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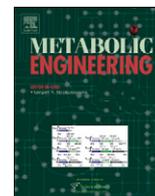
# Metabolic engineering of β-oxidation in *Penicillium chrysogenum* for improved semi-synthetic cephalosporin biosynthesis

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## ABSTRACT

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-ACA), adipoyl-7-desacetoxyaminocephalosporanic acid (ad-7-ADCA) and adipoyl-7-amino-3-carbamoyloxymethyl-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 *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). $^{13}$C-labeling studies (Thykaer et al., 2002) and genome-wide expression profiling (Harris et al., 2009b) suggested that adipic acid catabolism in *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 *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 carbon and energy sources (Mingrone and Castagneto, 2006). Their metabolism starts with activation into the corresponding dicarboxyloyl-CoA, followed by chain shortening of the resulting CoA ester via β-oxidation, using the same set of reactions as those involved in β-oxidation of mono-carboxylic acids (Ferdinandusse et al., 2004; Suzuki et al., 1989). β-oxidation yields acetyl-CoA and short-chain dicarboxyloyl-CoA compounds such as succinyl-CoA ($C_4$), which can enter central metabolism, and adipoyl-CoA ($C_6$) (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 *Aspergillus nidulans* (Maggio-Hall and Keller, 2004) but in *Saccharomyces cerevisiae*, β-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 an acyl-CoA compound, thereby forming a trans-2-enoyl-CoA, is catalyzed by an FAD-linked acyl-CoA dehydrogenase, which can donate electrons to the mitochondrial respiratory chain (Crane et al., 1955; Crane and Beinert, 1956). In peroxisomes, this oxidation is predominantly catalyzed by an $H_2O_2$-producing acyl-CoA oxidase (Inestrosa et al., 1979).

The initial step in β-oxidation of adipoyl-CoA is relevant for metabolic engineering of adipate metabolism in *P. chrysogenum*, as it directly competes with the production of cephalosporin precursors for available adipoyl-CoA. Moreover, it has been proposed that acyl-CoA dehydrogenases and oxidases have a strong impact on the kinetics and specificity of β-oxidation pathways (Ikeda et al., 1985). For example, while acyl-CoA dehydrogenases have different chain-length preferences, the acyl-CoA oxidases tend to show very low, if any, activity with short-chain substrates (Hiltunen and Qin, 2000). In several organisms, very long and/or branched fatty acids are initially shortened via peroxisomal β-oxidation (Poirier et al., 2006; Wanders et al., 2001). Once the size of the acyl-CoA ester cannot be further reduced by peroxisomal β-oxidation, it is transported to the mitochondria (reviewed by Ramsay (2000)), where it is further broken down.

In filamentous fungi, β-oxidation has been most extensively studied in *A. nidulans*. In studies using mutant strains, the mitochondrial and peroxisomal β-oxidation pathways were shown to be able to complement each other (Maggio-Hall and Keller, 2004). In *A. nidulans*, the transcription factors FarA and FarB are involved in the regulation of fatty acid catabolism and other peroxisomal functions. FarA has been implicated in the regulation of both long and 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 *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

*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,

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

| Strain | Genotype/Description | Reference |
|---|---|---|
| DS17690 | High penicillin producer | (Harris et al., 2009a) |
| Wisconsin54-1255/ATCC28089 | Ancestor of DS17690 | (Mac Donald et al., 1964) |
| DS54465 | *hdfAΔ* | (Snoek et al., 2009) |
| DS50661 | *[pcbAB–pcbC–penDE]Δ* | (Harris et al., 2009a) |
| DS49834 | *pcbC$_P$-cefEF-penDE$_T$ pcbC$_P$-cmcH-penDE$_T$* | (Harris et al., 2009b) |
| DsRedSKL | *pcbC$_P$-DsRed::SKL-penDE$_T$* | (Kiel et al., 2009) |
| DS68330 | *hdfAΔ Pc20g01800Δ-amdS* | This study |
| DS63170 | *hdfAΔ Pc20g07920Δ-amdS* | This study |
| DS66982 | *hdfAΔ Pc13g14410Δ-amdS* | This study |
| DS66984 | *hdfAΔ Pc22g25150Δ-amdS* | This study |
| DS66983 | *hdfAΔ Pc20g15640Δ-amdS* | This study |
| DsRed.SKL eGFP-Pc13g14410 | *pcbC$_P$-eGFP::Pc13g14410-penDE$_T$ amdS pcbC$_P$-DsRed::SKL-penDE$_T$* | This study |
| DsRed.SKL eGFP-Pc20g01800 | *pcbC$_P$-eGFP::Pc20g01800-penDE$_T$ amdS pcbC$_P$-DsRed::SKL-penDE$_T$* | This study |
| DsRed.SKL eGFP-Pc21g17590 | *pcbC$_P$-eGFP::Pc21g17590-penDE$_T$ amdS pcbC$_P$-DsRed::SKL-penDE$_T$* | This study |
| DsRed.SKL eGFP-Pc22g25150 | *pcbC$_P$-eGFP::Pc22g25150-penDE$_T$ amdS pcbC$_P$-DsRed::SKL-penDE$_T$* | This study |
| DS17690 Pc20g07920-eGFP | *pcbC$_P$-Pc20g07920::eGFP-penDE$_T$ amdS pcbC$_P$-DsRed::SKL-penDE$_T$* | This study |
| DS17690 Pc20g15640-eGFP | *pcbC$_P$-Pc20g15640::eGFP-penDE$_T$ amdS pcbC$_P$-DsRed::SKL-penDE$_T$* | This study |

The Netherlands) classical strain improvement program. Localizations 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 (*hdfAΔ*), 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, plasmid 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 *Hind*III, *Mph*1103I and *Cfr*42I 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 (*hdfAΔ*) 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 pairs Fwd07920/Rev07920 and Fwd01800/Rev01800 (Table S1). All fragments contained a 14 bp tail to facilitate directional and efficient cloning via the STABY method (Eurogentec, Maastricht, The Netherlands). Two variants of the standard STABY vector (pSTC1.3, Eurogentec), were constructed for individual cloning of both fragments. pSTamdSL was constructed by inserting a 1.8 kb fragment of the *amdS* expression cassette by PCR amplification of the last 2/3 of the *amdS* selection marker gene using oligonucleotides A3F and A3R from pHELY-A1 (van den Berg et al., 2004), and cloning it in the *Hind*III–*Bam*HI sites of pSTC1.3. pSTamdSR was constructed by insertion of an overlapping 2.4 kb fragment of the *amdS* expression cassette by PCR amplification of the *gpdA* promoter (*gpdA_P*) and part of the *amdS* gene, wherein the *Eco*RV sites were removed, using oligonucleotides A5F and A5R. The fragment was cloned into the *Hind*III–*Pme*I sites of pSTC1.3 (Table S1). Also, the strong terminator was inserted in front of the *gpdA_P*–*amdS*; the 0.7 kb *trpC* terminator was PCR amplified using oligonucleotides TCF and TCR from pAN7-1 (Punt and van den Hondel, 1992) and introduced via the *Sbf*I–*Not*I sites of *gpdA_P*–*amdS* in pSTamdSR. Both vectors contained an overlapping non-functional fragment of the fungal selection marker gene *amdS*, allowing recipient cells that recombine the two fragments into a functional selection marker to grow on agar media with acetamide as the sole nitrogen source (Tilburn et al., 1983). The PCR fragments were ligated overnight using T4 DNA ligase (Invitrogen) at $16^{\circ}$ C into their respective STABY-vector according to the supplier's manual and transformed to chemically competent CYS21 *E. coli* cells (Eurogentec). Transformants were used to PCR amplify the gene specific sequences fused to the non-functional *amdS* fragments using oligonucleotides TWF and TWR (Table S1). The obtained PCR fragments were combined and used to transform *P. chrysogenum* DS54465 (Snoek et al., 2009), yielding strains DS68330 (*amdS*::Pc20g01800) and DS63170 (*amdS*::Pc20g07920).

### 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, Pc13g14410 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 oxidases (Pc13g14410 and Pc20g01800) were amplified from the respective cDNA pools with the Expand High Fidelity PCR System (Roche, Paris, France) and specific primer pairs (Table S1). The amplified PCR products were cloned into pENTR/D-TOPO vector and transformed into *E. coli* TOP 10 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 recombined with pENTR41-pcbC_P-eGFP, pENTR23-His_8-penDE_T (Kiel et al., 2009) and pDEST R4-R3/AMDS-NotI (pDEST R4-R3 with *A. nidulans amdS* expression cassette and a *Not*I 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-ter fused eGFP gene. The corresponding pENTR clones were recombined with pENTR41-pcbC_P, pENTR23-eGFP-penDE_T and pDEST R4-R3/AMDS-NotI resulting in pEXP-Pc20g07920.eGFP and pEXP-Pc20g15640.eGFP. The expression (pEXP) vectors were linearized with *Sma*I (Pc13g14410, Pc20g01800 and Pc21g17590 containing vectors), with *Kpn*I (Pc22g25150 containing vector) and *Not*I (Pc20g07920 and Pc20g15640 containing vectors) and used to transform the *P. chrysogenum* strain DsRedSKL 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 (Pc13g14410Δ), DS66984 (Pc22g25150Δ), DS66983 (Pc20g15640Δ), DS68330 (*amdS*::Pc20g07920), DS68330 (*amdS*::Pc20g01800) and DS54465 (*hdfAΔ*) 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 (*hdfAΔ*) strain and the transformants using primers F14410 and R14410, F25150 and R25150, F15640 and R15640 (Table S1) to confirm the absence of 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 (Pc13g14410Δ), DS66984 (Pc22g25150Δ), DS66983 (Pc20g15640Δ) and DS54465 (hdfAΔ) 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 Pc22g 25150, were used 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 $^9$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 CPD-star (Roche, Paris, France). As expected, hybridization showed 3635 bp and 5158 bp fragments in *P. chrysogenum* strains DS54465 (hdfAΔ) and DS66982 (Pc13g14410Δ), respectively; 3208 bp and 1785 bp fragments in strains DS54465 (hdfAΔ) and DS66984 (Pc22g25150Δ), respectively, and 3361 bp and 4050 bp fragments in strains DS54465 (hdfAΔ) and DS66983 (Pc20g15640Δ), 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 (Pc13g14410Δ), DS66984 (Pc22g25150Δ), DS66983 (Pc20g15640Δ), DS68330 (amdS:: Pc20g01800), DS63170 (amdS::Pc20g07920) and DS54465 (hdfAΔ), γ-actin (with the primers F γ-actin gDNA and R γ-actin gDNA) and an intergenic target (using the primers F-IGR Pc20g07090 and R-IGR Pc20g07090) 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 (hdfAΔ), 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 Miniopticon$^{TM}$ system (Biorad) using the Bio Rad CFX manager software in which the $C(t)$ values were determined automatically by regression. The SensiMix$^{TM}$ 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% $Na_2SO_4$, 0.4% urea, 50 mM potassium-phosphate buffer pH 6.5, 0.05% phenoxyacetic acid and 4 ml l$^{-1}$ of a trace element solution). The trace-element solution contained per litre: 34.6 g EDTA·$2H_2O$, 43.76 g $Na_3$citrate·$2H_2O$, 24.84 g $FeSO_4$·$7H_2O$, 256.4 g $MgSO_4$·$7H_2O$, 12.4 mg $H_3BO_3$, 12.4 mg $Na_2MoO_4$·$2H_2O$, 0.64 g $CuSO_4$·$5H_2O$, 2.52 g $ZnSO_4$·$7H_2O$, 0.64 g $CoSO_4$·$7H_2O$, 3.04 g $MnSO_4$·$H_2O$ and 1.24 g $CaCl_2$, 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 $KH_2PO_4$, 3.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$·$7H_2O$, 7.5 g of glucose and 10 ml of trace element solution. The trace element solution contained 15 g l$^{-1}$ $Na_2EDTA$·$2H_2O$, 0.5 g l$^{-1}$ $CuSO_4$·$5H_2O$, 2 g l$^{-1}$ $ZnSO4$·$7H_2O$, 2 g l$^{-1}$ $MnSO_4$·$H_2O$, 4 g l$^{-1}$ $FeSO_4$·$7H_2O$ and 0.5 g l$^{-1}$ $CaCl_2$·$2H_2O$. 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 $CO_2$ 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 $H_2SO_4$ at a flow rate of 0.6 ml min$^{-1}$ (Nasution et al., 2008). Quantitative $^1$HNMR 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 $^1$HNMR experiments were performed at 600 MHz on a Bruker Avance 600 spectrometer (Bruker, Wormer, 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 $D_2O$ 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 × 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). eGFP 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 560-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 $E_m$ for Motif Elucidation (MEME—version 3.5.4) (Bailey and Elkan, 1995). Promoters (from −1000 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
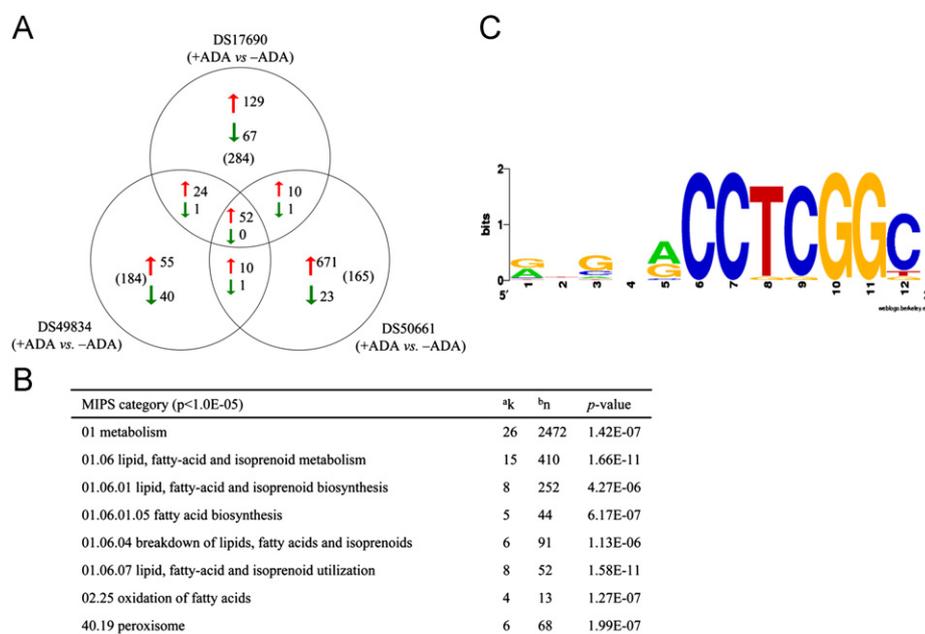


| MIPS category (p<1.0E-05) | [a]k | [b]n | *p*-value |
|---|---|---|---|
| 01 metabolism | 26 | 2472 | 1.42E-07 |
| 01.06 lipid, fatty-acid and isoprenoid metabolism | 15 | 410 | 1.66E-11 |
| 01.06.01 lipid, fatty-acid and isoprenoid biosynthesis | 8 | 252 | 4.27E-06 |
| 01.06.01.05 fatty acid biosynthesis | 5 | 44 | 6.17E-07 |
| 01.06.04 breakdown of lipids, fatty acids and isoprenoids | 6 | 91 | 1.13E-06 |
| 01.06.07 lipid, fatty-acid and isoprenoid utilization | 8 | 52 | 1.58E-11 |
| 02.25 oxidation of fatty acids | 4 | 13 | 1.27E-07 |
| 40.19 peroxisome | 6 | 68 | 1.99E-07 |

**Fig. 1.** Transcriptome analysis of *P. chrysogenum* strains DS17690, DS49834 (ad-7-ACCCA producing strain) and DS05661 (cluster free strain) in the presence and absence of adipic acid. A—Transcriptome comparison: Red arrows represent the number of genes with a higher transcript level and green arrows the genes that showed a lower transcript level in growth with ADA versus without. The total number of differentially expressed genes is mentioned in between brackets. B—MIPS functional categories overrepresented in the set of genes significantly differentially expressed in strains DS17690, DS49834 and DS50661 (FC > |2|, FDR=1%) grown in medium supplemented with adipic acid relative to medium not supplemented. [a]k represents the number of gene in MIPS category found in the differentially expressed genes and [b]n represents the number of genes of the same MIPS category found in the whole genome. Enrichment analysis p-value according to Fischer exact statistics. C—Enriched motifs identified in the upstream region of the 52 genes simultaneously expressed in all strains in the presence of ADA. Promoter analysis of the 1 kbp upstream region of groups of genes with similar transcriptional regulation was performed with a motif range from 4 to 12 nucleotides. Regulatory motifs with an *E*-value lower than $10^{-4}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).
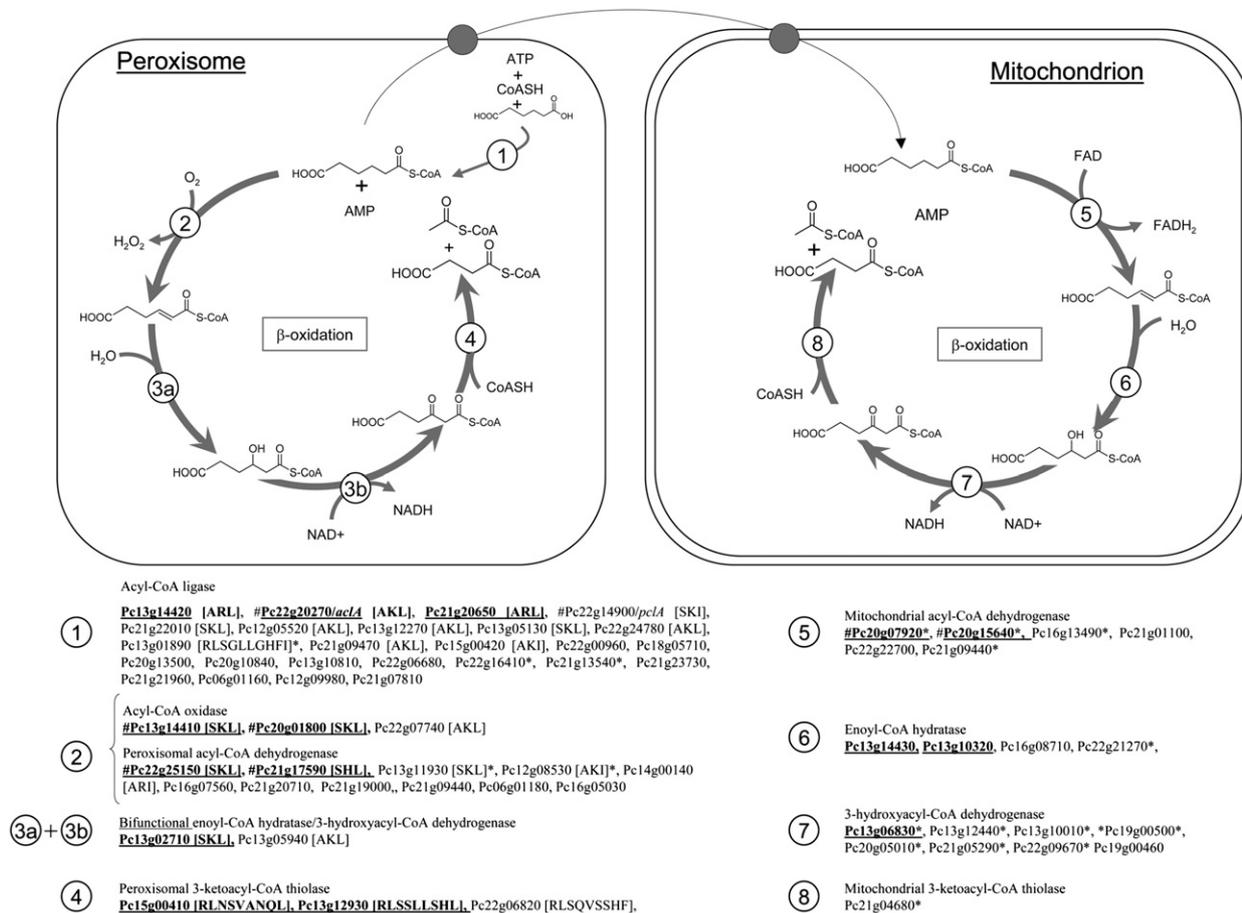
**Fig. 2.** Overview of β-oxidation in *P. chrysogenum*. Genes involved in adipic acid catabolism by β-oxidation and their putative placement in the metabolism of *P. chrysogenum*. (1) acyl-CoA ligase; (2) acyl-CoA oxidase and peroxisomal acyl-CoA dehydrogenase; (3a) enoyl-CoA hydratase activity of multifunctional enzyme; (3b) 3-hydroxyacyl:CoA dehydrogenase activity of multifunctional enzyme; (4) peroxisomal 3-keto-acyl-CoA thiolase; (5) mitochondrial acyl-CoA dehydrogenase; (6) enoyl:CoA hydratase; (7) 3-hydroxyacyl:CoA dehydrogenase; (8) mitochondrial 3-ketoacyl-CoA thiolase. The presence of a predicted peroxisomal targeting sequence (type 1 or 2) is indicated between brackets following the gene name. Genes of which the transcript levels were significantly and consistently changed in the presence of adipic acid in the DS17690, DS50661 and DS49834 strains are represented in bold and underlined. The # symbol indicates genes for which the gene products have been localized by eGFP tagging. The peroxisomal localization of Pc22g20720/AclA and Pc22g14900/PclA has been reported previously (Koetsier et al., 2010). Proteins encoded by the genes marked with an asterisk (*) were predicted to contain a mitochondrial targeting signal as defined by MITOPROT (Claros and Vincens, 1996).

[5′-CCKSGGB-3′] present in the upstream sequence (−1 kb) of 36 of these genes (Fig. 1; Table S2). This putative regulatory element 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 2).

### 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). Genes whose predicted protein sequences contained a peroxisomal targeting sequence (PTS1; Pc13g14410, Pc20g01800, Pc21g17590 and Pc22g25150) did not encode protein sequences with a mitochondrial targeting signal and therefore were fused to a DNA sequence encoding enhanced green fluorescent protein (eGFP) at their N-terminus. Genes whose predicted protein sequences contained a mitochondrial signal but lacked a PTS1-encoding sequence were fused to yield C-terminal fusions with eGFP (Pc20g07920 and Pc20g15640). A chimeric gene encoding a fusion protein containing the red fluorescent protein DsRed tagged with a canonical PTS1 (SKL) sequence was used as a marker for peroxisomes (Kiel et al., 2009). Mitochondria were marked with the fluorescent dye Mito-tracker®

**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 ([*pcbAB-pcbC-penDE*]Δ), DS49834 (*pcbC_P-cefEF-penDE_T pcbC_P-cmcH-penDE_T*) grown with (+) or without adipic acid (−). Transcript levels were determined with Affymetrix GeneChip DSM PENa520255F. 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.

| | Putative function | Sub. cell. loc. | DS17690 | | DS50661 | | DS49834 | | Similarity to |
| | | | − | + | − | + | − | + | |
| | | | ADA | ADA | ADA | ADA | ADA | ADA | |
|---|---|---|---|---|---|---|---|---|---|
| **Differentially expressed** | | | | | | | | | |
| Pc13g14410 | Oxidase | Perox (SKL) | 12.0 ± 0 | 437.9 ± 34 | 12.0 ± 0 | 1049.2 ± 136 | 12.0 ± 0 | 345.7 ± 96 | Hypothetical protein contig42.tfa_690wg—*A. fumigatus* |
| Pc20g01800 | Oxidase | Perox (SKL) | 125.4 ± 13 | 339.3 ± 40 | 62.5 ± 14 | 377.8 ± 73 | 112.6 ± 11 | 290.9 ± 50 | Hypothetical protein contig42.tfa_690wg—*A. fumigatus* |
| Pc20g07920 | Dehydrogenase | Mit # | 204.8 ± 47 | 1713.4 ± 155 | 157.7 ± 8 | 2137.6 ± 577 | 195.4 ± 28 | 1281.5 ± 200 | Glutaryl:CoA dehydrogenase GCHD—*H. sapiens* |
| Pc22g25150 | Dehydrogenase | Perox (SKL) | 96.9 ± 23 | 553.7 ± 55 | 85.9 ± 20 | 726.7 ± 58 | 92.6 ± 29 | 548.7 ± 110 | Acyl CoA dehydrogenase aidB—*E. coli* |
| Pc21g17590 | Dehydrogenase | Perox (SHL) | 838.7 ± 129 | 2142.6 ± 72 | 431.5 ± 117 | 2371.9 ± 364 | 698.9 ± 96 | 1510.2 ± 198 | Acyl-CoA dehydrogenase like An17g01150—*A. niger* |
| Pc20g15640 | Dehydrogenase | Mit # | 37.1 ± 6 | 229.0 ± 20 | 54.4 ± 18 | 312.3 ± 53 | 51.4 ± 8 | 220.3 ± 34 | Hypothetical protein—*Bradyrhizobium japonicum* |
| **Unchanged** | | | | | | | | | |
| Pc13g11930 | Dehydrogenase | nd | 51.4 ± 13 | 168.4 ± 14 | 149.2 ± 13 | 370.4 ± 31 | 70.8 ± 4 | 124.3 ± 45 | Acyl-CoA dehydrogenase aidB—*E. coli* |
| Pc12g08530 | Dehydrogenase | nd | 178.3 ± 13 | 226.3 ± 24 | 177.7 ± 44 | 258.2 ± 29 | 160.8 ± 23 | 264.8 ± 45 | Long-chain acyl-CoA dehydrogenase like An13g03940—*A. niger* |
| Pc16g07560 | Dehydrogenase | nd | 291.8 ± 156 | 528.4 ± 107 | 160.6 ± 43 | 243.1 ± 14 | 221.5 ± 76 | 344.8 ± 73 | Long-chain acyl-CoA dehydrogenase like An04g03290—*A. niger* |
| Pc21g20710 | Dehydrogenase | nd | 834.7 ± 166 | 1171.3 ± 163 | 669.4 ± 36 | 1240.9 ± 14 | 675.6 ± 39 | 1046.4 ± 69 | Isovaleryl-CoA dehydrogenase like An11g00400—*A. niger* |
| Pc16g13490 | Dehydrogenase | nd | 290.4 ± 10 | 242.0 ± 17 | 174.7 ± 29 | 303.2 ± 25 | 200.3 ± 29 | 295.2 ± 44 | Branched chain acyl-CoA dehydrogenase ACADSB—*H. sapiens* |
| Pc21g01100 | Dehydrogenase | nd | 97.0 ± 28 | 71.1 ± 8 | 61.9 ± 13 | 51.3 ± 5 | 69.7 ± 18 | 82.7 ± 17 | Isovaleryl-coenzyme A dehydrogenase like An07g04280—*A. niger* |
| Pc21g19000 | Dehydrogenase | nd | 93.7 ± 13 | 134.4 ± 24 | 103.2 ± 15 | 104.8 ± 17 | 82.4 ± 7 | 94.7 ± 17 | Hypothetical protein contig31_part_ii.tfa_2980wg—*A. fumigatus* |
| Pc14g00140 | Dehydrogenase | nd | 163.7 ± 72 | 320.7 ± 41 | 137.1 ± 24 | 188.4 ± 27 | 176.3 ± 56 | 223.9 ± 60 | Hypothetical protein contig 1 62 scaffold 4.tfa_510cg—*A. nidulans* |
| **Not expressed** | | | | | | | | | |
| Pc22g07740 | Oxidase | nd | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | Acyl-CoA oxidase tylP—*S. fradiae* |
| Pc22g22700 | Dehydrogenase | nd | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | Acyl-CoA dehydrogenase like protein An14g03240—*A. niger* |
| Pc21g09440 | Dehydrogenase | nd | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | Hypothetical protein contig.1.93_scaffold_6.tfa_70wg—*A. nidulans* |
| Pc06g01180 | Dehydrogenase | nd | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | 12.0 ± 0 | Hypothetical protein |
| Pc16g05030 | Dehydrogenase | nd | 12.0 ± 0 | 12.0 ± 0 | 14.3 ± 3 | 13.5 ± 2 | 15.8 ± 5 | 12.8 ± 4 | Acyl-CoA oxidase tylP—*S. fradiae* |

**Table 3**
Physiological and metabolites data obtained during aerobic glucose-limited chemostat cultivations supplemented with adipic acid ($D = 0.03$ h$^{-1}$, $T = 25$ °C, pH = 6.5) of the different *P. chrysogenum* strains. Data are presented as averages $\pm$ mean deviation from at least two independent replicate cultures.

| Strain | | g/g | mmol/g/h | | μmol/g/h | | | | Ratio |
|---|---|---|---|---|---|---|---|---|---|
| Sub. cell. localization | | $^{a}Y_{SX}$ | $^{b}q_{Glucose}$ | $^{b}q_{ADA}$ | $^{b}q_{ad-6-APA}$[c] | $^{b}q_{IPN}$[d] | $^{b}q_{6-APA}$[e] | $^{b}q_{8-HPA}$[f] | $q_{ad-6-APA}/q_{ADA}$ |
| DS17690 | | 0.41 ± 0.02 | 0.41 ± 0.02 | 0.054 ± 0.004 | 2.94 ± 0.27 | 0.78 ± 0.01 | 0.12 ± 0.03 | 0.64 ± 0.16 | 0.05 |
| DS66982 (Pc13g14410Δ) | Peroxisome | 0.38 ± 0.00 | 0.45 ± 0.00 | 0.031 ± 0.001 | 3.63 ± 0.43 | 1.45 ± 0.17 | 0.83 ± 0.12 | 0.00 ± 0.00 | 0.12 |
| DS68330 (Pc20g01800Δ) | Peroxisome | 0.36 ± 0.00 | 0.47 ± 0.00 | 0.019 ± 0.001 | 4.81 ± 0.50 | 0.99 ± 0.05 | 0.69 ± 0.06 | 0.09 ± 0.02 | 0.25 |
| DS63170 (Pc20g07920Δ) | Mitochondrion | 0.36 ± 0.02 | 0.47 ± 0.03 | 0.029 ± 0.012 | 11.00 ± 0.90 | 1.68 ± 0.25 | 1.10 ± 0.12 | 0.17 ± 0.01 | 0.38 |
| DS66984 (Pc22g25150Δ) | Peroxisome | 0.36 ± 0.01 | 0.47 ± 0.01 | 0.039 ± 0.004 | 3.77 ± 0.51 | 1.62 ± 0.24 | 0.91 ± 0.17 | 0.00 ± 0.00 | 0.10 |

[a] Biomass yield on glucose (g of biomass/g of glucose consumed).
[b] Biomass specifc production and consumption rate.
[c] Adipoyl-6-amino-penicillanic acid.
[d] Isopenicillin N.
[e] 6-amino-penicillanic acid.
[f] 8-hydroxy-penicillanic acid.

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 DsRed.SKL, indicating that these proteins are peroxisomal (Fig. 3A–D). Pc20g07920::eGfp and Pc20g15640::eGfp fusion proteins co-localized with the Mito-tracker® 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 data due to the recombinative transformation-associated loss of penicillin biosynthesis clusters (Harris et al., 2009a; Nijland et al., 2010). The strains were therefore analyzed by q-PCR, using the strains DS17690, DS54465 (hdfAΔ) (six to eight penicillin biosynthetic clusters), Wisconsin54-1255 (one penicillin biosynthetic cluster) and DS50562 (zero penicillin biosynthetic cluster) as references. The five deletion strains did not show significant changes in their penicillin biosynthetic gene cluster number relative to the DS17690 and DS54465 reference strains (data not shown) and were therefore used for further analysis.

To analyze the impact of the five genes on adipoylated β-lactam intermediates under industrially relevant conditions, the *P. chrysogenum* strains DS17690, DS68330 (amdS::Pc20g01800), DS63170 (amdS::Pc20g07920Δ), DS66982 (Pc13g14410Δ), DS66984 (Pc22g25150Δ) and DS66983 (Pc20g15640Δ) were grown in glucose-limited chemostat cultures that were supplemented with adipic acid. The deletion strain DS66983 (Pc20g15640Δ) did not grow with the usual filamentous morphology (dispersed hyphae) in the presence of adipic acid but instead consistently formed small pellets. Since this hindered the interpretation of the fermentation data, this strain was left out of the analysis. This left four deletion mutants that were quantitatively analyzed in glucose-limited

chemostat cultures. All of these deletion strains exhibited a lower biomass formation ($Y_{S/X}$) relative to the reference DS17690 strain (Table 3). This reduced biomass yield was correlated with a significantly (*t*-test, *p*-value < 0.01) lower consumption of adipic acid ($q_{ADA}$) in all four mutants. These results were in agreement with the hypothesis that β-oxidation enables *P. chrysogenum* to use adipic acid as a carbon and energy source and further suggests that all four acyl-CoA oxidases and dehydrogenases contribute to this pathway. However, of the four deletion mutants, only *P. chrysogenum* DS68330 (amdS::Pc20g01800) and DS63170 (amdS::Pc20g07920) showed a significant increase in the production of the semi-synthetic intermediate ad-6-APA (+2-fold and +4-fold, respectively). The mitochondrially located product of Pc20g07920, a putative acyl-CoA dehydrogenase, had the largest impact on ad-6-APA production (Table 3). Associated with their increased ad-6-APA biosynthesis, strains DS68330 (amdS::Pc20g01800) and DS63170 (amdS::Pc20g07920) also exhibited an improved incorporation of adipic acid into β-lactams. The ratio between the biomass specific ad-6-APA production rate ($q_{ad-6-APA}$) and the biomass specific adipic acid consumption rate ($q_{ADA}$) increased by almost 8-fold in *P. chrysogenum* DS63170 (Pc20g07920Δ) relative to the reference strain DS 17690 (0.38 compared to 0.05) (Table 3).

To explore the contribution of the putative mitochondrial acyl-CoA dehydrogenase Pc20g01800 and the putative peroxisomal acyl-CoA oxidase Pc20g07920 to the β-oxidation of different fatty acids, the strains *P. chrysogenum* DS68330 (amdS::Pc20g01800Δ) and DS63170 (amdS::Pc20g07920Δ) were grown in shake flasks with fatty acids as sole carbon source. *P. chrysogenum* DS17690 was unable to grow on short and medium chain fatty acids (from C$_4$ to C$_{14}$) as sole carbon source, but could grow on the long chain fatty acids oleic acid (C$_{18:1}$) and erucic acid (C$_{22:1}$). *P. chrysogenum* DS63170 (Pc20g07920Δ) retained the ability to grow on oleic acid, while the putative acyl-CoA oxidase mutant DS68330 (Pc20g01800Δ) did not show any growth on this substrate. On erucic acid, the reference strain DS17690 reached a much lower biomass concentration than on oleic acid. While both deletion mutants grew on this substrate, the final biomass concentrations reached during growth on erucic acid were less than 10% of the biomass reached by strain DS17690 (Table 4).

## 4. Discussion

### 4.1. Metabolic engineering of side-chain precursor degradation in P. chrysogenum

Metabolic engineering of *P. chrysogenum* for the production of semi-synthetic cephalosporins (Cantwell et al., 1992; Cantwell
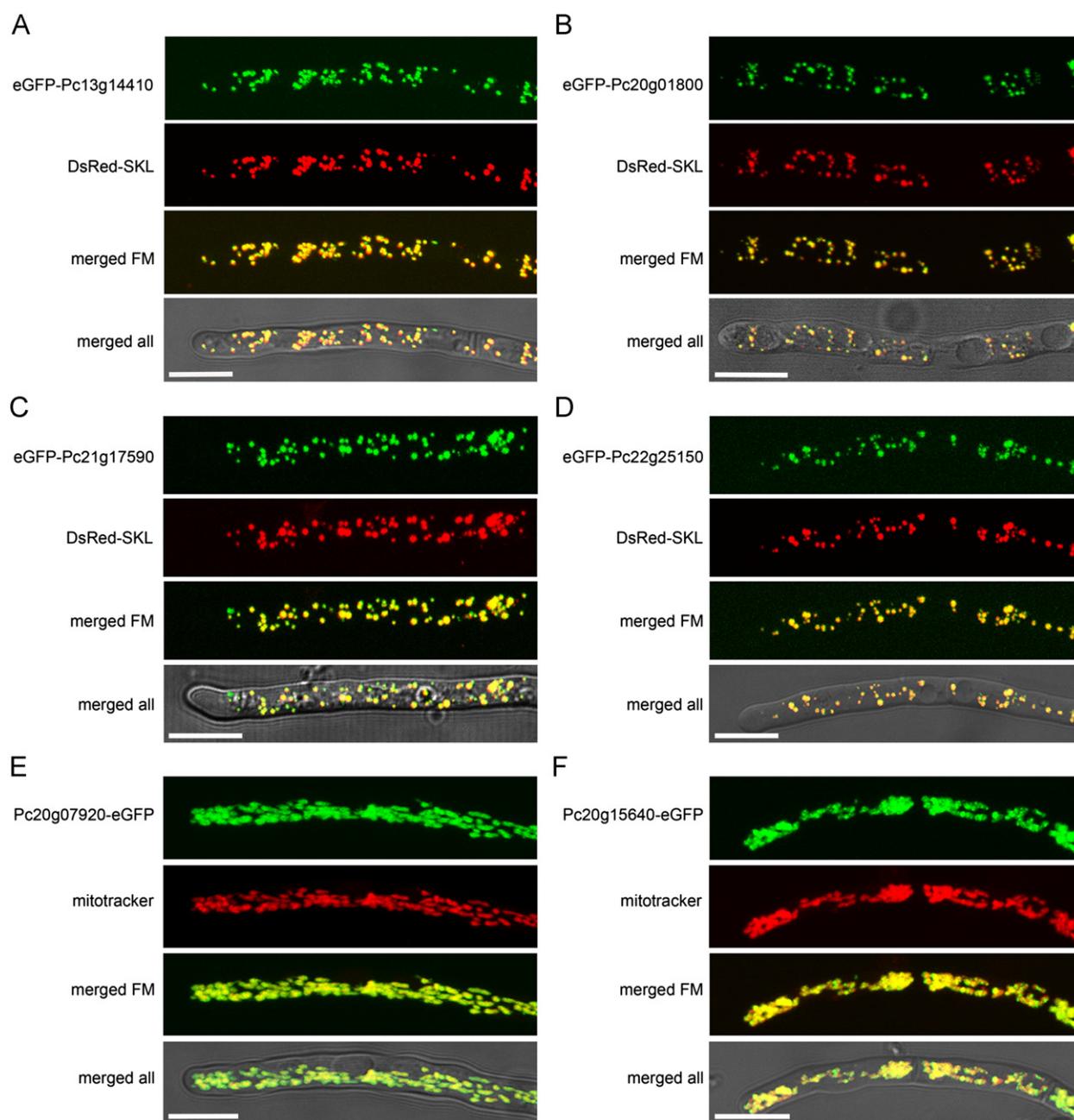
**Fig. 3.** Subcellular localization of acyl-CoA oxidases and dehydrogenases in *P. chrysogenum*. *P. chrysogenum* DsRed.SKL eGFP-Pc13g14410 (*panel A*), DsRed.SKL eGFP-Pc20g01800 (*panel B*), DsRed.SKL eGFP-Pc21g17590 (*panel C*), DsRed.SKL eGFP-Pc22g25150 (*panel D*), DS17690 Pc20g07920-eGFP (*panel E*) and DS17690 Pc20g15640-eGFP (*panel F*) cells were cultivated on penicillin production medium supplemented with phenoxyacetic 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 μm.

**Table 4**
Biomass dry weight concentrations (g l$^{-1}$) of *P. chrysogenum* grown in the presence of oleic (C18:1, $\omega$9) and erucic (C22:1, $\omega$9) acid as sole carbon source. Data are presented as averages $\pm$ mean deviation from three independent replicate shake flask cultures.

| Strain time [h] | Oleic acid (C18:1) | | Erucic acid (C22:1) | |
|---|---|---|---|---|
| | 166 | 286 | 214 | 305 |
| DS17690 | 1.52 ± 0.03 | – | 0.55 ± 0.13 | – |
| DS63170 (Pc20g07920Δ) | 0.00 ± 0.00 | 0.60 ± 0.16 | 0.05 ± 0.01 | 0.02 ± 0.02 |
| DS68330 (Pc20g01800Δ) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.04 ± 0.02 | 0.03 ± 0.02 |

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 β-lactam antibiotics and their precursors. Industrial fermentation constitutes a substantial contribution to the overall production cost of β-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,

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 chemostat-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 $\mu mol\, g^{-1}\, h^{-1}$ versus 20 $\mu mol\, g^{-1}\, h^{-1}$; 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 ($V_{max}$) of the competing reactions, but also by other properties (e.g. $K_m$ 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 ammonium-, 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.

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## Appendix A. Supporting information

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

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