wspólnie przez akademickie i przemysłowe środowiska naukowe, doprowadziły do scharakteryzowania enzymatycznych podstaw szlaku biosyntezy penicyliny (Rysunek 1).

Rozdział 1 podsumowuje obecną wiedzę na temat procesu produkcji antybiotyków β-laktamowych u *Penicillium chrysogenum,* ze szczególnym zwróceniem uwagi na lokalizację poszczególnych procesów w komórce, transport pomiędzy jej częściami jak i biogenezę peroksysomów. W szlaku biosyntezy penicyliny peroksysomy odgrywają ważną role, gdyż są w nich zlokalizowane ostatnie dwa enzymy szlaku biochemicznego. Biosynteza β-laktamów zachodzi w górnej części grzybni, w dwóch różnych częściach komórki jakimi są cytozol i peroksysomy. Proces rozpoczyna się w cytozolu poprzez kondensację trzech aminokwasów: kwasu L-α- aminoadypinowego, L-cysteiny i L-valiny w trójpeptyd δ-(L-α-aminoadypinowy)-L-cysteinowy-D-valinowy, ta reakcja jest katalizowana przez syntetazę δ-(L-α-aminoadypinowo)-L-cysteinowo-D-valinową (ACVS). Następnie, w reakcji katalizowanej przez syntetazę izopenicyliny N (IPNS) powstaje pierścień β-laktamowy. Ostatnie etapy biosyntezy penicyliny zachodzą w peroksysomach. Jest to aktywacja kwasu karboksylowego przy udziale koenzymu A, na przykład kwasu fenylooctowego (PAA) lub kwasu fenoksyoctowego (POA), odpowiednio do penicyliny G (penicylina benzylowa) bądź V (fenoksymetylowa). Aktywacja koenzymem A (dalej KoA) kwasu fenylooctowego (PAA) zachodzi przy udziale ligazy fenylooctowej KoA (PCL), w zależności od rodzaju kwasu karboksylowego, różne rodzaje ligaz mogą brać udział w procesie aktywacji łańcucha bocznego. W

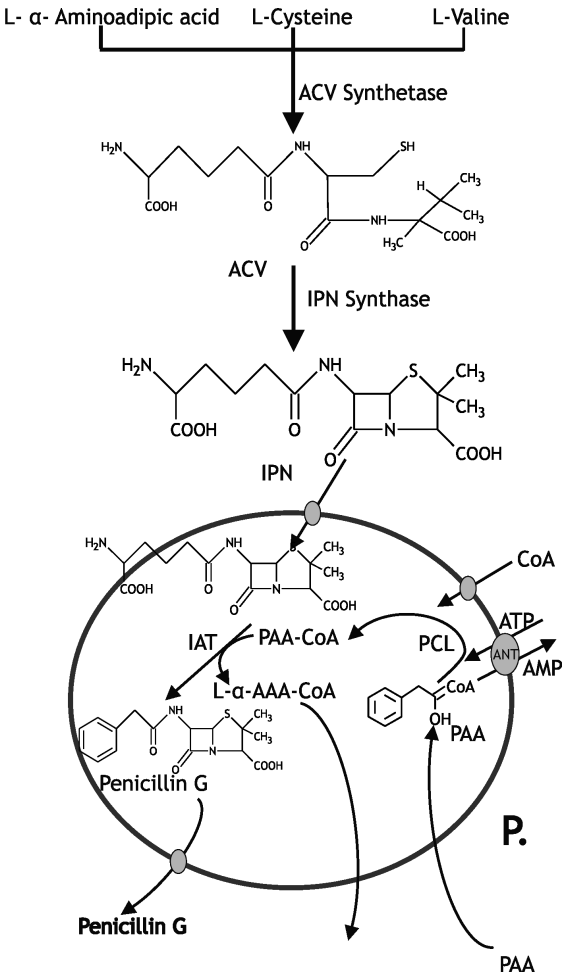

Figure 1. Schematyczna reprezentacji szlaku biosyntezy penicyliny w grzybni *Penicillium chrysogenum*.

ostatnim etapie biosyntezy następuje zamiana reszty aminoadypinowej izopenicyliny N na prekursor łańcucha bocznego (reszta fenylooactanowa lub fenoksyoctanowa). Ta reakcja jest katalizowana przez acylotransferazę izopenicyliny N (IAT), która jest również zlokalizowana w peroksysomach. Peroksysomy to małe organella otoczone pojedynczą błoną komórkową, zapewniające enzymom optymalne dla ich aktywności, lekko zasadowe środowisko. Ponieważ peroksysomy są kluczowe dla efektywnego procesu biosyntezy β-laktamów, również sposób ich powstawania wzbudza zainteresowanie. U *P. chrysogenum* zidentyfikowano całą gamę białek Pex, zbadano ich rolę w powstawaniu peroksysomów, jak i w syntezie antybiotyków (Bartoszewska et al., 2011a; Bartoszewska et al., 2011b; Meijer et al., 2010). Nadprodukcja białka Pex11

prowadzi do zwiększonej proliferacji peroksysomów i równocześnie do zwiększonej biosyntezy β-laktamów (Kiel et al., 2005; Meijer et al., 2010).

Zauważono, że pomimo zwiększonej liczby, zazwyczaj mniejszych peroksysomów, poziom enzymów biorących udział w biosyntezie penicyliny nie uległ zmianie przez nadprodukcję Pex11. Dlatego też stwierdzono, że wzrost biosyntezy penicyliny ma związek ze zwiększoną powierzchnią błony wzlędem całkowitej objętości peroksysomów, co może pozwolić na wyższy przepływ substratów i produktów.

Enzymy zaangażowane w biosyntezę penicylin są w większości kodowane przez pojedynczy klaster genowy, który w szczepach wysokoprodukcyjnych występuje w wielu kopiach. W przeciwieństwie do innych organizmów produkujących β-laktamy, klaster genowy biosyntezy penicyliny *P. chrysogenum* nie posiada żadnej ramki odczytu, która kodowałaby transportery (van den Berg et al., 2008). Molekularne podstawy transportu pośrednich jak i końcowych produktów biosyntezy poprzez błonę peroksysomów pozostają niewyjaśnione. Celem niniejszej pracy było zbadanie błony peroksysomów *P. chrysogenum w* kierunku identyfikacji transporterów oraz innych elementów zaangażowanych w biosyntezę β-laktamów.

Rozdział 2 opisuje identyfikację białek błony peroksysomów metodą proteomiczną przy użyciu frakcjonowania komórkowego. W użytym szczepie *P. chrysogenum* zachodziła ekspresja białka Pex11 połączonego z białkiem A, zlokalizowanym w błonie peroksysomów. Zadaniem białka A było umożliwienie specyficznego wyizolowania peroksysomów przy użyciu metody immunoizolacji. Do izolacji peroksysomów użyto wcześniej opisanych metod, zmodyfikowanych w celu izolacji błon (Kiel et al., 2009; Kikuchody, et al., 2004). Najpierw przygotowano protoplasty, które następnie zostały otwarte, aby uzyskać mieszaninę organelli komókowych. Większość nieuszkodzonych organelli została wyizolowana przy użyciu "wirowania różnicowego", następnie zastosowano separację organelli poprzez wirowanie na gradiencie cukrowym.

W kolejnych etapach frakcje zawierające peroksysomy zostały połączone, dokonano lizy przy użyciu wysoko-zasadowego buforu Tris. Tak uzyskaną próbkę poddano immunoizolacji używając kulek magnetycznych zespolonych z przeciwciałami . Frakcja błonowa wyizolowana przy pomocy metody immunoizolacji została zanalizowana przy użyciu SDS-PAGE i spektrometrii masowej.W ten sposób zidentyfikowano białka należące do błony mikrociałek, np. białko podobne do peroksysomowego białka błonowego PMP22 u *Arabidopsis thaliana*, jak i Pex11. Ponadto, znaleziono wiele białek mitochondrialnych, rybosomalnych, jak i rozpuszczalnych białek cytozolowych oraz światła peroksysomów. W czasie kiedy opisane eksperymenty były przeprowadzone, proteomika organelli grzybowych była we wczesnje fazie rozwoju. Stąd też napotkano tu na liczne utrudnienia techniczne, podczas oczyszczania nieuszkodzonych peroksysomów oraz odzyskania białek błonowych w ilościach potrzebnych do przeprowadzenia koniecznych analiz. Aby uzyskać około 0.5 mg całkowitej mieszaniny organelli użyto 2 litry kultury *P. chrysogenum,* która była hodowana przez 64-72 h. W tym czasie mogło dojść do znaczącej degradacji peroksysomów. Dlatego też moment

równowagi pomiędzy masą wzrostową jak i poziomem degradacji został zbadany. W późniejszych etapach przygotowywania próbki, przy izolowaniu i oczyszczaniu błon peroksysomów większość białek błonowych została utracona.

Zamrożony preparat błony peroksysomów ma gładką strukturę, co wskazuje na to, że liczba integralnych białek błonowych jest bardzo niska (Bartoszewska et al., 2011b). Poza tym, grzyby posiadają grubą ścianę komórkową wymagającą degradacji enzymatycznej (generowanie protoplastów), co wiążę sie z ryzykiem degradacji białek. Dodatkowo, komórki grzybów także posiadają wiele endogennych proteaz, w związku z czympodczas lizy komórkowejmoże nastąpić szybka degradacja białek. Dlatego też, metody izolacji i oczyszczania błon w przypadku grzybów muszą być wyjątkowo szybkie. Wykonanie opisanego protokołu trwa kilka dni, dlatego trudno jest uniknąć degradacji białek. Znaczące różnice występują wśród komórek zorganizowanych w grzybnię, co utrudnia izolację organelli komórkowych. Dla przykładu, biosynteza β-laktamów występuje głównie w apikalnej części komórki, uzyskanie biologicznej próbki zawierającej tylko tego rodzaju komórki jest dużym wyzwaniem. Przez obecnośc mikrotubul wzrost komórkowy jest spolaryzowany, stąd grupy peroksysomów ułożone są bardzo blisko innych organelli takich jak, mitochondria i retikulum endoplazmatyczne. Taka zależność powoduje, że uzyskanie czystej frakcji organelli jest skomplikowane, co jest również widoczne w wynikach eksperymentów proteomicznych.

Peroksysomy wywodzą się z retikulum endoplazmatycznego, dlatego też w pewnym okresie istnienia organelli, spodziewana jest podwójna lokalizacja białek. Poza wyżej wspomnianymi trudnościami, istnieją też ogólne wyzwania w ramach proteomiki białek błonowych, gdyż integralne białka błonowe są niełatwe do identyfikacji z powodu ich hydrofobowej natury i słabej dostępności do proteaz użytych w celu uzyskania peptydów, które mogą być zidentyfikowane w spektrometrii masowej. Dlatego też, proteomika białek błonowych pozostaje wyzwaniem dla przyszłych badań naukowych. Jednakże poznanie jak w czasie wzrostu zmienia się skład białek w peroksysomach i jaki ma to wpływ na produkcję β-laktamów pozostaje interesujące.

Przeprowadzona analiza proteomiczna nie wykazała żadnych nowych kandydatów na potencjalne błonowe białka transportowe, dlatego też rozpoczęto analizę genomu. Jak dotąd, w różnych oranizmach, rozpoczynając od drożdży po komórki ssacze, zidentyfikowano zaledwie kilka białek transportowychzlokalizowanych w błonie peroksysomów. Są to: transportery nukleotydów- Ant1p (ang. *adenine nucleotide transporter*) i transportery długich kwasów tłuszczowych ALDP (ang. *adenoleukodystrophy protein*). Na tych transporterach skupiono uwagę kolejnej części pracy. Zbadano ich lokalizację jak i rolę w syntezie β-laktamów u *P. chrysogenum*.

Rozdział 3 jest skoncentrowany na zapotrzebowaniu na źródło energii, jakim jest ATP (ang. *adenosine triphosphate)* w czasie końcowej fazy biosyntezy β-laktamów. Aktywacja bocznego łańcucha prekursorów w obecności ligazy KoA jest procesem zależnym od ATP, co sugeruje istnienie mechanizmu zapewniającego powstawanie lub transport ATP do peroksysomów. Genom *P. chrysogenum* koduje cztery homologii

Ant1p *Saccharomyces cerevisiae*, takie jak Pc13g03380, Pc21g09430, Pc12g12500 and Pc22g11190. Pochodzące z drożdży białko Ant1p funkcjonuje jako system pobierający nukleotydy adeniny w błonie peroksysomów i wymienia ATP (ang. *adenosine triphosphate*) na ADP (ang. *adenosine triphosphate*). W czasie wzrostu *P. chrysogenum* zachodzi ekspresja wszystkich czterech genów domniemanych homologów *Ant1p,* aczkolwiek w obecności kwasu fenylooctowego wzrasta ekspresja jedynie p*c13g03380.* To sugeruje funkcjonalny związek pomiędzy Ant1p a szlakiem degradacji kwasów fenylooctowego i fenoksyoctowego. Utylizacja kwasu oleinowego zachodzi w wyniku procesu degradacji poprzez β-oksydację, zachodzącą w peroksysomach i mitochondriach. Podobnie grzyby mogą również rozkładać kwas fenylooctowy poprzez „homogentisate pathway" w peroksysomach. Jendakże, u *P. chrysogenum* Wisconsin 54-1255 ten szlak jest zablokowany poprzez mutację w genie *pahA* kodującym hydroksydazę fenylooctową (Rodríguez-Sáiz et al., 2001).

Pc13g03380 został również zlokalizowany w błonie peroksysomów. Delecja jednego z genów miała znaczący wpływ na produkcję β-laktamów, redukując ją do bardzo niskich poziomów. Biorąc pod uwagę wszystkie dane, wyniki sugerują,że Pc13g03380 jest zaangażowany w transport ATP do peroksysomów, które jest wykorzystane do aktywacji łańcuchów bocznych kwasów karboksylowych w czasie biosyntezy penicyliny, jak pokazano na Rysunku 1. Godny uwagi jest fakt, że analiza delecyjna nasiliła problem usuwania genów u przemysłowych szczepów *P. chrysogenum*. Szczepy przemysłowe posiadają wiele kopii klastrów genowych szlaku biosyntezy penicyliny, które w czasie przeprowadzania transformacji mogą ulec wycięciu, co prowadzi do powstawania kolonii o zredukowanej produktywności. Ta wewnętrzna niestabilność genetyczna była w szczególności widoczna w mutancie genu *pc13g03380,* gdzie wszystkie pięć niezależnie wybranych klonów straciło sześć z ośmiu kopii klastrów genowych szlaku biosyntezy penicyliny. Zdarza się bardzo rzadko, że delecja jednego z genów powoduje usunięcie innego. Można spekulować, że mutacja, która spowodowała defekt w procesie powstawania penicyliny jest szczególnie wrażliwa na tą niestabilność. W wyniku usunięcia genu pc13g03380 dochodzi do zwiększenia poziomu 6-APA, co może działać toksycznie na komórki. Aby potwierdzić przedstawione hipotezy, konieczne są bardziej szczegółowe badania.

Rozdział 4 opisuje pracę wykonaną na homologach podrodziny ABC-D transporterów u *P. chrysogenum,* jako że białka transportujące ABC są typowo zlokalizowane w błonie peroksysomów. Pc13g11640 i Pc16g09390 są homologami białek Pxa1 i Pxa2 u *S. cerevisiae*. Są to białka transportujące, zlokalizowane w błonie peroksysomów, transportujące długie kwasy tłuszczowe. Pxa1 i Pxa2 są półtransporterami ABC, które działają jako hetero- lub homodimery biorące udział w transporcie długich kwasów tłuszczowych aktywowanych koenzymem A. Koenzym A jest też wymagany do aktywacji prekursorów łańcucha bocznego, jednakże sposób w jaki koenzym A dostaje się do peroksysomów nie jest jasny. Aby dokonać analizy jaki wpływ mają homologii białek Pxa1/2 na produkcję β-laktamów, przeprowadzono pojedynczą i podwójną delecję genów Pc13g11640 i Pc16g0939 u *P. chrysogenum.*

Analiza tych mutantów nie wykazała żadnego wpływu na biosyntezę β-laktamów. Nie zaobserwowano również efektu na utylizację kwasu oleinowego. Na podstawie uzyskanych wyników wywnioskowano, że musi istnieć alternatywne źródło dostarczające koenzym A do środka peroksysomów. Obydwa białka, Pc13g11640 i Pc16g09390 były funkcjonalne w delecyjnym mutancie *S. cerevisiae* Pxa1/Pxa2, co potwierdza ich rolę jako transporterów długich kwasów tłuszczowych.

Rozdział 5 Geny zaangażowane w biosyntezę penicyliny u *P. chrysogenum* są zorganizowane w klaster genowy biosyntezy penicyliny. Klasyczny program ulepszenia szczepów przemysłowych doprowadził do znacznego zwiększenia liczby klastrów genowych i jednocześnie wzrostu ilości syntetyzowanej penicyliny. W związku z tym, wprowadzenie dodatkowych amplikonów do szczepu wielokopijnego, jak i do szczepu posiadającego pojedynczy amplikon (Wisconsin 54-1255) powoduje wzrost w biosyntezie penicyliny (Theilgaard et al., 2001). Jak dotąd nie przeprowadzono dokładnych badań wyjaśniających efekt dawki genu. Stworzenie szczepu *P. chrysogenum,* gdzie gen *ku70* został zdeaktywowany, umożliwiło przeprowadzanie bezpośrednich delecji (Snoek et al., 2009). Jako, że Ku70 jest częścią systemu naprawy DNA poprzez łączenie niehomologicznych zakończeń (ang. *non-homologues end-joining pathway*; NHEJ), jego dezaktywacja doprowadziła do wzrostu efektywności mutacji genów przez homologiczną rekombinację. Szczep Δ*ku70* okazał się być doskonałym narzędziem, aby skonstruować i badać mutanty delecyjne szczepów przemysłowych. Ta metoda została użyta, aby studiować wpływ ilości klastrów genowych na szczepy przemysłoweoptymalizowane pod względem produkcji β-laktamów, jak zaprezentowano w **rozdziale 5.** Użyte szczepy różniły się ilością β-laktamowych klastrów genowych pomiędzy jedną a ośmioma kopiami, poziom białka i mRNA były ustalone dla kluczowych genów biosyntetycznych. Poziomy transkrypcji *pcbAB*, *pcbC* i *penDE* odpowiadały ilości kopii klastrów genowych. Co ciekawe, komórkowy poziom białka AT był z łatwością nasycony w przypadku niskiej ilości kopii klastrów genowych, natomiast poziom PCL spadał wraz ze wzrastającą ilością kopii. Nie zaobserwowano zależności pomiędzy ilością i morfologią peroksysomów a ilością obecnych klastrów genowych. Godne uwagi jest to, że produkcja β-laktamów wzrastała proporcjonalnie do ilości kopii klastrów genowych, aczkolwiek ten wzrost nie był liniowy i raczej wyrównany dla szczepów z niską ilością klastrów genowych To dowodzi, że istnieje znaczne ograniczenie w przypadku wielokopijnych szczepów przemysłowych i nie jest to spowodowane przez problemy w czasie transkrypcji genów, ale raczej ma związek z translacją, dojrzewaniem i/lub składaniem genów. Ten problem wydaje się mieć związek z poziomem acyltransferazy i prawdopodobnie jej importu do peroksysomów. Ponadto, dostępność koenzymu A, aktywującego łańcuch boczny, może również stanowić problem w przypadku szczepów wysokokopijnych, gdyż zaobserwowano spadek ekspresji PCL wraz ze wzrastającą ilością kopii genów, być może w skutek szybszego wyczerpania PAA, kluczowego w indukcji ekspresji PCL. Inne badania wykazały , że wymuszony wzrost ilości kopii *penDE* może zwiększać poziom produkcji

β-laktamów w wielokopijnych szczepach (Weber et al., 2012). Jednakże, to również może prowadzić do nadmiernej aktywności acyltransferazy, powodując akumulację 6-APA. Dlatego też wywnioskowano, że produkcja penicyliny w przemysłowych szczepach jest limtowana przez aktywność acyltransferazy i jest potrzebna równowaga pomiędzy różnymi enzymami po to, aby zmaksymalizować produkcję.

Aby dostarczyć szczegółowych informacji o tym, jak klasyczny program optymalizacji doprowadził do sukcesu szczepy pochodzące od *P. chrysogenum*, konieczne jest przeprowadzanie eksperymentów ilościowych, stanowiących podstawę do zrozumienia molekularnych mechanizmów wysokoprodukcyjnych szczepów β-laktamowych.

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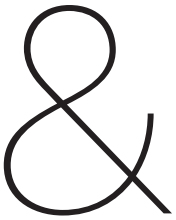

I arrived to Groningen as a Socrates-Erasmus student, excited to join a project in the Department of Molecular Microbiology, at the Rijksuniversiteit in Groningen. My plan was to stay here for a short period of time, however, a short adventure turned out to become our life.

Charmed by the atmosphere and excited by the challenge to join a scientific project concerning the proteomics of the microbody membrane from *Penicillium chrysogenum,* I came back to Groningen as a PhD student.

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