Metabolic engineering of β-oxidation in *Penicillium chrysogenum* for improved semi-synthetic cephalosporin biosynthesis

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**A B S T R A C T**

Industrial production of semi-synthetic cephalosporins by *Penicillium chrysogenum* requires supplementation of the growth media with the side-chain precursor adipic acid. In glucose-limited chemostat cultures of *P. chrysogenum*, up to 88% of the consumed adipic acid was not recovered in cephalosporin-related products, but used as an additional carbon and energy source for growth. This low efficiency of side-chain precursor incorporation provides an economic incentive for studying and engineering the metabolism of adipic acid in *P. chrysogenum*. Chemostat-based transcriptome analysis in the presence and absence of adipic acid confirmed that adipic acid metabolism in this fungus occurs via β-oxidation. A set of 52 adipate-responsive genes included six putative genes for acyl-CoA oxidases and dehydrogenases, enzymes responsible for the first step of β-oxidation. Subcellular localization of the differentially expressed acyl-CoA oxidases and dehydrogenases revealed that the oxidases were exclusively targeted to peroxisomes, while the dehydrogenases were found either in peroxisomes or in mitochondria. Deletion of the genes encoding the peroxisomal acyl-CoA oxidase Pc20g01800 and the mitochondrial acyl-CoA dehydrogenase Pc20g07920 resulted in a 1.6- and 3.7-fold increase in the production of the semi-synthetic cephalosporin intermediate adipoyl-6-APA, respectively. The deletion strains also showed reduced adipate consumption compared to the reference strain, indicating that engineering of the first step of β-oxidation successfully redirected a larger fraction of adipic acid towards cephalosporin biosynthesis.

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1. Introduction

The filamentous fungus *Penicillium chrysogenum* is the major industrial producer of penicillin antibiotics. Metabolic engineering has expanded the product range of *P. chrysogenum* to include other β-lactams. In particular, expression of heterologous genes has enabled the production of industrially relevant precursors for the production of semi-synthetic cephalosporins, such as adipoyl-7-aminocephalosporanic acid (ad-7-ADCA) and adipoyl-7-amino-3-carbamoyloxyethyl-3-cephem-4-carboxylic acid (ad-7-ACCCA) (Crawford et al., 1995; Harris et al., 2009b; Robin et al., 2003; Thykaer et al., 2002). These cephalosporin precursors are produced as adipoylated molecules and, during their production, adipic acid (hexane-1,6-dioic acid) has to be added to growth media. After activation of adipic acid by a peroxisomally located acyl-CoA ligase (Koetsier et al., 2010), the formed adipoyl-CoA replaces the α-amino-adipate moiety of isopenicillin N in a reaction catalyzed by the isopenicillin N acyltransferase encoded by the penDE gene. Adipoyl-6-aminopenicillanic acid (ad-6-APA) then enters the engineered cephalosporin pathway.

In practice, not all adipic acid added to culture media is used as a side-chain precursor for the production of cephalosporin molecules. For example, in a quantitative analysis of an engineered
ad-7-ACCCA producing \textit{P. chrysogenum} strain, only 12% of the adipic acid consumed by the cultures could be recovered as ad-7-ACCCA and adipoylated cephalosporin intermediates. The remaining 88% was used as an additional energy source for fungal growth (Harris et al., 2009b). 13C-labeling studies (Thykaer et al., 2002) and genome-wide expression profiling (Harris et al., 2009b) suggested that adipic acid catabolism in \textit{P. chrysogenum} occurred via β-oxidation. However, the genes involved in this pathway have not been functionally analyzed and an understanding of the mechanism of adipic acid degradation in \textit{P. chrysogenum} is needed to redirect adipic acid metabolism towards product formation by metabolic engineering.

In nature, dicarboxylic acids derive from ω-oxidation, which together with β-oxidation, is responsible for the metabolism of medium and (very) long chain monocarboxylic fatty acids in mammals (Ferdinandusse et al., 2004; Jin and Tserng, 1989; Pettersen and Stokke, 1973; Sanders et al., 2006; Verkade and Van Der Lee, 1934). The dicarboxylic acids formed in ω-oxidation can, subsequently, act as energy sources (Maggio-Hall and Keller, 2004). In the fungus \textit{Aspergillus nidulans} metabolism has been demonstrated in the fungus \textit{A. nidulans}. Their metabolism starts with activation to the corresponding dicarboxyloyl-CoA, followed by chain shortening of the resulting CoA ester via ω-oxidation, using the same set of reactions as those involved in β-oxidation of monocarboxylic acids (Ferdinandusse et al., 2004; Suzuki et al., 1989). β-oxidation yields acetyl-CoA and short-chain dicarboxyloyl-CoA compounds such as succinyl-CoA (C\textsubscript{4}), which can enter central metabolism, and adipoyl-CoA (C\textsubscript{6}) (Hiltunen and Qin, 2000). In humans, adipic acid is excreted in urine, but several studies demonstrate that both rats and humans are capable of further metabolizing adipoyl-CoA via β-oxidation, yielding acetyl-CoA and succinyl-CoA (Bates et al., 1991; Bates, 1989, 1990; Rusoff et al., 1960; Svendsen et al., 1985).

In higher eukaryotes, β-oxidation of monocarboxylic fatty acids occurs in peroxisomes as well as in mitochondria. A similar compartmentation has been demonstrated in the fungus \textit{Aspergillus nidulans} (Maggio-Hall and Keller, 2004) but in Saccharomyces cerevisiae, ω-oxidation exclusively occurs in peroxisomes (Trotter, 2001). An important difference between peroxisomal and mitochondrial β-oxidation pathways concerns the first reaction step. In mitochondria the initial oxidation step, which results in the introduction of a double bond into the acyl-CoA compound, thereby forming a trans-2-enoyl-CoA, is catalyzed by an FAD-linked acyl-CoA dehydrogenase, while FarB is predominantly controlling the metabolism of short chain fatty acid catabolism, while FarB is predominantly controlling the metabolism of short chain fatty acids (Hynes et al., 2006; Kiel and van der Klei, 2009; Reiser et al., 2010).

The goal of the present study was to investigate the pathway of adipic acid metabolism in \textit{P. chrysogenum}, to identify target genes for metabolic engineering strategies to reduce adipic acid degradation and to quantitatively analyze adipic acid degradation and cephalosporin production by strains in which key genes in adipic acid metabolism have been deleted. Identification of target genes for metabolic engineering was based on a transcriptome analysis of chemostat cultures grown in the absence and presence of adipic acid.

### 2. Materials and methods

#### 2.1. Strains

\textit{P. chrysogenum} strains used in this study are listed in Table 1. The penicillin high producing strain DS17690 (Harris et al., 2006; Harris et al., 2009a; Kleijn et al., 2007; Nasution et al., 2006; Zhao et al., 2008) resulted from the DSM (DSM-Anti-Infectives, Delft, Netherlands), which was used as the parental strain for metabolic engineering.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS17690</td>
<td>High penicillin producer</td>
<td>(Harris et al., 2009a)</td>
</tr>
<tr>
<td>Wisconsin54-1255/ATCC28089</td>
<td>Ancestor of DS17690</td>
<td>(Mac Donald et al., 1964)</td>
</tr>
<tr>
<td>DS64465</td>
<td>hdfA\textsubscript{1}</td>
<td>(Snoek et al., 2009)</td>
</tr>
<tr>
<td>DS50061</td>
<td>penDE::eGFP::{pachB-\text{pachC}-penDE}\textsubscript{4}</td>
<td>(Harris et al., 2009a)</td>
</tr>
<tr>
<td>DS49884</td>
<td>pch\textsubscript{G}-cefET\textsubscript{1}, pch\textsubscript{G}-cmcHI\textsubscript{1}-penDE\textsubscript{1}</td>
<td>(Harris et al., 2009b)</td>
</tr>
<tr>
<td>DsRed\textsubscript{DS}</td>
<td>pch\textsubscript{G}-DsRed::SKL\textsubscript{1}-penDE\textsubscript{1}</td>
<td>This study</td>
</tr>
<tr>
<td>DS681330</td>
<td>hdfA\textsubscript{1} Pc20g01800\textsubscript{1}-amdS</td>
<td>This study</td>
</tr>
<tr>
<td>DS65170</td>
<td>hdfA\textsubscript{1} Pc20g07920\textsubscript{1}-amdS</td>
<td>This study</td>
</tr>
<tr>
<td>DS65068</td>
<td>hdfA\textsubscript{1} Pc13g14410\textsubscript{1}-amdS</td>
<td>This study</td>
</tr>
<tr>
<td>DS65068K</td>
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<td>This study</td>
</tr>
<tr>
<td>DS65068Y</td>
<td>hdfA\textsubscript{1} Pc20g15640\textsubscript{1}-amdS</td>
<td>This study</td>
</tr>
<tr>
<td>DsRed\textsubscript{DS}.SL eGFP::Pc13g14410</td>
<td>pch\textsubscript{G}-eGFP::Pc13g14410\textsubscript{1}-penDE\textsubscript{1} amdS pch\textsubscript{G}-DsRed::SKL\textsubscript{1}-penDE\textsubscript{1}</td>
<td>This study</td>
</tr>
<tr>
<td>DsRed\textsubscript{DS}.SL eGFP::Pc20g01800</td>
<td>pch\textsubscript{G}-eGFP::Pc20g01800\textsubscript{1}-penDE\textsubscript{1} amdS pch\textsubscript{G}-DsRed::SKL\textsubscript{1}-penDE\textsubscript{1}</td>
<td>This study</td>
</tr>
<tr>
<td>DsRed\textsubscript{DS}.SL eGFP::Pc21g17590</td>
<td>pch\textsubscript{G}-eGFP::Pc21g17590\textsubscript{1}-penDE\textsubscript{1} amdS pch\textsubscript{G}-DsRed::SKL\textsubscript{1}-penDE\textsubscript{1}</td>
<td>This study</td>
</tr>
<tr>
<td>DsRed\textsubscript{DS}.SL eGFP::Pc22g25150</td>
<td>pch\textsubscript{G}-eGFP::Pc22g25150\textsubscript{1}-penDE\textsubscript{1} amdS pch\textsubscript{G}-DsRed::SKL\textsubscript{1}-penDE\textsubscript{1}</td>
<td>This study</td>
</tr>
<tr>
<td>DS17690 Pc20g07920-eGFP</td>
<td>pch\textsubscript{G}-eGFP::Pc20g07920::eGFP\textsubscript{1} penDE\textsubscript{1} amdS pch\textsubscript{G}-DsRed::SKL\textsubscript{1}-penDE\textsubscript{1}</td>
<td>This study</td>
</tr>
<tr>
<td>DS17690 Pc20g15640-eGFP</td>
<td>pch\textsubscript{G}-eGFP::Pc20g15640::eGFP\textsubscript{1} penDE\textsubscript{1} amdS pch\textsubscript{G}-DsRed::SKL\textsubscript{1}-penDE\textsubscript{1}</td>
<td>This study</td>
</tr>
</tbody>
</table>
The Netherlands) classical strain improvement program. Localization of GFP fusion proteins were performed using strain DS17690 or its derivative DsRed.SKL (Kiel et al., 2009) in which expression cassettes had been ectopically integrated. All gene deletions and interruptions were performed in *P. chrysogenum* DS54465 (*hdfA1*), which has a high frequency of homologous recombination (Snoek et al., 2009). Requests for the academic use of *P. chrysogenum* strains listed in Table 1, under a materials transfer agreement, should be sent to Prof. R.A.L. Bovenberg, DSM Biotechnology Center, Alexander Fleminglaan 1, 2613 AX Delft, The Netherlands. *Escherichia coli* DH5α was used as host strain for high frequency transformation, pLAD DNA amplification (Sambrook and Russel, 2001) if not mentioned otherwise.

2.2. Strain construction

2.2.1. Deletion strains

Genomic DNA fragments used in the construction of gene-deletion cassettes for Pc13g14410, Pc22g25150, Pc20g15640, Pc20g07920 and Pc20g01800 deletion strains were amplified from genomic DNA of *P. chrysogenum* Wisconsin54-1255 using Phusion Hot-Start Polymerase (Finnzymes, Landsmeer, The Netherlands) and the oligonucleotides listed in Table S1. Plasmid construction was performed with the Multisite Gateway® Three-Fragment Vector Construction Kit (Invitrogen, Breda, The Netherlands) as previously described (Gombert et al., 2011). The destination vectors pDEST43-KO Pc13g14410, pDEST43-KO Pc22g25150 and pDEST43-KO Pc20g15640 contained HindIII, Mph1103I and CfoI restriction sites, respectively, that were used to cut out the deletion cassettes. Subsequently, Pc13g14410, Pc22g25150 and Pc20g15640 deletion cassettes were transformed to *P. chrysogenum* DS54465 (*hdfA1*) protoplasts (Snoek et al., 2009) using acetamide as selection marker (Kolar et al., 1988) resulting in strains DS66982, DS66984 and DS66983, respectively (Table 1).

For the deletion of the Pc20g01800 and Pc20g07920 genes, their promoters were PCR amplified using primer pairs FP07920/RP07920 and FP01800/RP01800, respectively, and their open reading frames were PCR amplified using primer directed enzymes Fwd07920/Rev07920 and Fwd01800/Rev01800 (Table S1). All gene deletions and interruptions were performed in *P. chrysogenum* strains listed in Table 1, under a materials transfer agreement, should be sent to Prof. R.A.L. Bovenberg, DSM Biotechnology Center, Alexander Fleminglaan 1, 2613 AX Delft, The Netherlands. *Escherichia coli* DH5α was used as host strain for high frequency transformation, pLAD DNA amplification (Sambrook and Russel, 2001) if not mentioned otherwise.

2.2.2. GFP-tagged strains

The cDNA pools were prepared from total RNA isolated from chemostat cultures as previously described (Harris et al., 2009a). Pc22g25150, Pc20g07920, Pc20g15640, Pc20g01800 cDNAs were prepared from mRNA samples of *P. chrysogenum* DS17690 grown in glucose-limited chemostat with adipic acid. Pc21g17590 cDNA was prepared from a sample of the strain Wisconsin 1255-54 grown in glucose-limited chemostat with phenylacetate (van den Berg et al., 2008) and Pc20g01800 cDNA was prepared from a sample of *P. chrysogenum* Wisconsin 1255-54 grown in glucose-limited chemostat cultures without phenylacetate (van den Berg et al., 2008). cDNAs encoding putative acyl-CoA dehydrogenases (Pc20g07920, Pc20g15640, Pc21g17590 and Pc22g25150) and acyl-CoA oxides (Pc13g14410 and Pc20g01800) were amplified from the respective cDNA pools with the Expand High Fidelity PCR System (Roche, France) and specific primer pairs (Table S1). The amplified PCR products were cloned into pENTR/D-TOPO vector and transformed into *E. coli* TOP10 cells using the pENTR directional TOPO cloning kit (Invitrogen). The six cloned cDNA sequences were sent for sequencing (BaseClear, Leiden, The Netherlands) to verify correct strain construction.

The construction of the fused genes was carried out using MultiSite Gateway Technology (Invitrogen) following the manufacturer’s instructions. For Pc13g14410, Pc20g01800, Pc21g17590, and Pc22g25150, the corresponding pENTR clones were recombinated with pENTR41-pcbC-eGFP, pENTR23-His6-penDET (Kiel et al., 2009) and pDEST R4-R3/AMDS-NotI resulting in pEXP-r4-r3 with A. nidulans *amdS* expression cassette and a NotI restriction site) resulting in pEXP-eGFP, Pc13g14410, pEXP-eGFP-Pc20g01800, pEXP-eGFP-Pc21g17590, pEXP-eGFP-Pc22g25150, respectively. For Pc20g07920 and Pc20g15640 the stop codon of the gene was removed, in order to enable translation of the C-terminus fused eGFP gene. The corresponding pENTR clones were recombinated with pENTR41-pcbC, pENTR23-eGFP-penDET and pDEST R4-R3/AMDS-NotI resulting in pEXP-Pc20g07920.eGFP and pEXP-Pc20g15640.eGFP. The expression (pEXP) vectors were linearized with Smal (Pc13g14410, Pc20g01800 and Pc21g17590 containing vectors), with KpnI (Pc22g25150 containing vector) and NotI (Pc20g07920 and Pc20g15640 containing vectors) and used to transform the *P. chrysogenum* strain DsRed.SKL or DS17690 (Table 1) as previously described (Cantoral et al., 1987).

2.3. Strain construction confirmation

2.3.1. Diagnostic PCR

Genomic DNA of strains DS66982 (Pc13g14410), DS66984 (Pc22g25150), DS66983 (Pc20g15640) and DS54465 (hdfA1) was isolated using the E.Z.N.A. Fungal DNA kit (Omega Bio-tek, Amsterdam, The Netherlands). The *amdS* gene in the transformants was amplified using primers F-amds and R-amds (Table S1) to confirm its presence in the transformants. The Pc13g14410, Pc22g25150 and Pc20g15640 genes were amplified in the DS54465 (hdfA1) strain and the transformants using primers F14410 and R14410, F25150 and R25150, F15640 and R15640 (Table S1) to confirm the absence of the each of these genes after transformation. The correct inactivation of Pc20g07920 and Pc20g01800 was confirmed with primer pairs Pc20g07920-P1/P2 and Pc20g01800-P1/P2, respectively (Table S1).
2.3.2. Southern blot/hybridization

Genomic DNA (2.5 μg) of the transformants DS66982 (Pc13g14410.1), DS66984 (Pc22g25150.1), DS66983 (Pc20g15640.1) and DS54465 (hdfA.1) was digested using the restriction enzymes BamHI, EcoRI and EcoRV, respectively, followed by electrophoresis on a 0.8% agarose gel and blotting onto a Zeta-Probe membrane (Biorad, Hercules, CA) (Sambrook and Russel, 2001). The 3′ flanking region of Pc13g14410 and Pc20g15640 and the 5′ flanking region of Pc22g25150, were used as probes, after labeling with digoxigenin using the PCR DIG Probe Synthesis Kit (Roche) according to the manufacturer’s instructions. Hybridization was done overnight at 42 °C in hybridization buffer (50% formamide, 5x Saline-Sodium Citrate (SSC) buffer), 2% blocking reagent (Roche), 0.1% Na-lauroylsarcosyl, 0.02% Sodium Dodecyl Sulfate (SDS)). Membranes were washed twice with 2x SSC, 0.1% SDS for 15 min and twice with 0.2x SSC, 0.1% SDS. Digoxigenin-labeled probes were detected by chemiluminescence using CDP-star (Roche, Paris, France). As expected, hybridization showed 3635 bp and 5158 bp fragments in P. chrysogenum strains DS54465 (hdfA.4) and DS66982 (Pc13g14410.1), respectively; 3208 bp and 1785 bp fragments in strains DS54465 (hdfA.4) and DS66984 (Pc22g25150.1), respectively, and 3361 bp and 4050 bp fragments in strains DS54465 (hdfA.4) and DS66983 (Pc20g15640.1), respectively.

2.3.3. Penicillin gene cluster copy number determination by quantitative PCR analysis

To analyze the number of penicillin biosynthetic gene clusters in the transformed strains P. chrysogenum DS66982 (Pc13g14410.1), DS66984 (Pc22g25150.1), DS66983 (Pc20g15640.1), DS68330 (amds::Pc20g01800), DS63170 (amds::Pc20g07920) and DS54465 (hdfA.4), γ-actin (with the primers F γ-actin gDNA and R γ-actin gDNA) and an intergenic target (using the primers F-IGR Pc20g07920 and R-IGR hdfA::Pc20g07920) were used as reference templates in qPCR (Table S1). The primers for pcbAB (F-pcbAB and R-pcbAB) and pcbC (F-pcbC and R-pcbC) (Table S1) were used to assess the cluster copy number in genomic DNA. P. chrysogenum strains DS54465 (hdfA.4), Wisconsin54-1255 and DS50652 (lacking all penicillin biosynthesis gene cluster) (Table 1) were used as controls containing 8, 1 and 0 penicillin gene clusters, respectively. The gene copy numbers were analyzed on a MiniopticTM system (Biorad) using the Bio Rad CFX manager software in which the C(t) values were determined automatically by regression. The SensiMixTM SYBR mix (Bioline, Alphen aan den Rijn, The Netherlands) was used as a master mix for qPCR with 0.4 μM primers and 40 ng gDNA in a 25 μl reaction volume. Copy numbers of the penicillin gene cluster were calculated from duplicate experiments.

2.4. Media and culture conditions

P. chrysogenum strains expressing fluorescent proteins were grown in liquid culture with penicillin production medium (7.5% lactose, 0.5% ammonium-acetate, 0.4% Na2SO4, 0.4% urea, 50 mM potassium-phosphate buffer pH 6.5, 0.05% phenoxacyctic acid and 4 ml l−1 of a trace element solution). The trace-element solution contained: 24.6 g EDTA−2H2O, 43.76 g Na2citrate−2H2O, 24.84 g FeSO4⋅7H2O, 256.4 g MgSO4⋅7H2O, 12.4 mg H3BO3, 12.4 mg Na2MoO4⋅2H2O, 0.64 g CuSO4⋅5H2O, 2.52 g ZnSO4⋅7H2O, 0.64 g CoSO4⋅7H2O, 3.04 g MnSO4⋅H2O and 1.24 g CaCl2, pH 6.5. Batch cultures were incubated at 25 °C for 40 h.

Chemostat cultures of P. chrysogenum were performed in a glucose-limited defined mineral medium that contained, per litre of demineralized water: 0.8 g KH2PO4, 3.5 g (NH4)2SO4, 0.5 g MgSO4⋅7H2O, 7.5 g of glucose and 10 ml of trace element solution. The trace element solution contained 15 g l−1 Na2EDTA⋅2H2O, 0.5 g l−1 CuSO4⋅5H2O, 2 g l−1 ZnSO4⋅7H2O, 2 g l−1 MnSO4⋅H2O, 4 g l−1 FeSO4⋅7H2O and 0.5 g l−1 CaCl2⋅2H2O. The synthesis of semi-synthetic β-lactam intermediates was induced by the addition of 5 g l−1 of adipic acid to the medium. The pH of the reservoir medium was set at 5.5 with KOH. Aerobic chemostat cultures were grown in a 3 l bioreactor (Applikon, Schiedam, The Netherlands) at a dilution rate of 0.03 h−1, as described previously (Harris et al., 2009b). Chemostat cultures were assumed to be in steady state when at least 5 volume changes had passed since the initiation of continuous feeding and, moreover, the variation of culture dry weight and off-gas CO2 measurements were lower than 4% over two consecutive volume changes.

Shake flask cultures were grown on a defined mineral medium that, in comparison with the chemostat medium, had the following modifications: the medium was supplemented with 0.05 M MES buffer and the glucose was replaced with several fatty acids at a concentration of 1.44 g l−1. The concentrations used were 0.03 M butyric acid, 0.02 M hexanoic acid, 0.015 M caprylic acid, 0.012 M capric acid, 0.01 M lauric acid, 0.0086 M myristic acid, 0.0067 M oleic acid, 0.0055 M erucic acid and 0.02 M adipic acid. Capric, lauric, myristic, oleic and erucic acid were solubilized with 1% (v/v) Tergitol NP 40. The pH was adjusted to 6.5 with KOH prior to cultivation.

2.5. Analytical methods

Biomass dry weight was measured by filtering 10 ml culture samples over pre-weighed glass fiber filters (Type A/E, Pall Life Sciences, East Hills, NY). The filters were washed with demineralized water, dried in a microwave oven (20 min at 600 W) and subsequently weighed. Measurements were performed in duplicate.

Glucose and adipic acid titers in culture supernatant and media were analyzed by HPLC (Waters Alliance 2695 Separation Module supplied with a Waters 2487 Dual Absorbance Detector and a Waters 2410 Refractive Index Detector—Waters, Milford, MA) using a Biorad HPX87H column (Biorad) eluted at 60 °C with 0.5 mM H2SO4 at a flow rate of 0.6 ml min−1 (Nasution et al., 2008). Quantitative 1H NMR was used to measure extracellular concentration of ad-6-APA (adipoyl-6-aminopenicillanic acid), IPN (isopenicillin N), 6-APA (6-aminopenicillanic acid) and 8-HPA (8-hydroxy-penicillanic acid) from P. chrysogenum cultures. Quantitative 1H NMR experiments were performed at 600 MHz on a Bruker Avance 600 spectrometer (Bruker, Worfem, The Netherlands). To a known quantity of filtrate, a known quantity of internal standard (maleic acid), dissolved in phosphate buffer, was added prior to lyophilization. The residue was dissolved in D2O and measured at 300 K. The delay between scans (30 s) was more than five times T1 of all compounds, so the ratio between the integrals of the compounds of interest and the integral of the internal standard was an exact measure for the quantity of the penicillins and cephalosporins.

2.6. Protein localization experiments

Confocal laser scanning microscopy was performed using a Carl Zeiss LSM510 with a 63 x 1.40 NA Plan Apochromat objective (Carl Zeiss, Sliedrecht, The Netherlands) and photomultiplier tubes (Hamamatsu Photonics, Herrsching am Ammersee, Germany). Images were acquired using AIM 4.2 software (Carl Zeiss, Sliedrecht, The Netherlands). GFP fluorescence was visualized by excitation of the cells with a 488-nm argon ion laser (Lasos, Jena, Germany) and emission was detected using a 500–530-nm bandpass emission filter. DsRed and Mitotracker Orange signals were visualized by excitation with a 543-nm helium neon laser (Lasos, Jena, Germany) and emission was detected using a 660–700-nm longpass emission filter. To visualize mitochondria, Mitotracker Orange (Invitrogen) was added to a final concentration of...
1 nM to the medium. Images were taken after incubation for 20 min at 25 °C.

2.7. Transcriptome data

Chemostat cultures of \( P. \) chrysogenum DS50661 grown in the presence of adipic acid were sampled by rapidly filtering 60 ml of culture broth over a glass fiber filter (Type A/E, Pall Life Sciences, East Hills, NY). Filter and mycelium were immediately wrapped in aluminum foil, quenched in liquid nitrogen and stored at −80 °C. Samples were processed as previously described (Harris et al., 2009a; van den Berg et al., 2008). Acquisition and quantification of array images and data filtering were performed using Affymetrix GeneChip Operating Software (GCOS version 1.2). Arrays were globally scaled to a target value of 100, applying the average signal from all genes (global scaling). Arrays were analyzed as previously described (Harris et al., 2009a). Significant changes in expression were statistically assessed by comparing replicate array experiments, using the software Significance Analysis of Microarray (SAM version 2.0) (Tusher et al., 2001). The fold-change of 2 was used and a maximum false discovery rate set to 1%. Gene clusters were assessed for enrichment in MIPS (Munich Information Center for Protein Sequences) categories (version 1.3) by employing hypergeometric distribution with a p-value cut-off of \( 10^{-3} \) (Harris et al., 2009a; Knijnenburg et al., 2009; Kresnowati et al., 2006). Transcriptome data of strains DS50661, DS17690 and DS49834 were derived from the accession series GSE12632 (Harris et al., 2009a), GSE12617 (Koetsier et al., 2010) and GSE12612 (Harris et al., 2009b), respectively.

Promoter analysis was performed using the web-based software Multiple Em for Motif Elucidation (MEME—version 3.5.4) (Bailey and Elkan, 1995). Promoters (from −1,000 to 0) of each set of co-regulated genes were analyzed for over-represented tetra- to dodeca-nucleotides. Consensus sequences were represented by the web base application WebLogo (version 2.8.2) (Crooks et al., 2004).

3. Results

3.1. Resolving adipic acid catabolism in \( P. \) chrysogenum

Previous \(^{13}\)C flux (Harris et al., 2009b; Thykaer et al., 2002) and transcriptome (Harris et al., 2009b; Thykaer et al., 2002) analyses already suggested an involvement of β-oxidation in adipic acid metabolism by \( P. \) chrysogenum. To further refine these data on \( P. \) chrysogenum cultures grown in the presence and absence of adipic acid (Harris et al., 2009b), the transcriptome analysis was expanded to include all relevant datasets derived from \( P. \) chrysogenum strains: DS17690 (high-producing penicillin strain) (Koetsier et al., 2010), DS49834 (a strain producing the cephalosporin precursor ad-7-ACCCA) and DS50661 (a derivative of DS17690 that is unable to produce β-lactams due to the absence of the pcbAB, pcbC and penDE genes) (Harris et al., 2009b)). A three-way comparison of the different strains led to the identification of 52 genes whose transcripts were consistently up-regulated in all three strains when they were grown in the presence of adipic acid (corresponding to 0.4% of the entire \( P. \) chrysogenum genome) (Fig. 1; Table S2). No genes were identified whose transcript levels were consistently down-regulated in adipic-acid supplemented cultures of all three strains.

The group of 52 genes transcriptionally up-regulated in the presence of adipic acid was subjected to functional category enrichment using Fisher’s exact test. This analysis revealed a clear enrichment (p value < \( 10^{-5} \)) of metabolic genes located in the peroxisome and involved in β-oxidation (Fig. 1). A search for overrepresented sequences in 5′-non-coding regions of these 52 genes revealed an overrepresented putative cis-regulatory motif.
In the upstream sequence (−1 kb) of 36 of these genes (Fig. 1; Table S2), the putative regulatory element [5'-CCKSGGB-3'] present in the upstream sequence is highly similar to the FarA and FarB binding site (5'-CCTCGG-3') in *A. nidulans*. FarA and FarB are transcription factors involved in the activation of fatty acid metabolism (Hynes et al., 2006). Coincidently, Pc20g07170, which has a strong similarity to *A. nidulans* FarB (E-value 4E⁻⁰⁷, 56% sequence identity) was also among the 52 genes that were transcriptionally up-regulated in the presence of adipic acid (Table S2).

Of 52 genes whose transcript level was increased in the presence of adipic acid, 20 were previously described as having a putative function in peroxisomal or mitochondrial β-oxidation (Harris et al., 2009b; van den Berg et al., 2008) (Fig. 2). Activation of adipic acid to the corresponding CoA-thioester via acyl-CoA ligase is essential for its incorporation in the β-lactam backbone (Koetsier et al., 2010). Therefore, the initial oxidation step in β-oxidation, in which adipoyl-CoA is oxidized to a trans-2-enoyl-CoA, would provide the most logical target for minimization of adipic acid catabolism. The *P. chrysogenum* genome harbors 19 putative structural genes for acyl-CoA oxidases and dehydrogenases (van den Berg et al., 2008). However, only two putative acyl-CoA oxidases and four acyl-CoA dehydrogenases showed a consistent up-regulation in the presence of adipic acid in chemostat-based transcriptome analyses of three *P. chrysogenum* strains (Table S2).

### 3.2. Subcellular localization of putative acyl-CoA oxidases and dehydrogenases

The subcellular localization of the six putative acyl-CoA oxidases and acyl-CoA dehydrogenases whose structural genes were consistently up-regulated in the presence of adipic acid was investigated to assess their possible involvement in peroxisomal or mitochondrial β-oxidation. The putative location of these and other *P. chrysogenum* proteins was previously assessed by *in silico* studies, via identification of putative peroxisomal targeting sequences (PTS) (Kiel et al., 2009). This analysis was combined with the systematic search prediction for mitochondrial targeting sequence using Mitoprot (Claros and Vincens, 1996). Proteins encoded by the genes marked with an asterisk (*) were predicted to contain a mitochondrial targeting signal as defined by MITOPROT (Claro and Vincens, 1996). Proteins encoded by the genes marked with a # symbol indicates genes for which the gene products have been localized by eGFP tagging. The putative peroxisomal localization of Pc22g07200/AcA1 and Pc22g14900/Pc1A has been reported previously (Koetsier et al., 2010).
Table 2
Transcript level of putative *P. chrysogenum* acyl-CoA oxidases and acyl-CoA dehydrogenases in glucose-limited chemostat cultures of *P. chrysogenum* strains DS17690, DS50661 (p*cbAB*-p*cbC*-p*penD*E), DS49834 (p*cbG*-c*efIF*-p*cbC*-c*mcH*-p*penD*E) grown with (+) or without adipic acid (−). Transcript levels were determined with Affymetrix GeneChip DSM FENA52025SF. Data represent the average ± mean deviation of three independent chemostat cultures. # indicates the presence of a predicted mitochondrial targeting sequence at the C-ter of the given protein. nd: not determined.

<table>
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<th>Putative function</th>
<th>Sub. cell. loc.</th>
<th>ADA</th>
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<th>ADA</th>
<th>ADA</th>
<th>ADA</th>
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</tr>
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<td>Pc11g14410 Oxidase</td>
<td>Perox (SKL)</td>
<td>12.0 ± 0</td>
<td>437.9 ± 34</td>
<td>12.0 ± 0</td>
<td>1049.2 ± 136</td>
<td>12.0 ± 0</td>
<td>345.7 ± 96</td>
</tr>
<tr>
<td>Pc20g01800 Oxidase</td>
<td>Perox (SKL)</td>
<td>125.4 ± 13</td>
<td>339.3 ± 40</td>
<td>62.5 ± 14</td>
<td>377.8 ± 73</td>
<td>112.6 ± 11</td>
<td>290.9 ± 50</td>
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<td>Pc20g07920 Dehydrogenase</td>
<td>Mit #</td>
<td>204.8 ± 47</td>
<td>1713.4 ± 155</td>
<td>157.7 ± 8</td>
<td>2137.6 ± 577</td>
<td>195.4 ± 28</td>
<td>1281.5 ± 200</td>
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<tr>
<td>Pc22g25190 Dehydrogenase</td>
<td>Perox (SKL)</td>
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<td>553.7 ± 55</td>
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<td>2142.6 ± 72</td>
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<td>698.9 ± 96</td>
<td>1510.2 ± 198</td>
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<td>229.0 ± 20</td>
<td>54.4 ± 18</td>
<td>312.3 ± 53</td>
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<td>220.3 ± 34</td>
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<td>Pc13g11930 Dehydrogenase</td>
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<td>51.4 ± 13</td>
<td>168.4 ± 14</td>
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<td>188.4 ± 27</td>
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<td>Pc22g22700 Dehydrogenase</td>
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<td>Pc06g01180 Dehydrogenase</td>
<td>nd</td>
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<td>12.0 ± 0</td>
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<td>Pc16g05030 Dehydrogenase</td>
<td>nd</td>
<td>12.0 ± 0</td>
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<td>13.5 ± 2</td>
<td>15.8 ± 5</td>
<td>12.8 ± 4</td>
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Similarity to
- Hypothetical protein contig42.tfa_690wg—*A. fumigatus*
- Hypothetical protein contig42.tfa_690wg—*A. fumigatus*
- Acyl-CoA dehydrogenase aidB—*E. coli*
- Acyl-CoA dehydrogenase *A. niger*
- Hypothetical protein—*Bradyrhizobium japonicum*
- Acyl-CoA dehydrogenase aidB—*E. coli*
- Acyl-CoA dehydrogenase *A. niger*
- Long-chain acyl-CoA dehydrogenase *A. niger*
- Isovaleryl-CoA dehydrogenase *A. niger*
- Branched chain acyl-CoA dehydrogenase *A. niger*
- Isovaleryl-coenzyme A dehydrogenase *A. niger*
Orange. Confocal Laser Scanning Microscopy showed that green fluorescence from the eGFP fusion proteins containing PTS sequences (Pc13g14410, Pc20g01800, Pc21g17590 and Pc22g25150) co-localized with the red fluorescence of DsRedSKL, indicating that these proteins are peroxisomal (Fig. 3A–D). Pc20g07920::eGFP and Pc20g15640::eGFP fusion proteins co-localized with the Mitotracker-Orange fluorescence, indicating a mitochondrial localization (Fig. 3E, F). All putative acyl-CoA oxidases localized exclusively to the peroxisome, while acyl-CoA dehydrogenases were either found peroxisomes or in mitochondria. These results suggest that both peroxisomal and mitochondrial β-oxidation pathways are involved in adipic acid metabolism by P. chrysogenum.

3.3. Inactivation of the acyl-CoA oxidase Pc20g01800 and the acyl-CoA dehydrogenase Pc20g07920 leads to improved ad-6-APA production.

To investigate the role in adipic acid catabolism of the 6 putative acyl-CoA oxidases and dehydrogenases identified by the transcriptome analysis, mutants of the corresponding genes were constructed. Pc21g17590, which exhibited the lowest up-regulation in the presence of adipic acid (Table 2), was not included in the rest of the study. A rigorous copy number evaluation of the penicillin biosynthetic genes was carried out by quantitative PCR for each deletion mutant to avoid a mis-interpretation of the fermentation data due to the recombinative transformation-by quantitative PCR for each deletion mutant to avoid a mis-interpretation of the fermentation data due to the recombinative transformation.
et al., 1990; Harris et al., 2009b; Queener et al., 1994; Velasco et al., 2000) has transformed this fungus into a platform organism for production of \(\beta\)-lactam antibiotics and their precursors. Industrial fermentation constitutes a substantial contribution to the overall production cost of \(\beta\)-lactam antibiotics (Elander, 2003). In fact, reduced degradation of the side-chain precursor phenylacetic acid by a point mutation in the \(P. \) chrysogenum \(pahA\) gene, which encodes phenylacetic acid hydroxylase, was an important step in the classical strain improvement process for penicillin \(G\) production (Rodriguez-Saiz et al., 2001, 2005). While adipate is a bulk chemical used in the production of nylon and polyurethane, it is considerably more expensive than glucose and,

<table>
<thead>
<tr>
<th>Strain time [h]</th>
<th>Oleic acid (C18:1)</th>
<th>Erucic acid (C22:1)</th>
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<td>166</td>
<td>1.52 ± 0.03</td>
<td>0.55 ± 0.13</td>
</tr>
<tr>
<td>286</td>
<td>0.60 ± 0.16</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>214</td>
<td>0.02 ± 0.02</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>305</td>
<td>0.00 ± 0.00</td>
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Fig. 3. Subcellular localization of acyl-CoA oxidases and dehydrogenases in \(P. \) chrysogenum. \(P. \) chrysogenum DsRed.SKL eGFP-Pc13g14410 (panel A), DsRed.SKL eGFP-Pc20g01800 (panel B), DsRed.SKL eGFP-Pc21g17590 (panel C), DsRed.SKL eGFP-Pc22g25150 (panel D), DS17690 Pc20g07920-eGFP (panel E) and DS17690 Pc20g15640-eGFP (panel F) cells were cultivated on penicillin production medium supplemented with phenoxycetic acid for 40 h and analyzed by confocal laser scanning microscopy. Panels A–D: in all cases GFP and DsRed fluorescence co-localized indicating that the GFP fusion proteins are sorted into peroxisomes. In panels E–F the GFP fluorescence co-localized with Mitotracker Orange fluorescence, demonstrating mitochondrial sorting. The bar represents 10 \(\mu\)m.
in contrast to glucose, is made from petrochemical feedstock. Therefore, degradation of adipic acid by *P. chrysogenum* negatively affects the economics and the carbon footprint of the fermentative production of cephalosporin precursors.

Despite recent progress in the genetic modification of *P. chrysogenum* (Snoek et al., 2009), the introduction and analysis of gene inactivation still require a significant input of time and resources. In the present study, we therefore prioritized putative genes encoding the first step in the degradation of adipoyl-CoA, the direct precursor for production of cephalosporins, by a chromatin-based transcriptome analysis (Daran-Lapujade et al., 2009). Inactivation of two of the identified target genes (Pc20g01800 and Pc20g07920) was shown to cause a strong decrease of adipic acid degradation, and to lead to an increased production of adipoylated intermediates of cephalosporin.

In a strain in which Pc20g07920, encoding a putative mitochondrial acyl-CoA dehydrogenase, was inactivated, the efficiency of adipic acid incorporation into cephalosporin intermediates was increased from 0.05 to 0.38 (Table 3). In strain DS63170 (amds::Pc20g07920), the productivity of the cephalosporin biosynthesis intermediate ad-6-APA was in the same order of magnitude as the productivity of penicillin G in the high-producing strain DS17690 (11 μmol g⁻¹ h⁻¹ versus 20 μmol g⁻¹ h⁻¹; Table 3 and (Harris et al., 2009a)). Surprisingly, gene inactivation of Pc20g01800, encoding a peroxisomal acyl-CoA oxidase, which led to a stronger decrease of the rate of adipic acid degradation than the gene inactivation of Pc20g07920, had a smaller impact on the production of cephalosporin intermediates. Both AclA, an acyl-CoA ligase that can activate adipic acid, and isopenicillinN acyltransferase (IAT), a key enzyme in cephalosporin biosynthesis that uses adipoyl-CoA as a substrate, are peroxisomal proteins (Muller et al., 1991; Muller et al., 1992). This observation may be related to the fact that competition for a common substrate is not solely determined by the capacities (Vmax) of the competing reactions, but also by other properties (e.g. km for adipoyl-CoA). Furthermore, the competition between different cellular processes and compartments for adipoyl-CoA is likely to be affected by the capacity and kinetic properties of transport processes of adipoyl-CoA across peroxisomal and mitochondrial membranes (e.g. via a carnitine shuttle) (Swiegers et al., 2001, 2002).

To further improve the efficiency of adipic acid incorporation into desired products, this proof-of-principle study can be followed up by combinatorial studies, in which several combinations of the putative acyl-CoA dehydrogenase and acyl-CoA oxidase genes identified in this study are deleted. While none of the deletion mutants examined in this study exhibited growth defects or morphological changes during growth on glucose in the absence of adipic acid (data not shown), the single deletion of the mitochondrial putative acyl-CoA dehydrogenase gene Pc20g15640 led to clear and reproducible morphological changes in submerged cultures with adipic acid. Further research should establish whether Pc20g15640 plays a role in the β-oxidation of adipic acid and, if so, whether this role can be separated from its impact on morphology.

### 4.2. β-oxidation in *P. chrysogenum*

In the well studied eukaryotic model organism *Saccharomyces cerevisiae* the pathway for β-oxidation of acyl-CoA compounds comprises only five structural genes: *POX1* (acyl-CoA oxidase), *FOX2* (bi-functional enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase), *POT1* (3-oxoacyl-CoA thiolase) and *DCI1* and *ECI1* (enoyl-CoA isomerase). All five gene products are localized in peroxisomes. In contrast, the *P. chrysogenum* genome contains no fewer than 19 putative structural genes for the first step of β-oxidation, whose gene products are either localized in peroxisomes or mitochondria (Table 2). Also for other steps in the β-oxidation pathway, multiple putative genes can be identified in the *P. chrysogenum* genome (Fig. 2).

The importance of β-oxidation in *P. chrysogenum* is further illustrated by comparing its transcriptional regulation. In *S. cerevisiae*, β-oxidation is transcriptionally repressed when excess glucose is supplied as the carbon source (Gaby et al., 1957; Kolkman et al., 2006; Stanway et al., 1995; Tai et al., 2005; Wang et al., 1992) and transcriptionally induced by a set of transcriptional regulators (Adr1, Oaf1 and Pif1) (Gurvitz et al., 2001; Simon et al., 1995; Trotter, 2001) when fatty acids are available. In contrast, *P. chrysogenum* genes that, based on sequence similarity are predicted to encode β-oxidation proteins are not strictly subject to a similar glucose catabolite repression. Of the six acyl-CoA oxidase and dehydrogenase genes investigated in this study (Table 2), five showed significant transcript levels in ammonia-, sulfate- and phosphate-limited chemostat cultivations grown at high residual glucose concentrations (circa 25 g l⁻¹). The only gene (out of the six studied) not expressed in any glucose excess conditions was Pc13g14410 that also was not expressed in glucose-limited conditions (Table 2). Pc13g14410 was the sole acyl-CoA oxidase whose expression was strictly adipate-dependent (T. Veiga and J.-M. G. Daran, unpublished data).

Interestingly, the strongest impact on adipic acid catabolism was found when Pc20g01800, encoding a peroxisomal acyl-CoA oxidase, was deleted. However, the impact of the inactivation of Pc20g07920, encoding a mitochondrial acyl-CoA dehydrogenase, together with the morphological changes observed in the presence of adipic acid upon deletion of Pc20g15640 (whose sequence suggests a similar role), indicate that in *P. chrysogenum* peroxisomal and mitochondrial β-oxidation pathways are both involved in adipic acid metabolism. These results therefore indicate that, in contrast to the predominantly mitochondrial β-oxidation of short-chain monocarboxylates in higher eukaryotes (Houten and Wanders, 2010; Van Veldhoven, 2010) and *A. nidulans* (Maggio-Hall and Keller, 2004), peroxisomal β-oxidation has also a significant impact on β-oxidation of the short-chain dicarboxylic acid, adipic acid.

The higher complexity of the *P. chrysogenum* β-oxidation pathway gene catalog and regulation suggests that its physiological functions are broader than the metabolism of linear fatty acids. Involvement in adipic acid metabolism, the subject of the present study, is just one of many possible roles in carbon metabolism. For example, in *A. nidulans*, deletion of a single acyl-CoA dehydrogenase impaired growth on short chain fatty acids as well as on the branched-chain amino acids isoleucine and valine (Maggio-Hall et al., 2008; Maggio-Hall and Keller, 2004), indicating a role in the metabolism of branched-chain carbon skeletons. Peroxisomal β-oxidation has been also implicated in biotin biosynthesis in *A. nidulans* (Magliano et al., 2011). The availability of a well annotated genome sequence and efficient gene deletion tools make *P. chrysogenum* an interesting platform for further studies on the physiological roles and metabolic compartmentation of β-oxidation in filamentous fungi.

### Acknowledgments

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Sustainability). This project was carried out within the research program of the Kluiver Centre for Genomics of Industrial Fermentation. We thank Marcel van den Broek for his assistance with the promoter analysis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymgen.2012.02.004.

References


