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Synthesis of *Penicillium chrysogenum* acetyl-CoA:isopenicillin *N* acyltransferase in *Hansenula polymorpha*: First step towards the introduction of a new metabolic pathway

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Abstract

The enzyme acetyl-CoA:isopenicillin *N* acyltransferase (IAT) is a peroxisomal enzyme that mediates the final step of penicillin biosynthesis in the filamentous fungi *Penicillium chrysogenum* and *Aspergillus nidulans*. However, the precise role of peroxisomes in penicillin biosynthesis is still not clear. To be able to use the power of yeast genetics to solve the function of peroxisomes in penicillin biosynthesis, we introduced IAT in the yeast *Hansenula polymorpha*. To this purpose, the *P. chrysogenum pen*DE gene, encoding IAT, was amplified from a cDNA library to eliminate the three introns and introduced in *H. polymorpha*. In this organism IAT protein was produced as a 40 kDa pre-protein and, as in *P. chrysogenum*, processed into an 11 and 29 kDa subunit, although the efficiency of processing seemed to be slightly reduced relative to *P. chrysogenum*. The *P. chrysogenum* IAT, produced in *H. polymorpha*, is normally localized in peroxisomes and in cell-free extracts IAT activity could be detected. This is a first step towards the introduction of the penicillin biosynthesis pathway in *H. polymorpha*.

Keywords: Yeast; Penicillin synthesis; Peroxisomes; Acyltransferase

1. Introduction

Penicillin, an important and the most-widely known member of the β-lactam antibiotics, is produced as a secondary metabolite by the filamentous fungi *Penicillium chrysogenum* and *Aspergillus nidulans*. Because of its importance in the clinical market, it is the subject of extensive research. As a result, the biosynthetic pathway of penicillin is almost completely elucidated (for review see [2,14,6,24]). One of the most striking features of this pathway is that it is partly compartmentalized, because the enzyme acetyl-CoA:isopenicillin *N* acyltransferase (IAT) [13,1,10] is localized in peroxisomes [20,28].

The gene for IAT, *pen*DE, encodes a protein of 357 amino acids. This approximately 40 kDa pre-protein is auto catalytically processed into two non-identical polypeptides of 102 and 255 amino acids (approx. 11 and 29 kDa, respectively) [19,16,15]. Enzymatically active IAT exists as a hetero-dimer of the 11 and 29 kDa peptides [29,17,21,10]. The 29 kDa subunit contains a typical C-terminal PTS1 peroxisomal targeting sequence, namely ARL-COOH in *P. chrysogenum* and ANI-COOH in *A. nidulans* [19,16,15]. When the ARL sequence in *P. chrysogenum* is removed, IAT is mislocalized to the

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cytosol and no penicillin is produced [15]. This implies that the peroxisomal localization of IAT is apparently essential for penicillin biosynthesis.

Studies on peroxisome biogenesis and proliferation in filamentous fungi were initiated in *A. nidulans* since this organism is readily accessible to classical and molecular genetic analyses [23,7,22]. Mutants were isolated that could not grow on media with carbon sources requiring functional peroxisomes (e.g. oleate). Only one mutant carried a mutation in a gene that seems to affect peroxisome biogenesis [7].

A more rapid and feasible approach to study the function of peroxisomes in β-lactam production is to use the power of yeast genetics. In yeast species like *Saccharomyces cerevisiae* and *Hansenula polymorpha* peroxisome proliferation and function can be readily prescribed by manipulation of the growth conditions. For both organisms various genes are characterized that play a crucial role in peroxisome biogenesis, maintenance and function (for review see [26]). However, to be able to use yeast as a model organism for studies on the role of peroxisomes in the β-lactam biosynthetic pathway, enzymes involved in this pathway first have to be introduced. In this paper, we report the expression of IAT in *H. polymorpha* as a first step towards the introduction of a new metabolic pathway in yeast.

2. Materials and methods

2.1. Strains, media and growth conditions

H. polymorpha NCYC495 *ade11.1 leu1.1* [9] and *pen*DE transformants derived from this strain were grown in batch cultures at 37 °C on YPD medium containing 1% yeast extract, 1% peptone and 1% glucose, on selective minimal media containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco) supplemented with 1% glucose (YND), or on mineral medium [25] supplemented with 0.5% (w/v) glucose or 0.5% (v/v) methanol. When needed leucine (20 mg l^{-1}) or adenine (30 mg l^{-1}) was added to the mineral media. For plates the media were supplemented with 1.5% (w/v) granulated agar. For cloning purposes, *Escherichia coli* DH5α was used and grown as described [18].

2.2. Expression of the P. chrysogenum penDE gene in H. polymorpha

The *P. chrysogenum pen*DE gene was amplified from the *P. chrysogenum* G cDNA library with the primers 5′-AGGAAGCTT**ATG**CTTCACATCCTCTGTC-3′ introducing a *Hin*dIII site immediately upstream of the start codon (in bold) and 5′-CCCGTCGAC**TCA**AAGCCTGGCGTTGAG-3′ introducing a *Sal*I site directly downstream of the stop codon (in bold). The amplified fragment was cloned using *Hin*dIII and *Sal*I into *Hin*dIII/*Sal*I digested pHIPX4 [8]. The final plasmid pHppenDE contains the *P. chrysogenum pen*DE gene behind the *H. polymorpha* alcohol oxidase promoter and *S. cerevisiae LEU*2 as a marker. It was linearized with *Sph*I for integration in the alcohol oxidase promoter and used to transform *H. polymorpha* NCYC495 *ade11.1 leu1.1*. Transformants were selected on YND plates containing adenine.

2.3. Biochemical methods

Crude extracts from *H. polymorpha* cells were prepared as described before [4]. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed by established procedures. Proteins on Western blots were detected using the chromogenic (NBT-BCIP) Western blotting kit (Roche Molecular Biochemicals, Almere, The Netherlands) after decoration with polyclonal antibodies against the *P. chrysogenum* penicillin biosynthesis enzymes (obtained from DSM, Delft, The Netherlands).

2.4. IAT activity

Whole cell lysates were prepared as described previously [27] in 50 mM HEPES buffer (pH 7.5) containing protease inhibitors (Complete™, Roche Molecular Biochemicals), 7.7 mM DTT and mM phenylacetyl-CoA. The reaction was started by adding 0.46 mM 6-aminopenicillanic acid (6-APA). The reaction was stopped by the addition of methanol. The formation of penicillin G was determined using HPLC.

2.5. Electron microscopy

Cells were fixed and prepared for electron microscopy as described previously [27]. Immunolabeling was performed on ultrathin sections of Unicryl-embedded cells, using specific antibodies against *P. chrysogenum* IAT and gold-conjugated goat-anti-rabbit antibodies (GAR-gold) [27].

3. Results

3.1. Introduction of the P. chrysogenum penDE gene in H. polymorrpha

From the three genes encoding the penicillin biosynthetic enzymes, the *pen*DE gene is the only one containing three introns. The gene was amplified from a *P. chrysogenum* cDNA library to eliminate these introns and subsequently cloned behind the methanol-inducible *H. polymorpha* alcohol oxidase promoter. The alcohol oxidase promoter sequence was used to integrate the

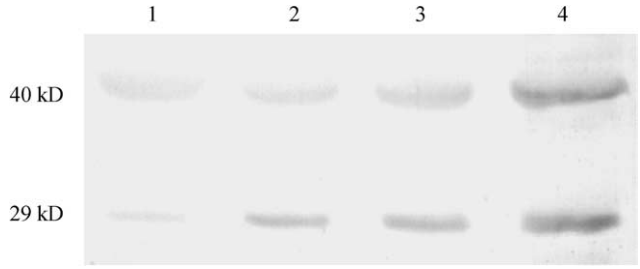

Fig. 1. Synthesis of IAT protein in methanol-grown *H. polymorpha* cells. Samples were collected, TCA-precipitated and subsequently used for the preparation of crude extracts. Equal amounts of protein were loaded per lane. Western blots were decorated using α-IAT antibodies. Data indicate that in cells containing a single copy (lane 1), two copies (lane 2), three copies (lane 3) or a multi-copy integration (lane 4), the IAT protein was synthesized, with the highest levels in the multi-copy integrant. In extracts of all transformants the 40 kDa pre-protein and the 29 kDa subunit were detected.

gene into the *H. polymorpha* genome, using *S. cerevisiae LEU*2 as marker. The presence of the gene in the *H. polymorpha* genome was analyzed by PCR (data not shown). Transformants with a single, two, three and multi-copy (>3) integrations were obtained.

*H. polymorpha pen*DE transformants were grown on methanol to induce the expression of the *pen*DE gene and crude cell extracts of these cells were analyzed by Western blotting, decorated with α-IAT antibodies (Fig. 1). The wild-type control strain showed no cross-reaction with these antibodies whereas in extracts of the transformants 1–4 (lanes 2–5) two distinct protein bands were detected. The highest band represents the 40 kDa IAT pre-protein, the lower band the 29 kDa subunit. The α-IAT antibodies do not cross react with the 11 kDa subunit. The difference in protein levels between the various transformants reflects the difference in copy number (1, 2, 3 and multi-copy).

3.2. IAT is enzymatically active in H. polymorpha

To determine if the heterologous produced IAT was enzymatically active, crude cell extracts were prepared from cells of the *H. polymorpha pen*DE multi-copy transformants grown on methanol. After adding phenyl acetyl-CoA (PA-CoA) and 6-aminopenicillanic acid (6-APA) to the cell lysate, the formation of penicillin G was determined. The specific IAT enzyme activity detected in the multi-copy transformant amounted to 11.8 nmol mg^{-1} min^{-1}. The IAT activity detected in crude extracts of a *P. chrysogenum* strain that produces high levels of penicillin is 0.72 nmol mg^{-1} min^{-1} [3].

3.3. IAT is localized to peroxisomes in H. polymorpha

To localize IAT in *H. polymorpha pen*DE transformants, immunocytochemistry has been performed. Analysis of ultrathin sections of methanol-grown cells, labeled using α-IAT antibodies and GAR-gold, showed that the α-IAT specific labeling is exclusively located on peroxisome profiles (Fig. 2). The IAT protein appears to cluster outside of the alcohol oxidase crystal. Using ultrathin sections of wild-type *H. polymorpha* cells no labeling could be detected (data not shown), demonstrating that the labeling is specific for *P. chrysogenum* IAT.

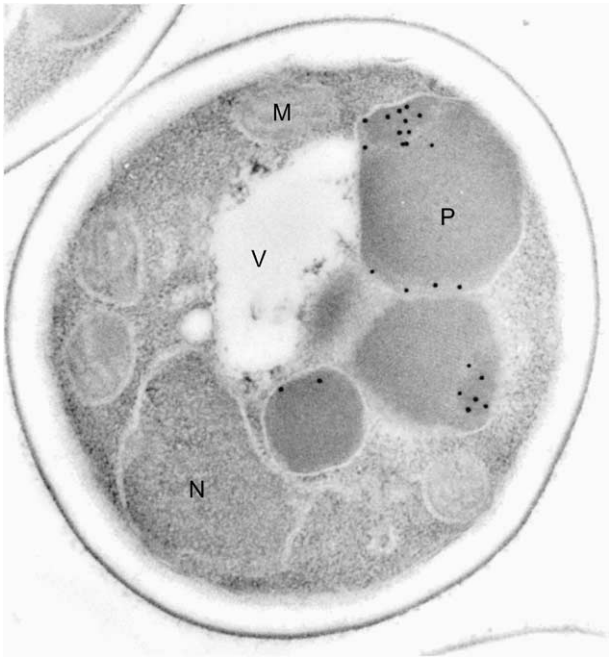

Fig. 2. Immunocytochemical localisation of IAT in *H. polymorpha pen*DE. The *H. polymorpha pen*DE transformant with the highest IAT level was grown on methanol. α-IAT-dependent labelling was localized exclusively on the peroxisome profiles. *P*, peroxisome; *V*, vacuole; *M*, mitochondrion; *N*, nucleus.

4. Discussion

In this paper, we describe the introduction and expression of the *P. chrysogenum pen*DE gene, encoding the third enzyme in the penicillin biosynthetic pathway IAT, into the yeast *H. polymorpha*. This is an initial step towards the introduction of a new metabolic pathway in *H. polymorpha* in order to facilitate research on the precise role of peroxisomes in this pathway.

Muller et al. [15] have demonstrated that peroxisomes are essential for the biosynthesis of penicillin. However, as yet still little is known about the precise role of the organelles in β-lactam biosynthesis. Obtaining mutants of filamentous fungi that are affected in peroxisome biogenesis is laborious and difficult. For *A. nidulans* it has been shown that cells of this fungus can grow on oleic acid as sole carbon source, a process associated with peroxisome proliferation [23]. De Lucas et al. [7] isolated a series of *A. nidulans* mutants that were not able to

grow on oleic acid as sole carbon source, but only one of these mutants displayed a phenotype that resembled peroxisomal protein import-defective mutants of *H. polymorpha*. In these mutants, only few peroxisomes were present, representing only 0.9% of the cytoplasmic volume compared to 4.5% in wild-type cells. The peroxisomal matrix protein catalase was localized in these peroxisomes, whereas other matrix proteins, 3-hydroxyacetyl-CoA dehydrogenase and isocitrate lyase, were mislocalized to the cytosol. Although peroxisome biogenesis seems to be affected in this mutant, the penicillin production rate was comparable to that of the wild-type control. This could mean that in *A. nidulans* the role of peroxisomes in penicillin biosynthesis is not essential and that *A. nidulans* IAT is functional in the cytosol. On the other hand, the *A. niger* mutant may have only been defective for 3-hydroxyacetyl-CoA dehydrogenase and isocitrate lyase import. Since these enzymes are essential for growth on oleic acid but not for penicillin biosynthesis, this may explain why the ole$^-$ strains still produce penicillin. Alternatively, a small portion of IAT is correctly located in peroxisomes and is sufficient to produce wild-type levels of penicillin in *A. nidulans*.

A second approach to study the role of peroxisomes in penicillin biosynthesis is to disrupt PEX genes that are involved in the biogenesis of these organelles. [11,12] have cloned several *P. chrysogenum* PEX genes (*PEX1 PEX5* and *PEX6*). However, despite numerous attempts, it was not possible to obtain stable disruption mutants. Probably, in fungi peroxisomes/peroxins are also essential for caryogamy [5].

Taken together, severe blocks in peroxisome biogenesis in filamentous fungi may lead to undesirable side effects, eventually resulting in non-viable cells. Yeast species like *H. polymorpha* or *S. cerevisiae* would be perfect model organisms for studying the role of peroxisomes, due to availability of many viable mutants that are affected in peroxisome biogenesis. However, these organisms lack the β-lactam biosynthetic pathway.

Of the three penicillin biosynthesis enzymes, most problems could be expected with the introduction of IAT in a host organism other than *P. chrysogenum* and *A. nidulans*. Firstly, the *pen*DE gene encoding IAT contains three introns. To introduce the gene in *H. polymorpha* we amplified it from a cDNA library to prevent problems with the splicing. However, for some genes the removal of their natural introns somehow blocks the exit of functional mRNA products to the cytosol. Since we observed production of the IAT protein, the absence of the introns does not interfere with the expression of the *pen*DE gene in *H. polymorpha*.

IAT is only enzymatically active as a hetero-dimer of the 11 and 29 kDa subunits, products of the autocatalytic processing of the 40 kDa pre-protein. It has already been shown that this autocatalytic processing also occurs when *pen*DE is heterologously expressed in *E. coli* and *Cephalosporium acremonium,* and when it is transcribed/translated in vitro [29,17,21]. Indeed, also in *H. polymorpha* the protein is processed. Compared to *P. chrysogenum*, where the 40 kDa pre-protein can hardly be detected, the processing in *H. polymorpha* seems to be less efficient since still a prominent amount of the protein is present as the 40 kDa pre-protein. The same had also been shown for the expression in *E. coli* [17,21]. Factors like growth temperature and internal pH might be of influence on the efficiency of processing. In this respect, it may be relevant to mention that the acidic internal pH of *H. polymorpha* peroxisomes (pH 5.5–6.0) is low relative to the value reported for *P. chrysogenum* (pH 7.5).

Finally, the localization of IAT in *H. polymorpha* was peroxisomal, as it is in *P. chrysogenum*, and IAT activity was detected in crude cell lysate of the *H. polymorpha pen*DE multi-copy transformant. This activity is approximately 16 times enhanced relative to the activity detected in crude extracts of a *P. chrysogenum* strain that produces high levels of penicillin. This implies that in the *H. polymorpha* transformant a higher ratio of produced IAT to total protein content is obtained.

Hence, this implies that we have successfully introduced *P. chrysogenum* IAT into *H. polymorpha*, a first step towards the introduction of a heterologous metabolic route for penicillin biosynthesis into the yeast *H. polymorpha*. Introduction of the two other key enzymes of penicillin biosynthesis, ACVS and IPNS, will allow studies of the function of peroxisomes in penicillin biosynthesis in *H. polymorpha.*

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