Reprogramming *Hansenula polymorpha* for penicillin production: expression of the *Penicillium chrysogenum pcl* gene

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Received 18 November 2006; revised 2 February 2007; accepted 3 February 2007. First published online 10 April 2007. DOI:10.1111/j.1567-1364.2007.00228.x

**Editor:** Gerd Gellissen

**Keywords**

β-lactam antibiotics; filamentous fungi; genetic engineering; microbody; PTS1 receptor.

**Abstract**

We aim to introduce the penicillin biosynthetic pathway into the methylotrophic yeast *Hansenula polymorpha*. To allow simultaneous expression of the multiple genes of the penicillin biosynthetic pathway, additional markers were required. To this end, we constructed a novel host–vector system based on methionine auxotrophy and the *H. polymorpha* MET6 gene, which encodes a putative cystathionine β-lyase. With this new host–vector system, the *Penicillium chrysogenum pcl* gene, encoding peroxisomal phenylacetyl-CoA ligase (PCL), was expressed in *H. polymorpha*. PCL has a potential C-terminal peroxisomal targeting signal type 1 (PTS1). Our data demonstrate that a green fluorescent protein–PCL fusion protein has a dual location in the heterologous host in the cytosol and in peroxisomes. Mutation of the PTS1 of PCL (SKI-COOH) to SKL-COOH restored sorting of the fusion protein to peroxisomes only. Additionally, we demonstrate that peroxisomal PCL–SKL produced in *H. polymorpha* displays normal enzymatic activities.

**Introduction**

Penicillin and its derivatives are the oldest known chemotherapeutic agents that are used against various bacteria. Penicillin belongs to the β-lactam family of antibiotics. The penicillin biosynthetic pathway in the filamentous fungus *Penicillium chrysogenum* has been well characterized both genetically and biochemically (Liras & Martin, 2006). In *P. chrysogenum*, this pathway is partly compartmentalized, and involves the action of the nonribosomal peptide synthetase δ-(1-α aminoacidyl)-l-cysteinyl-d-valine synthase and isopenicillin-N-synthase, which are present in the cytosol. These enzymes catalyze the first steps in β-lactam biosynthesis. In addition, the enzymes required in the final steps in penicillin biosynthesis, acyl-CoA:isopenicillin-N-acyltransferase (IAT) and phenylacetyl-CoA ligase (PCL), are located in microbodies (reviewed by van de Kamp et al., 1999).

Microbodies (peroxisomes, glyoxysomes, glycosomes, Woronin bodies; in the remainder of the text, designated peroxisomes) are important organelles that are present in all eukaryotic cells. These organelles contain a protein-rich matrix consisting of enzymes involved in highly diverse metabolic pathways, such as plasmalogens biosynthesis in mammals, photorespiration in plants, glycolysis in trypanosomes, the primary metabolism of various unusual carbon sources, as well as penicillin biosynthesis in certain filamentous fungi (van den Bosch et al., 1992).

We aim to introduce the penicillin biosynthetic pathway in the methylotrophic yeast *Hansenula polymorpha*. Yeast species have the advantage of being versatile, and easy to handle and cultivate. The use of a yeast model system provides information on the actual requirements of the penicillin biosynthetic pathway. Additionally, it enables easy manipulation of the penicillin biosynthetic pathway for the production of novel antibiotics. *Hansenula polymorpha* has been successfully developed as a host for the production of foreign proteins (Hollenberg & Gellissen, 1997). *Hansenula polymorpha* has the added advantage that the number and volume fraction of peroxisomes can be readily regulated, allowing better control over peroxisomal enzymes and their function (van der Klei et al., 1991).

Proper sorting of the peroxisome-borne enzymes IAT and PCL to the correct destination in the yeast host is of crucial importance in this metabolic reprogramming scheme. This has already been achieved for *P. chrysogenum* IAT. When produced in *H. polymorpha*, IAT is correctly sorted to peroxisomes and functionally active (Lutz et al., 2005). In the current work, we introduced the *P. chrysogenum pcl* gene
Expression of \textit{P. chrysogenum} pcl in \textit{H. polymorpha} using a novel host–vector system, and determined the subcellular location and activity of the heterologously produced protein.

**Materials and methods**

**Microorganisms and growth conditions**

The strains used in this study are listed in Table 1. All \textit{H. polymorpha} strains are derivatives of NCYC495 (Gleeson & Sudbery, 1988), and were grown at 37 °C in either (1) rich complex media (YPD) containing 1% yeast extract, 1% peptone and 1% glucose, (2) selective media containing 0.67% yeast nitrogen base without amino acids (DIFCO) supplemented with 0.5% glucose (YND) or 0.5% methanol (YNM), or (3) mineral medium (MM) as described by Van Dijken et al. (1976), supplemented with 0.25% ammonium sulfate, using 0.5% glucose or 0.5% methanol as carbon source. For growth on plates, 2% granulated agar was added to the media. Whenever necessary, media were supplemented with 30 μg mL\(^{-1}\) leucine, 30 μg mL\(^{-1}\) uracil, 20 μg mL\(^{-1}\) adenine, and 20 μg mL\(^{-1}\) methionine. For biochemical analysis, selected strains were pre-cultured for at least three rounds in MM containing glucose, and subsequently shifted to MM containing methanol to induce expression of genes under the control of the alcohol oxidase (AO) promoter.

To obtain a fourfold auxotrophic \textit{H. polymorpha} strain, we crossed NCYC495 ade11.1 leu1.1 ura3 (Haan et al., 2002) with NCYC495 ade11.1 leu1.1 met6, using the procedure described by Gleeson & Sudbery (1988). This resulted in the isolation of strain NCYC495 ade11.1 leu1.1 met6 ura3.

**Miscellaneous DNA techniques**

The plasmids and primers used in this study are listed in Tables 1 and 2, respectively. All DNA manipulations were carried out according to standard methods (Sambrook et al., 1989). Hansenula polymorpha cells were transformed by electroporation (Faber et al., 1994). Chromosomal DNA was extracted from YPD-grown \textit{H. polymorpha} cells as described by Sherman et al. (1986), but included an additional protein precipitation step using 5 M sodium chloride prior to DNA precipitation. DNA-modifying enzymes were used as recommended by the supplier (Roche, Almere, the Netherlands).

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Genotype/description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>\textit{E. coli}</td>
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<td></td>
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<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169Δ(aΔ80lacZAM15)</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td></td>
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<tr>
<td>\textit{H. polymorpha}</td>
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<tr>
<td>NCYC495 ade11.1 leu1.1 ura3</td>
<td>Adenine, leucine and uracil auxotrophic strain</td>
<td>Haan et al. (2002)</td>
</tr>
<tr>
<td>NCYC495 ade11.1 leu1.1 met6</td>
<td>Adenine, leucine and methionine auxotrophic strain</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>NCYC495 ade11.1 leu1.1 ura3 met6</td>
<td>Segregant of cross between NCYC495 ade11.1 leu1.1 ura3 and NCYC495 ade11.1 leu1.1 met6</td>
<td>This study</td>
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<tr>
<td>\textit{P. chrysogenum}</td>
<td></td>
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<tr>
<td>DS17690</td>
<td>High penicillin-producing strain</td>
<td>DSM antiinfectives, Delft, the Netherlands</td>
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<tr>
<td>\textit{Plasmids}</td>
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<tr>
<td>pYT3-MET6</td>
<td>Plasmid complementing the methionine auxotrophy of \textit{H. polymorpha} ade11.1 leu1.1 met6; amp^R</td>
<td>This study</td>
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<tr>
<td>pBluescript II SK^+</td>
<td>\textit{E. coli} cloning vector; amp^R</td>
<td>Stratagene Inc., San Diego, CA</td>
</tr>
<tr>
<td>pHIPX4</td>
<td>\textit{H. polymorpha} integrating plasmid; Sc-LEU2; kan^R</td>
<td>Gietl et al. (1994)</td>
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<tr>
<td>pHIPX4-HNBE SX</td>
<td>Derivative of pHIPX4 with alternative polylinker; Sc-LEU2; kan^R</td>
<td>Laboratory collection</td>
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<tr>
<td>pSKM66</td>
<td>pBluescript II SK^+ containing the \textit{H. polymorpha} MET6 gene; amp^R</td>
<td>This study</td>
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<tr>
<td>pHIPM4</td>
<td>\textit{H. polymorpha} integrating plasmid; Hp-MET6; kan^R</td>
<td>This study</td>
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<td>pHIPM4-PCL</td>
<td>pHIPM4 containing \textit{P. chrysogenum} pcl; Hp-MET6; kan^R</td>
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<tr>
<td>pHIPM4-GFP-PCL</td>
<td>pHIPM4 containing fusion gene between GFP and \textit{P. chrysogenum} pcl; Hp-MET6; kan^R</td>
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<td>This study</td>
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<td>pHIPM4-GFP-PCL-SKL</td>
<td>pHIPM4 containing fusion gene between GFP and \textit{P. chrysogenum} pcl.SKL; Hp-MET6; kan^R</td>
<td>This study</td>
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The novel *H. polymorpha* integration vector pHIPM4 was constructed as follows. First, a 2.0-kb DNA fragment comprising the *H. polymorpha* MET6 gene, including its regulatory sequences, was isolated by PCR with primers MET6F and MET6R (Table 2), using pYT3-MET6 as template, and cloned into SmaI-digested pBluescript II SK+-. From the resulting plasmid, designated pSKMET6, a 2.0-kb BamHI fragment containing HpMET6 was isolated and cloned into BglII-digested pHIPX4-HNBEX [a derivative of pHIPX4 (Gietl et al., 1994)], thereby replacing the ScLEU2 marker in this plasmid. The resulting plasmid was designated pHIPM4 (Fig. 2).

For the construction of plasmid pHIPM4-PCL, a BamHI site was introduced upstream of the *P. chrysogenum* pcl gene by PCR with primers PCL-1F and PCL-3R, using DNA from a *P. chrysogenum* cDNA library (Kiel et al., 2000) as template. The resulting 1.7-kb DNA fragment was digested with BamHI and cloned between the HindIII and NcoI sites of pHIPM4, resulting in plasmid pHIPM4-PCL. The resulting plasmid, designated pHIPM4-GFP.PCL, contains an in-frame GFP–pcl fusion gene.

To enable optimization of the targeting of PCL to peroxisomes, we mutagenized the PTS1 signal of PCL (SKI-COOH) into SKL-COOH. To this end, the *P. chrysogenum* pcl gene was amplified by PCR with primers PCL-1F and PCL-4R, using pHIPM4-PCL as template. The resulting 1.7-kb DNA fragment, containing the pcl.SKL coding sequence, was inserted between the BamHI and SmaI sites of pHIPM4, resulting in plasmid pHIPM4-PCL.SKL. In addition, the same fragment was inserted between the BamHI and SmaI sites of pHIPM4-GFP.PCL, resulting in plasmid pHIPM4-GFP.PCL.SKL.

Integration of pHIPM4-derived plasmids into the MET6 locus of the *H. polymorpha* genome was achieved by transforming EcoRI-linearized plasmid DNA. Correct integration and copy number determination was analyzed by Southern blotting (data not shown).

**Biochemical methods**

Crude extracts of *H. polymorpha* and *P. chrysogenum* cells were prepared with glass beads basically as described by Waterham *et al.* (1994), using TANG buffer (50 mM Tris-HCl, pH 7.5, 0.02% sodium azide, 200 mM NaCl, 10% glycerol) instead of phosphate buffer. For Western blots, extracts of *H. polymorpha* and *P. chrysogenum* cells were prepared using the trichloroacetic acid (TCA) method (Baerends *et al.*, 2000). Protein concentrations were determined using the Bio-Rad Protein Assay system and bovine serum albumin as standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting were performed using established procedures. Western blots were decorated with polyclonal antibodies raised in rabbit against a maltose binding protein (MBP)–PCL fusion protein produced in *E. coli* (a gift of M. Koetsier, Biochemical Laboratory, GBB, University of Groningen, the Netherlands).

**Determination of PCL enzymatic activity**

PCL activity was determined in crude cell extracts by measuring the rate of conversion of cinnamic acid to cinnamoyl-CoA at 30 °C, following the procedure of M. Koetsier *et al.* (unpublished results).

**Morphologic analysis**

Fluorescence microscopy studies were performed using a Zeiss Axioskop microscope (Carl Zeiss, Göttingen, Germany).

**Results**

**Cloning of *H. polymorpha* MET6**

Introduction of the *P. chrysogenum* penicillin biosynthetic pathway into *H. polymorpha* involves expression of the multiple *P. chrysogenum* genes of this pathway (Brakhage, 2007 Federation of European Microbiological Societies Published by Blackwell Publishing Ltd. All rights reserved
amino acids with a predicted molecular mass of 42 kDa. From a methionine prototrophic transformant, a plasmid with an insert of 6.1 kb was rescued in 1995). From this methionine auxotrophic strain, we transformed H. polymorpha MET6 with an integrated vector, designated pHIPM4 (Fig. 2). This vector contains a single copy of the Pcl expression cassette, designated PclIC, was analyzed further. Western blots, using crude extracts prepared from methanol-grown cells, decorated with α-PCL antibodies, demonstrated (Fig. 3, lanes 1–3) that strain PclIC produces high levels of a protein identical in size to P. chrysogenum Pcl that was absent in wild-type controls. From this, we conclude that the novel host–vector system enables efficient production of heterologous proteins.

Localization of P. chrysogenum PCL in H. polymorpha

In P. chrysogenum, PCL localizes to peroxisomes (W.H. Meijer et al., unpublished results), probably via its PTS1 signal, SKI-COOH. In order to determine the subcellular location of PCL in H. polymorpha, we constructed strain GFP.PCL expressing a PAOX-driven GFP–pel fusion gene. Methanol-grown cells of this strain were analyzed by fluorescence microscopy. The data (Fig. 4a and b) indicate that in H. polymorpha, GFP–PCL has a dual localization. GFP fluorescence was observed in peroxisomes as a characteristic rim that surrounds the AO crystal (Veenhuis et al., 2000), but was also detected in the cytosol. To sort PCL completely to peroxisomes of H. polymorpha, the native PTS1 signal of PCL (SKI-COOH) was changed to SKI-COOH, which is a more efficient PTS1 signal (Reumann, 2004). In the resulting strain, GFP.PCLSKL, GFP fluorescence was confined to peroxisomes (Fig. 4c and d).

PCLSKL produced in H. polymorpha is active

For enzymatic activity determinations, the pclSKL gene was inserted into plasmid pHIPM4 and integrated with varying copy numbers into H. polymorphaNCYC495 ade11.1 leu1.1 ural3 met6. Strains containing one, two and multiple (more than three) copies of the expression cassette were selected. Western blot analysis demonstrated that these strains produced enhanced levels of PCLSKL protein, corresponding to the number of expression cassettes integrated (Fig. 3, lanes 4–6). PCL is involved in the activation of aromatic monocarboxylic acids by covalently adding CoA to the substrate (Ward & KE, 2005). We determined the enzymatic activity of PCLSKL oxidase terminator (TAMO). Integration of pHIPM4 into the H. polymorpha genome can be facilitated by linearization in either the PAOX or MET6 regions.

To fully explore the potential of pHIPM4, a fourfold auxotrophic strain, NCYC495 ade11.1 leu1.1 ural3 met6, was constructed. To demonstrate the use of this new host–vector system in metabolic engineering of H. polymorpha, we inserted the gene encoding P. chrysogenum PCL downstream from the PAOX in pHIPM4. The resulting plasmid, pHIPM4-PCL, was then integrated at the MET6 locus of strain NCYC495 ade11.1 leu1.1 ural3 met6. Initially, a strain containing a single copy of the PCL expression cassette, designated PclIC, was analyzed further. Western blots, using crude extracts prepared from methanol-grown cells, decorated with α-PCL antibodies, demonstrated (Fig. 3, lanes 1–3) that strain PclIC produces high levels of a protein identical in size to P. chrysogenum PCL that was absent in wild-type controls. From this, we conclude that the novel host–vector system enables efficient production of heterologous proteins.

Construction of an H. polymorpha strain producing P. chrysogenum PCL

We utilized the H. polymorpha MET6 gene with its endogenous promoter and terminator regions as auxotrophic marker in the construction of a novel H. polymorpha integration vector, designated pHIPM4 (Fig. 2). This vector allows high-level expression of genes by the strong, inducible AO promoter (PAOX), and also contains the amine
in methanol-grown cells of strains PCLSKL,1c, PCLSKL,2c and PCLSKL,mc with cinnamic acid as substrate, using H. polymorpha host cells and P. chrysogenum DS17690 cells as controls. The results (Table 3) indicated that crude extracts of the recombinant H. polymorpha strains displayed PCL enzymatic activity levels that increased with increasing copy numbers of the pclSKL expression cassette. PCL enzymatic activity was absent in the control host strain. The PCL activities detected in the H. polymorpha recombinant strains were enhanced relative to the activities in P. chrysogenum cells.

Discussion
Penicillin is normally produced by the filamentous fungus P. chrysogenum. Introduction of the penicillin biosynthetic pathway into a yeast species is beneficial for overcoming the...
difficulties of using *P. chrysogenum* in bulk fermentations, avoiding strain instabilities, reducing intracellular proteases, etc. The methylotrophic yeast *H. polymorpha* has been successfully developed as a host for the production of foreign proteins (Gellissen & Hollenberg, 1997). High-level protein production is facilitated by the availability of strong, inducible promoters (P\textsubscript{AOX}, P\textsubscript{FMD}) (van Dijk et al., 2000; Gellissen & Veenhuis, 2001). We aimed to utilize the favorable properties of *H. polymorpha*, via metabolic reprogramming, for synthesis of β-lactam antibiotics.

Penicillin biosynthesis requires the activity of multiple enzymes that are not available in *H. polymorpha*. To enable the use of multiple expression cassettes in this yeast, we first constructed a new host–vector system that consisted of an *H. polymorpha* strain carrying four auxotrophic markers and the novel integration vector pHIPM4, which contains the *H. polymorpha* MET6 gene. This novel host–vector system provides an additional tool for efficient metabolic engineering of *H. polymorpha*. Its suitability was demonstrated by the efficient production of *P. chrysogenum* PCL.

Our data indicate that the new vector can integrate in single and multiple copies into the *H. polymorpha* genome.
Southern blot analysis has shown that among 32 randomly picked transformants, in 22 cases the expression cassette had correctly integrated into the \textit{MET}6 locus. Of these, 64\% contained one copy, 18\% two copies and 18\% multiple copies (three or more) of the expression cassette (data not shown). Thus, integration of varying copies of pHIPM4-derivatives allows efficient regulation of the expression level of a heterologous gene, as demonstrated by our expression data for \textit{pcl–SKL} (Table 3 and Fig. 3).

In \textit{P. chrysogenum}, the last steps in penicillin production, side chain activation and attachment of the activated side chain to the \ellactam backbone, are performed by the peroxisomal enzymes PCL and IAT, respectively (Muller \textit{et al.}, 1992; Lamas-Maceiras \textit{et al.}, 2006). Both proteins contain a PTS1 (PCL, SKI-COOH; IAT, ARL-COOH), indicating that sorting to \textit{P. chrysogenum} peroxisomes requires the PTS1 receptor, Pex5p (Holroyd \& Erdmann, 2001). Previously, we have demonstrated that IAT produced in \textit{H. polymorpha} completely localizes to peroxisomes (Lutz \textit{et al.}, 2005), implying efficient recognition of its PTS1 by \textit{H. polymorpha} Pex5p. In contrast, our localization studies of GFP–PCL provide evidence that this protein is not efficiently sorted to peroxisomes of \textit{H. polymorpha}. In \textit{P. chrysogenum}, PCL completely localizes to peroxisomes (W.H. Meijer \textit{et al.}, unpublished results). This suggests that \textit{P. chrysogenum} Pex5p binds the PTS1 of PCL much more effectively than its \textit{H. polymorpha} ortholog. Such differences in recognition of PTS1 sequences by different Pex5 proteins have been observed before (van der Klei \textit{et al.}, 1995; Neuberger \textit{et al.}, 2003). Alternatively, endogenously produced PCL may contain (an) additional binding site(s) for \textit{P. chrysogenum} Pex5p that enables its efficient sorting to \textit{P. chrysogenum} peroxisomes, and that is not recognized by \textit{H. polymorpha} Pex5p. An analogous situation was observed in an \textit{H. polymorpha} pex5 strain producing \textit{P. chrysogenum} Pex5p (Kiel \textit{et al.}, 2004). In this study, \textit{P. chrysogenum} Pex5p was unable to sort endogenous AO into peroxisomes, whereas other PTS1 proteins (including GFP–SKL) were efficiently imported. Presumably, \textit{P. chrysogenum} Pex5p does not recognize the highly efficient, alternative PTS that resides in the AO molecule (Gunkel \textit{et al.}, 2004; Kiel \textit{et al.}, 2004). Thus, both PTS1 receptors have certain unique features. Nevertheless, efficient sorting of PCL to peroxisomes of \textit{H. polymorpha} could be established when the PTS1 signal of the protein was changed to the canonical SKL-COOH. This confirms that the low efficiency of targeting of PCL in \textit{H. polymorpha} was not caused by significant structural changes in the C-terminus of the protein that may have precluded binding of \textit{H. polymorpha} Pex5p.

Our data show that \textit{H. polymorpha} strains containing varying copies of the \textit{pcl–SKL} expression cassette may produce significantly higher levels of enzymatic activity than observed in \textit{P. chrysogenum}. Previously, it was shown that overproduction of PCL in \textit{P. chrysogenum} significantly increased penicillin production (Lamas-Maceiras \textit{et al.}, 2006). This suggests that the endogenous levels of PCL in \textit{P. chrysogenum} may not be optimal for efficient penicillin production. In \textit{H. polymorpha}, such an optimization of PCL–SKL levels can easily be obtained, because multiple expression systems are available.

In conclusion, our data demonstrate that, in addition to \textit{P. chrysogenum} IAT, PCL–SKL can also be produced in \textit{H. polymorpha} in a functionally active form, and is properly targeted to peroxisomes. This provides new possibilities for the metabolic reprogramming of \textit{H. polymorpha} to produce penicillin.

Acknowledgements

L. Gidijala and J.A.K.W. Kiel are financially supported by the Netherlands Ministry of Economic Affairs and the B-Basic partner organizations (www.b-basic.nl) through B-Basic, a public–private NWO-ACTS programme (ACTS, Advanced Chemical Technologies for Sustainability). We thank Martijn Koetsier for the gift of \alpha-MBP–PCL antibodies. We gratefully acknowledge Martijn Koetsier and Elena Kurbatova for expert technical assistance.

References


