



Functional characterization of the oxaloacetase encoding gene and elimination of oxalate formation in the β -lactam producer *Penicillium chrysogenum*

A.K. Gombert^{a,b,c}, T. Veiga^{a,b}, M. Puig-Martinez^{a,b}, F. Lamboo^{a,b}, J.G. Nijland^{b,d}, A.J.M. Driessen^{b,d}, J.T. Pronk^{a,b}, J.M. Daran^{a,b,*}

^a Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628BC Delft, The Netherlands

^b Kluyver Centre for Genomics of Industrial Fermentations, Julianalaan 67, 2628BC Delft, The Netherlands

^c Department of Chemical Engineering, University of São Paulo, C.P. 61548, 05424-970 São Paulo, SP, Brazil

^d Department of Molecular Microbiology, University of Groningen, Nijenborg 7, 9751 AG Groningen, The Netherlands

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ABSTRACT

Penicillium chrysogenum is widely used as an industrial antibiotic producer, in particular in the synthesis of β -lactam antibiotics such as penicillins and cephalosporins. In industrial processes, oxalic acid formation leads to reduced product yields. Moreover, precipitation of calcium oxalate complicates product recovery. We observed oxalate production in glucose-limited chemostat cultures of *P. chrysogenum* grown with or without addition of adipic acid, side-chain of the cephalosporin precursor adipoyl-6-aminopenicillanic acid (ad-6-APA). Oxalate accounted for up to 5% of the consumed carbon source. In filamentous fungi, oxaloacetate hydrolase (OAH; EC3.7.1.1) is generally responsible for oxalate production. The *P. chrysogenum* genome harbours four orthologs of the *A. niger oahA* gene. Chemostat-based transcriptome analyses revealed a significant correlation between extracellular oxalate titers and expression level of the genes Pc18g05100 and Pc22g24830. To assess their possible involvement in oxalate production, both genes were cloned in *Saccharomyces cerevisiae*, yeast that does not produce oxalate. Only the expression of Pc22g24830 led to production of oxalic acid in *S. cerevisiae*. Subsequent deletion of Pc22g24830 in *P. chrysogenum* led to complete elimination of oxalate production, whilst improving yields of the cephalosporin precursor ad-6-APA.

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1. Introduction

Eight decades after Fleming's discovery of penicillin, *Penicillium chrysogenum* remains the preferred organism for industrial production of penicillins (Demain and Elander, 1999; Elander, 2003; van den Berg et al., 2008). The high penicillin productivity and yield reached by current industrial strains is the result of many decades of intensive strain improvement by mutagenesis and selection (Newbert et al., 1997). These programmes also contributed to improved fermentation properties of this filamentous fungus in large-scale submerged fermentation processes (Kossen, 2000). The availability of the complete genome sequence of *P. chrysogenum* (van den Berg et al., 2008) now enables studies into the molecular basis of improved productivity in industrial strains, e.g. by using transcriptomics (Harris et al., 2009a,b; van den Berg et al., 2008) and proteomics (Jami et al., 2010). In addition, a full complement of genetic tools has been developed during recent years allowing an efficient gene deletion and functional analysis.

Metabolic engineering has endowed high-producing strains with the ability to produce semi-synthetic cephalosporins that are not naturally produced by *P. chrysogenum* (Crawford et al., 1995; Harris et al., 2009b; Nijland et al., 2008; Ullan et al., 2007). Cephalosporins have stronger antimicrobial activity towards Gram-negative bacteria and are less easily degraded by β -lactamases than penicillins. Further industrial applications of *P. chrysogenum* are likely to be developed in the near future, not only due to its advantageous fermentation properties, but also because of its potential as a producer of antimicrobial, antioxidant, and haemolytic compounds (Nakashima et al., 2008).

A common feature of filamentous fungi is the formation and excretion of oxalic acid (Currie and Thom, 1915; Dutton and Evans, 1996). In natural environments, this metabolite can confer advantages such as facilitation of the colonization of plants via sequestration of calcium from plant cell walls (Chen et al., 2010; Rio et al., 2008), release of soil nutrients by increasing their solubility (Cunningham and Kuiack, 1992; Gadd, 1999), detoxification of heavy metals (Fomina et al., 2005; Purchase et al., 2009), as an end-product of an energy-conserving metabolic pathway (Munir et al., 2001), and initiation of lignocellulose degradation (Dutton and Evans, 1996). Oxalate also contributes to the tolerance of the

* Corresponding author at: Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628BC Delft, The Netherlands.

E-mail address: j.g.daran@tudelft.nl (J.M. Daran).

industrial fungal species *Aspergillus niger* and *P. chrysogenum* towards the fungicide copper oxychloride (Gharieb et al., 2004). Degradation of oxalate by increasing the population size of oxalate degrading bacteria or overexpressing oxalate oxidase in plants has been shown to reduce plant pathogenicity of several fungi (Dong et al., 2008; Schoonbeek et al., 2007).

In industrial applications of filamentous fungi, oxalate is a frequently encountered by-product in submerged fermentations, especially at non-acidic pH (Kubicek et al., 1988). In industrial settings, oxalate production is highly undesirable, since diversion of carbon substrate to oxalate causes decreased product yields. Furthermore, additional downstream processing measures are required to remove calcium-oxalate crystals and to reduce oxalate levels in food and pharmaceutical products to acceptable levels (Pedersen et al., 2000a; Ruijter et al., 1999).

Oxalate formation in filamentous fungi occurs via a one-step cytosolic reaction catalysed by oxaloacetate hydrolase (OAH). This enzyme cleaves oxaloacetate into acetate and oxalate. Structural genes for OAH have already been cloned and characterized in *A. niger* (Pedersen et al., 2000b) and *Botrytis cinerea* (Han et al., 2007). Joosten et al. (2008) propose that the presence of a specific serine residue is essential for the enzymatic activity of OAH homologues (Joosten et al., 2008).

Although oxalate formation by *Penicillium* species was already described almost a century ago (Currie and Thom, 1915), oxalate formation in this genus has not yet been genetically characterized. In glucose-limited chemostat cultures of *P. chrysogenum* DS17690, an industrial penicillin producer, we observed that oxalate was present in culture supernatants unless phenylacetic acid, the side chain precursor of penicillin G, was included in growth media. The aim of this study was to identify the gene(s) responsible for oxalate formation in *P. chrysogenum* and, using this knowledge, to eliminate oxalate production by metabolic engineering.

2. Materials and methods

2.1. Strains and maintenance

The strains used in this study are listed in Table 1. *P. chrysogenum* DS17690 is a penicillin high-producing strain formerly used in industry by DSM-Anti-Infectives (Delft, The Netherlands). DS17690 strain request under material transfer agreement could be sent to Prof. RAL Bovenberg, DSM Anti-Infectives, P.O. Box 425, Delft NL-2600 AK, the Netherlands. Strain DS666248 (Pc22g24830Δ) was derived from the strain DS54465 (*hdfAΔ*) (Snoek et al., 2009), which has a high frequency of homologous recombination. All *P. chrysogenum* strains were kept on rice. All *Saccharomyces cerevisiae* strains (Table 1) used are derived from the CEN.PK strain family (van Dijken et al., 2000). Stock cultures were prepared on synthetic medium supplemented with 20 g l⁻¹

glucose (Hazelwood et al., 2010). When mid-log phase was reached, sterile glycerol was added to 30% glycerol (vol/vol) and 2 ml aliquots were stored at −80 °C.

2.2. Recombinant DNA technology

P. chrysogenum strain construction: *Escherichia coli* DH5α was used as host strain for high frequency transformation. Plasmid DNA amplification (Sambrook et al., 1989) and plasmid construction were performed with the Multisite Gateway® Three-Fragment Vector Construction Kit (Invitrogen, Breda, The Netherlands). Vector pDONR™ P4-P1R (Invitrogen, Breda, The Netherlands) was used for cloning of the 1436 bp 5' flanking region of the Pc22g24830 gene using primers attB4F Pc22g24830 *Nru*I and attB1R Pc22g24830 yielding pDONR41-5'FR 22g24830 (Table 2). In pDONR™ P2-P3R (Invitrogen, Breda, The Netherlands) the 1472 bp 3' flanking region of the Pc22g24830 gene was cloned using primers attB2F Pc22g24830 and attB3R Pc22g24830 *Xho*I yielding pDONR23-3'FR 22g24830. The pDONR™221 gateway vector was used to clone the *amdS* gene under control of the *gpdA* promoter and the *penDE* terminator which were amplified from plasmid pBlueAMDS with primers attB1F AMDS and attB2R AMDS. Combining these pDONR™ vectors with the pDEST™ P4-R3 in the LR reaction of the Multisite Gateway® Three-Fragment Vector Construction Kit a destination vector (pDest43-KO-22g24830) was made with both flanking regions of the Pc22g24830 gene of about 1500 bp and the *amdS* gene in between as selection marker. The pDest43-KO-22g24830 has two restriction enzymes sites (*Nru*I and *Xho*I) with which the deletion cassette was cut from the vector. Subsequently, pDest43-KO-22g24830 was transformed in the strain DS54465 (*hdfAΔ*) (Snoek et al., 2009) resulting in strain DS666248.

S. cerevisiae strain construction: Pc18g05100 and Pc22g24830 cDNAs were PCR amplified using primer pairs Pc18g05100_FWD/Pc18g05100_REV and Pc22g24830_FWD/Pc22g24830_REV respectively (Table 2) and a total RNA sample isolated from glucose-limited chemostat with adipic acid of the *P. chrysogenum* DS17690 strain (Harris et al., 2009b). PCR products were cloned in plasmid pENTR™/D-TOPO (Invitrogen, Breda, The Netherlands). For each cDNA, two independent entry clones were sequenced to verify the fidelity of the PCR reactions. The obtained entry clones were further recombined in an LR clonase™ reaction with the destination plasmid PVV214 (Van Mullem et al., 2003), which carries a *PGK1* promoter and *CYC1* terminator upstream and downstream of the att recombination sites, respectively. The expression vector pUDE05100 carries the Pc18g05100 cDNA placed under the control of the *PGK1* promoter. Similarly the plasmid pUDE24830 overexpressed Pc22g24830. Plasmids pUDE05100 and pUDE24830 were transformed with the lithium acetate protocol (Gietz and Schiestl, 2007) into *S. cerevisiae* CEN.PK113-5D, resulting in strains IME043 and IME046 respectively (Table 1).

Table 1
Strains used in this work.

Organism	Strain	Genotype ^a	Reference
<i>P. chrysogenum</i>	DS17690	High penicillin producer	Harris et al. (2009a)
<i>P. chrysogenum</i>	DS54465	<i>hdfAΔ</i>	Snoek et al. (2009)
<i>P. chrysogenum</i>	DS666248	<i>hdfAΔ</i> Pc22g24830Δ- <i>amdS</i>	This work
<i>P. chrysogenum</i>	DS50661	<i>pcbABΔ</i> <i>pcbCA</i> <i>penDEΔ</i>	Harris et al. (2009a)
<i>P. chrysogenum</i>	DS49834	<i>pcbABΔ</i> <i>pcbCA</i> <i>penDEΔ</i> <i>pcbC_{pr}-cefEF-penDE_{ter}</i> <i>pcbC_{pr}-cmcH-penDE_{ter}</i>	Harris et al. (2009b)
<i>S. cerevisiae</i>	CEN.PK113-5D	MATa <i>ura3-52</i>	P. Kotter ^a
<i>S. cerevisiae</i>	IME043	MATa <i>ura3-52</i> pUDE5100 (<i>URA3</i> <i>PGKp1_p::Pc18g05100::CY1_t</i>)	This work
<i>S. cerevisiae</i>	IME046	MATa <i>ura3-52</i> pUDE24830 (<i>URA3</i> <i>PGKp1_p::Pc22g24830::CY1_t</i>)	This work
<i>S. cerevisiae</i>	IME047	MATa <i>ura3-52</i> pVV214 (<i>URA3</i> , <i>PGKp1_p::CY1_t</i>)	This work

^a Institut für Molekulare Biowissenschaften, J.W. Goethe Universität Frankfurt, Frankfurt, Germany.

Table 2

Primers used in this study.

Primer	Sequence (5' → 3')
Pc18g05100_FWD	CACCACAATAATGGCTCTTCCATCG
Pc18g05100_REV	GTTATTCTCTTCCAAATCCACACC
Pc22g24830_FWD	CACCAACATGACTATCACAATCACCG
Pc22g24830_REV	CTAGACACCTCGGCAAAACC
attB4F Pc22g24830 <i>NruI</i>	GGGGACAACCTTTGTATAGAAAAGTTGCAGCATCCGGTTGGTCG TCGCG
attB1R Pc22g24830	GGGACTGCTTTTGTACAAACTTG CGTAGTATCCATCCTTCTCGACGG
attB2F Pc22g24830	GGGGACAGCTTTCTGTACAAAAGTGGG TGTCTAGGAATATATGATGGCG
attB3R Pc22g24830 <i>XhoI</i>	GGG GACAACCTTTGTATAATAAAGTTGGAATTGTGGCGTCACAACTCTGAGG
attB1F AMDS	GG GACAAGTTTGTACAAAAAGCAGGCTCGCAGGAATTCGAGCTCTGTAC
attB2R AMDS	GGGGACCACTTTGTACAAGAAAGCTGGGTCTC GCTCGTACCATGGGTTGAG
<i>F-ams</i>	GAAAGTCCAGACGCTGCTGCG
<i>R-ams</i>	CCCTGGTG CATATGTTAGCTG
F24830	AAGCTGACTAAGGACCGCTGC
R24830	CTACTCCATCGGCTTGTAGGCG
<i>F-γ-actin</i> gDNA	TTCTTGG CCTCGAGTCTGGCGG
<i>R-γ-actin</i> gDNA	GTGATCTCTTCTGCATAC GGTCC
F-IGR Pc20g07090	GTTCTATAGGA CGTAGCTCCGC
R-IGR Pc20g07090	AAATCAGCTCTACTAGCGATC CGC
F-pcbAB	CACTTGACGTTGCGCACCGTGC
R-pcbAB	CTGGTGGGTGAGAA CCTGACAG
F-pcbC	AGGGTTACCTCGATATCGAGGCG
R-pcbC	GTCGCCGTACGAGATTGGCC

2.3. Media and cultivation conditions

P. chrysogenum strains were grown in aerobic glucose-limited chemostats in 3 l bioreactors (Applikon, Schiedam, The Netherlands). The media contained the following components (per litre): 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 Cu₂SO₄·5H₂O, 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. To initiate production of cephalosporin intermediates, 5.0 g l⁻¹ of adipic acid (ADA) was added to the medium followed by recalibration of the medium pH to 6.5 with KOH.

For the chemostat growth, in the initial batch phase, the working volume was 1.5 l and the stirrer speed was set to 350 rpm upon inoculation and increased to 500 rpm and further onto 750 rpm, to

maintain the dissolved oxygen level above 50%. The oxygen level was continuously monitored with an oxygen electrode (Applisens, Schiedam, The Netherlands). The fed-batch phase began at the CO₂ concentration of 0.3%. The switch to continuous culture was carried out when the working volume reached 1.8 l. During the different cultivation phases temperature was controlled at 25 °C and the pH was maintained at 6.5, with the automatic addition of 2 M NaOH (ADI 1031 biocontroller, Applikon, Schiedam, The Netherlands). The bioreactor was sparged with air at a flow rate of 900 ml/min, using a Brooks mass-flow controller (Brooks Instruments, Hatfield, PA). For analysis purposes, the off-gas was cooled by a condenser (4 °C) and dried with a Perma Pure dryer (MD-110-48P-4, Permapure, Toms River, NJ). Concentrations of CO₂ and O₂ in the off-gas were measured with a NGA 2000 Rosemount analyser (Rosemount Analytical, Solon, OH). Off-gas flow rate was measured

Table 3

Physiological and metabolites data obtained during aerobic glucose-limited chemostat cultivations ($D = 0.03 \text{ h}^{-1}$, $T = 25 \text{ °C}$, $\text{pH} = 6.5$) of different *Penicillium chrysogenum* strains under several side chain precursor feeding regimes.

Strain	Yields and rates					Extracellular concentration of β-lactam intermediates (μM)				Reference
	Side chain	Y_{SX} (g/g) ^a	q_{GLUCOSE} (mmol/g/h) ^b	$q_{\text{SIDE CHAIN}}$ (mmol/g/h) ^b	$q_{\text{OXALIC ACID}}$ (mmol/g/h) ^b	ad-6-APA ^c	IPN ^d	6-APA ^e	8-HPA ^f	
DS17690	PAA	0.35 ± 0.01	0.48 ± 0.02	0.024 ± 0.002	0.0010 ± 0.0005	BDL ^g	N.D. ^h	N.D. ^h	N.D. ^h	van den Berg et al. (2008)/This study Koetsier et al. (2010) This study
	ADA	0.41 ± 0.02	0.41 ± 0.02	0.054 ± 0.004	0.07 ± 0.01	304.3 ± 28.3	81.0 ± 0.9	12.0 ± 3.2	66.2 ± 16.1	
	None	0.39 ± 0.00	0.43 ± 0.01	NA ⁱ	0.06 ± 0.01	BDL ^g	109.4 ± 1.3	15.4 ± 8.2	223.0 ± 20.4	
DS50661	PAA	0.36 ± 0.01	0.45 ± 0.01	0.006 ± 0.00	0.0012 ± 0.0001	BDL ^g	BDL ^g	BDL ^g	BDL ^g	Harris et al. (2009a)/This study Harris et al. (2009b)/This study Harris et al. (2009a)/This study
	ADA	0.47 ± 0.02	0.36 ± 0.05	0.11 ± 0.008	0.05 ± 0.01	BDL ^g	BDL ^g	BDL ^g	BDL ^g	
	None	0.39 ± 0.01	0.41 ± 0.01	NA ⁱ	0.03 ± 0.00	BDL ^g	BDL ^g	BDL ^g	BDL ^g	
DS49834	ADA	0.36 ± 0.01	0.42 ± 0.03	0.038 ± 0.006	0.06 ± 0.00	234 ± 13.5	45.2 ± 1.9	6.9 ± 2.0	36.8 ± 5.9	Harris et al. (2009b)/This study Harris et al. (2009b)/This study
	None	0.35 ± 0.01	0.44 ± 0.01	NA ⁱ	0.05 ± 0.01	BDL	70.8 ± 1.1	8.2 ± 3.8	190.5 ± 12.1	
DS666248	ADA	0.44 ± 0.03	0.39 ± 0.03	0.047 ± 0.028	BDL ^g	423.9 ± 87.5	135.9 ± 74.2	87.5 ± 45.4	N.D. ^h	This study
ΔPc22g24830 / <i>PcoahA</i>	None	0.40 ± 0.02	0.42 ± 0.02	NA ⁱ	BDL ^g	BDL ^g	45.9 ± 4.3	13.3 ± 1.9	33.6 ± 4.1	This study

^a Biomass yield on glucose (g of biomass/g of glucose consumed).

^b Biomass specific production and consumption rate.

^c Adipoyl-6-amino-penicillanic acid.

^d Isopenicillin N.

^e 6-Amino-penicillanic acid.

^f 8-Hydroxy-penicillanic acid.

^g Below detection limit (<5 μM).

^h Not determined.

ⁱ Not applicable.

with a SAGA digital flowmeter (Ion Science, Cambridge, UK). In the continuous culture, the dilution rate was set to 0.03 h^{-1} , and the feed medium was supplied continuously by a peristaltic pump (Cole Parmer, Vernon-Hill, IL). The working volume was adjusted discontinuously, by periodically removing approximately 1% of the culture volume using a specifically designed overflow effluent device (Gulik et al., 1989). Silicone antifoam (Bluestar Silicone, Lyon, France) was added discontinuously to prevent excess foaming. Chemostat cultures were assumed to be in steady-state after at least five volume changes and when culture dry weight and off-gas CO_2 analyses differed by less than 4% over two consecutive volume changes.

Shake-flask cultivation of *S. cerevisiae* was performed in 500 ml flasks filled with 100 ml chemically defined medium (Hazelwood et al., 2010; Verduyn et al., 1992). The medium was supplemented with either 3.0 g l^{-1} urea or 13.3 g l^{-1} aspartate, providing the same molar amount of nitrogen.

2.4. Analytical determinations

Biomass dry weight was measured by filtering culture samples (10 ml) over pre-weighed glass fibre filters (Type A/E, Pall Life Sciences, East Hills, NY). The filters were then washed with demineralised water, dried in a microwave oven for 20 min at 600 W and reweighed. Glucose, adipic acid and oxalate concentrations in media and culture supernatants were analysed 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) containing a Biorad HPX87H column (Biorad, Hercules, CA) eluted at $60\text{ }^\circ\text{C}$ with $0.5\text{ mM H}_2\text{SO}_4$ at a flow rate of 0.6 ml min^{-1} . Quantitative ^1H NMR was used to measure extracellular concentration of penicillins (G and V), ad-6-APA, IPN, 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

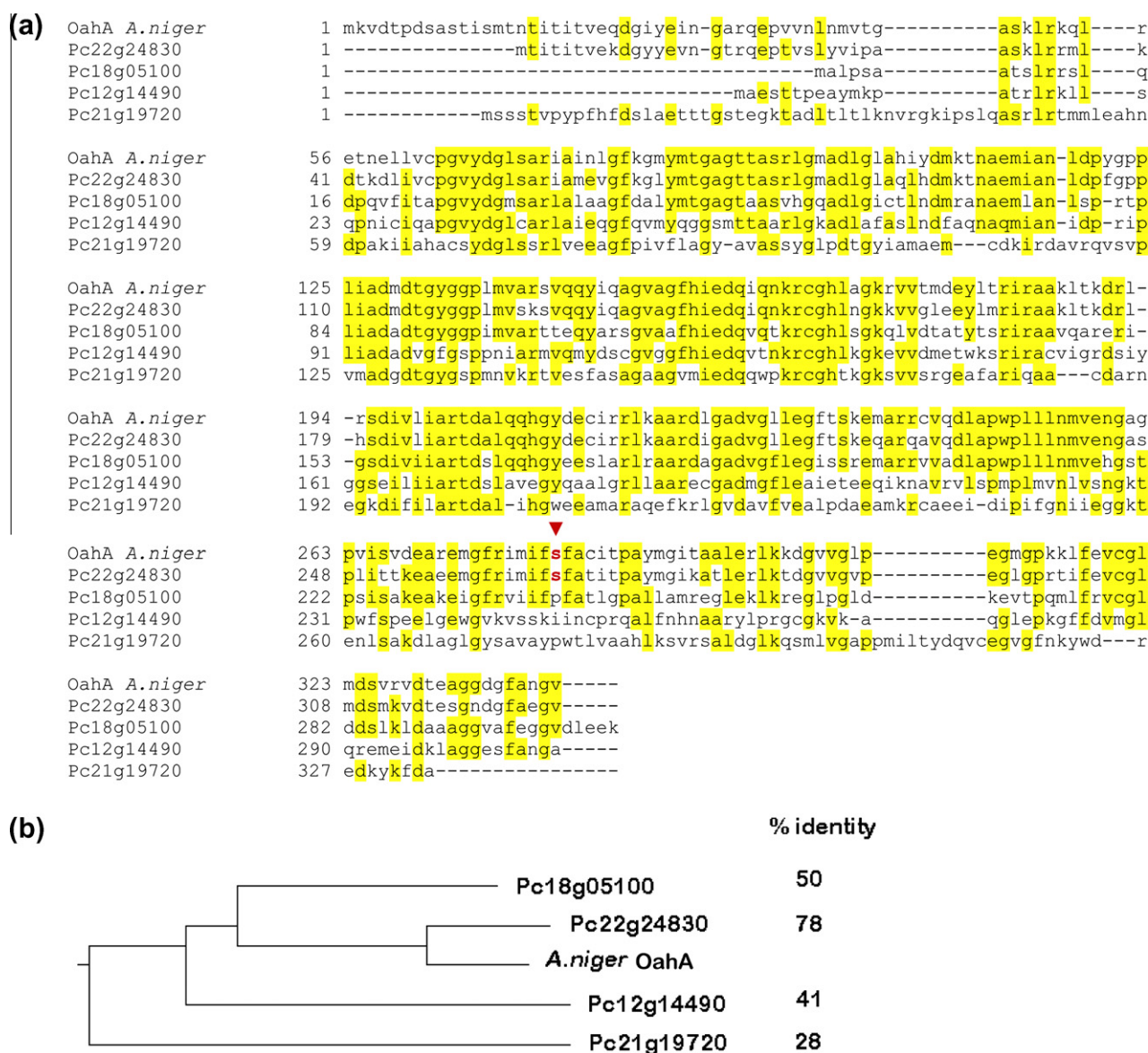


Fig. 1. (a) Alignment sequence of the four putative *P. chrysogenum* oxaloacetate hydrolases amino acid sequences. The red arrow indicates the conserved serine residue 281 and 266 in *A. niger* OahA sequence and in the Pc22g24830 amino acid sequence, respectively, characteristic of oxaloacetase hydrolases. (b) The phylogenetic tree is based on a Clustal-X alignment of the primary amino acid sequences of *P. chrysogenum* Pc12g1490, Pc18g05100, Pc21g19720, Pc22g24830 and *A. niger* OahA and was constructed using TREECON for Windows. The percentage of identity of the amino acid sequences was based on pair-wise alignment of the *P. chrysogenum* amino acid sequences with the *A. niger* OahA protein sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(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 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 (Koetsier et al., 2010).

2.5. Preparation of cell extracts

For preparation of cell extracts of *P. chrysogenum* and *S. cerevisiae*, cultures samples were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA and stored at −20 °C. Before cell disruption, cells were thawed at room temperature and the pellets were washed twice in ice-cold 100 mM phosphate buffer containing 2 mM Mg²⁺ and 2 mM dithiothreitol. Cell suspension samples (1 ml) were transferred to a pre-cooled safe-lock tube with 0.75 g of glass beads (425–600 µm) and introduced into a Fast Prep machine (Thermo Scientific, Erembodegem, The Netherlands) and subjected to 4 bursts of 20 s at speed setting 6. The tube was cooled on ice for 60 s between consecutive bursts. Each extract centrifuged for 20 min at 4 °C and 36,000g. The supernatants were used as cell extracts and kept on ice until enzyme-activity assays were performed. Protein measurements were performed according to the Lowry method (Lowry et al., 1951).

2.6. Oxaloacetase activity assay

Oxaloacetate acetyl hydrolase (EC 3.7.1.1) activity was measured using a modification of the direct optical determination of

oxaloacetate (OAA) at 255 nm as described in (Lenz et al., 1976). The disappearance of the enol tautomer of OAA was monitored at 255 nm ($\epsilon = 1.1 \text{ mM}^{-1} \text{ cm}^{-1}$) at 25 °C in a Hitachi model 100–60 spectrophotometer (Hitachi, Tokyo, Japan), using quartz cuvettes. The 1 ml reaction mixture contained 100 mM imidazole-HCl (pH 7.5), 0.9 mM MnCl₂·2H₂O, 1 mM OAA, 20 µL of cell extract (controls with different volumes of cell extracts confirmed linearity between enzyme activity and amount of cell extract). The reaction was started by addition of the cell extract.

2.7. Transcriptome data

Transcriptome data were derived from the accession series, GSE9825 (van den Berg et al., 2008), GSE12632 (Harris et al., 2009a), GSE12617 (Koetsier et al., 2010) (<http://www.ncbi.nlm.nih.gov/geo/>).

2.8. Sequence analysis of oxaloacetase homologues in *P. chrysogenum*

The 341 amino acids sequence of the *A. niger* oxaloacetase protein (UniProt entry Q7Z986) was employed as a query to interrogate the translated genome of *P. chrysogenum* Wisconsin 54-1255 (van den Berg et al., 2008) using the tblastn algorithm (Altschul et al., 1997).

3. Results

3.1. Oxalate formation and identification of putative oxaloacetase hydrolase genes in *P. chrysogenum*

Oxalate formation by *Penicillium* species was first reported almost a century ago (Currie and Thom, 1915). To investigate oxalate

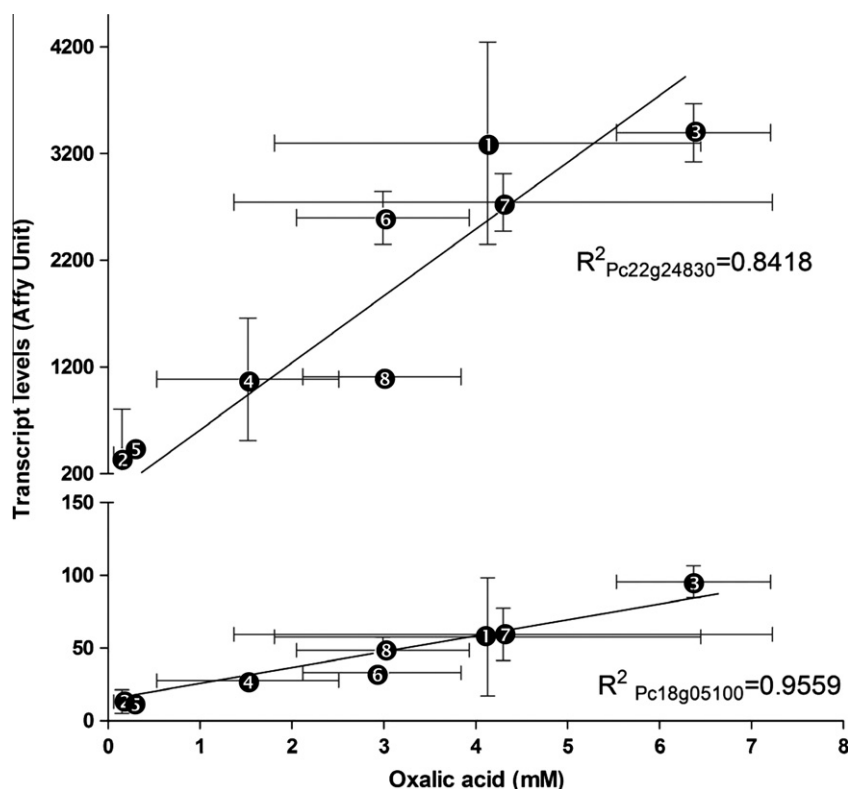


Fig. 2. Correlation between expression of Pc18g05100 and Pc22g24830 and oxalate production in three *P. chrysogenum* strains grown in glucose-limited chemostat with different side chain precursors. The strain DS50661 is a derivative from the strain DS17690 that misses the penicillin biosynthetic genes (*pcbAB*, *pcbC* and *penDE*). The strain DS49834 is derivative from the strain DS50661 that overexpressed the *A. chrysogenum* *cefEF* gene encoding an expandase/hydroxylase, and the *S. clavuligerus* *cmhC* gene encoding a 3'-hydroxymethylcephem-O-carbamoyltransferase. ① DS17690 grown without side chain precursor, ② DS17690 grown with PAA, ③ DS17690 grown with ADA ④ DS50661 grown without side chain precursor, ⑤ DS50661 grown with PAA, ⑥ DS50661 grown with ADA, ⑦ DS49834 grown without side chain precursor, ⑧ DS49834 grown with ADA. Error bars for both gene expression and oxalate are shown and were derived from at least triplicate experiments.

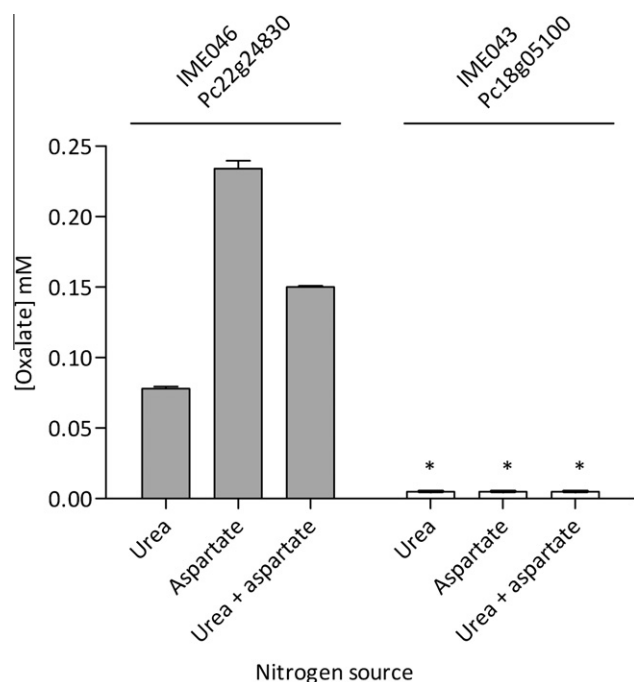


Fig. 3. Extracellular oxalate accumulation by *S. cerevisiae* strains expressing Pc22g24830/*PcoahA* and Pc18g05100 cDNA from *P. chrysogenum*. Oxalate concentrations were measured after 12 h of cultivation. All cultures were inoculated with the same amount of cells and sampled in mid-log phase. Data are presented as average \pm mean deviation of results from two independent cultures for each strain. *Below detection ($<5 \mu\text{M}$).

production by the penicillin high-producing strain *P. chrysogenum* DS17690, glucose-limited chemostat cultures were grown in the presence or absence of phenylacetate and adipate, which are side chain precursors for penicillin-G and the semi-synthetic cephalosporin precursor ad-6-APA side chain precursors, respectively. In chemostat cultures grown with or without adipate *P. chrysogenum* DS17690 produced oxalate at concentrations up to 7 mM, which corresponds to 5% of the consumed glucose carbon (Table 3). Similarly oxalate formation was observed in glucose-limited chemostat cultures of the strains DS50661 (completely lacks the β -lactams biosynthetic genes (Harris et al., 2009b)) and DS49834 (produces a cephamycin intermediate ad-7-ACCCA (Harris et al., 2009b)), when grown in the presence and absence of adipate (Table 3). Remarkably, in contrast, addition of the penicillin-G side chain precursor phenylacetate led to an at least 60-fold lower oxalate production (Table 3). These results indicate that while oxalate formation may not represent a major problem in industrial penicillin production, it is likely to cause a significant loss of substrate carbon in industrial cephalosporin fermentation, when the growth media are supplemented with adipate.

In filamentous fungi, oxalate formation involves the oxaloacetate hydrolase (OAH) that catalyses the conversion of oxaloacetate into acetate and oxalate. A genome-wide search of the *P. chrysogenum* protein database (comprising 13,670 proteins; Genbank accession number: AM920416 to AM920464 (van den Berg et al., 2008)) for putative oxaloacetate hydrolases was performed by a BlastP analysis (Altschul et al., 1997). Four *P. chrysogenum* ORFs were identified whose predicted protein sequences were highly similar (E value $< 1.0\text{E}-20$) to the *A. niger* OahA amino acid sequence (Fig. 1). All four proteins shared similarity with phosphoenol-pyruvate phosphonmutase and related enzyme family (pfam PF00463). Of these four ORFs, only Pc22g24830 contained a conserved serine at position 266, equivalent to Ser281 in the *A. niger* oxaloacetate hydrolyase, which has been demonstrated to

be essential for enzymatic activity (Han et al., 2007) (Fig. 1). To assess the possible involvement of the four OahA homologues, chemostat-based transcriptome data of *P. chrysogenum* DS17690 (van den Berg et al., 2008), DS50661 (Harris et al., 2009a) and DS49834 (Harris et al., 2009b; Koetsier et al., 2010) obtained at various side-chain regimes, were compared to the oxalate titers measured under the same conditions (Table 3). Of the four putative oxaloacetate hydrolase genes, only two (Pc22g24830 and Pc18g05100) were expressed in the chemostat cultures. The strong reduction of oxalate production in culture grown in the presence of phenylacetate was correlated with a significant repression of Pc18g05100 and Pc22g24830 expression (Fig. 2). Based on these results, Pc22g24830 and Pc18g05100 were further investigated as candidate structural genes for oxaloacetate hydrolase.

3.2. Cloning and functional characterization of two putative oxaloacetate hydrolases from *P. chrysogenum*

To functionally analyse Pc18g05100 and Pc22g24830, their cDNAs were cloned from mRNA samples isolated from glucose-limited chemostat cultures of *P. chrysogenum* DS17690 grown in presence of adipate. To verify whether any mutations in these ORFs had been introduced during the 20 years extensive strain improvement program that separates strain DS17690 from the sequenced strain Wisconsin 1255-54 (van den Berg et al., 2008), the two cDNAs were sequenced. This showed that the DNA sequences of the two ORFs were identical in these two strains (data not shown).

Both cDNAs were expressed in *S. cerevisiae*, which does not normally produce oxalate, under the control of the strong, constitutive *PGK1* promoter (Van Mullem et al., 2003). In shake flask cultures grown on a glucose synthetic medium, only the *S. cerevisiae* strain containing the Pc22g24830 showed detectable production of oxalate (up to 0.08 mM, Fig. 3). Oxalate concentration increased by three fold when aspartate rather than urea was provided as sole nitrogen source, presumably by increasing the availability of intracellular oxaloacetate (Fig. 3). Consistent with the oxalate production profiles, oxaloacetate hydrolase activity was found in cell extracts of *S. cerevisiae* IME046 (*PGK1p::Pc22g24830*) but not in strain IME043 (*PGK1p::Pc18g05100*) (Table 4). Based on these results, Pc22g24830 was renamed *PcoahA*.

3.3. Elimination of oxalate formation in *P. chrysogenum* and its impact on cell physiology

Having established that Pc22g24830/*PcoahA* encodes a functional oxaloacetate hydrolase, we investigated whether this is the sole gene responsible for oxalate formation by *P. chrysogenum*. To this end, a deletion mutant of *PcoahA* was constructed in *P. chrysogenum* DS54465 (DS17690 *hdfAΔ*) (Snoek et al., 2009), a strain facilitating homologous recombination, resulting in the strain DS666248 (*hdfAΔ PcoahAΔ-amds*). The *PcoahA* deletion strain was grown in glucose-limited chemostat cultures with and without adipic acid. Under these conditions, deletion of *PcoahA* had no major impact on biomass yield on glucose (Y_{SX}), specific rate of glucose consumption (q_{GLUCOSE}) or specific rate of adipic acid consumption ($q_{\text{SIDE-CHAIN}}$) (Table 3). However, oxalate formation was completely abolished by deleting *PcoahA*. Oxaloacetate hydrolase activity assays in cell extracts of *P. chrysogenum* strains DS17690, DS54465 and DS666248 confirmed that, under the conditions tested, *PcoahA* (Pc22g24830) was the only oxaloacetate hydrolase encoding gene (Table 4). The formation of β -lactam intermediate ad-6-APA increased marginally but significantly, in the deletion strain relative to the reference (+1.36-fold, with a p -value < 0.01 , Table 3).

Table 4

Oxaloacetate hydrolase activity in cell-free extract of *S. cerevisiae* and *P. chrysogenum*. *S. cerevisiae* strains were grown in shake flask (SF) on chemically defined medium with aspartate as sole nitrogen source. *P. chrysogenum* strains were grown in chemostat cultures (CC) with or without (w/o) side chain precursor phenylacetate (PAA). Data are presented as average \pm mean deviation of results from at least two independent cultures for each strain.

Strain	Genotype	Culture format	Activity (U/mg protein)
<i>S. cerevisiae</i>	IME046	PGK1 _p ::Pc22g24830::CYC1 _t	SF
	IME043	PGK1 _p ::Pc18g05100::CYC1 _t	SF
	IME047	PGK1 _p ::::CYC1 _t	SF
<i>P. chrysogenum</i>	DS17690	CC + PAA	0.27 \pm 0.02
	DS17690	CC w/o	0.00 \pm 0.00
	DS666248	Pc22g24830 Δ	0.00 \pm 0.00
		CC w/o	0.29 \pm 0.01

4. Discussion

Although oxalate production is a well-known and extensively studied subject in filamentous fungi (Currie and Thom, 1915; Kubicek et al., 1988; Ruijter et al., 1999), oxalate production by penicillin-producing *P. chrysogenum* has hitherto received little attention. The present study shows that oxalate can be a major byproduct in aerobic, sugar-limited cultures of a penicillin-G high-producing *P. chrysogenum* strain. The growth conditions in such cultures are highly similar to those in the large-scale industrial fed-batch cultures used for commercial penicillin production. The observed reduction of oxalate production during growth in the presence of the penicillin-G side-chain precursor phenylacetic acid could be linked to transcriptional down-regulation of the newly identified oxaloacetase structural gene Pc22g24830/*PcoahA* and this explains why oxalate production has probably not been a major problem in industrial penicillin-G production.

In studies on high-producing strains of *P. chrysogenum* such as the DS17690 strain used in this study, the question whether an observed phenotype is a 'natural' property or has been acquired during classical strain improvement is legitimate. Chemostat-based transcript analysis of Pc22g24830/*PcoahA* in the strains NRRL1951 and Wisconsin 1255-54, which are early ancestors of the DS17690 strain (van den Berg et al., 2008) revealed that repression of *PcoahA* expression by PAA already occurs in the early strains of the lineage (Fig. 4). This suggests that this repression may be a fortuitous characteristic of the natural isolate from which the strain improvement programme started. So far, the culture parameter most

closely involved in regulation of oxaloacetase expression in filamentous fungi is culture pH (Kubicek et al., 1988; Ruijter et al., 1999). In *A. niger* the promoter of the *oahA* gene contains a putative FacB cis-regulatory elements (Pedersen et al., 2000b). In *A. nidulans* FacB is involved in control of the expression of acetate utilization genes Todd, (1998). This was further correlated with the maximal expression of *A. niger oah* transcript and oxaloacetase activity during growth on acetate (Pedersen et al., 2000b). Similarly the *PcoahA* 5'UTR contains a FacB-like cis-element (-611 TCCCGTGAAAAGGA-597). In contrast to *PcoahA*, genes involved in acetate utilization (i.e. *facA* that encodes an acetyl-CoA ligase) show a different expression profile in presence and absence of phenylacetate (Haris et al., 2009a), suggesting that the regulation of *PcoahA* does not depend on *PcfacB* at least in the conditions described in this study. Further research should establish by which mechanism PAA controls expression of *PcoahA* in *P. chrysogenum*.

While the repression of oxalate production by PAA is convenient for Penicillin-G production, the observed product levels in cultures grown without side chain precursor indicate that it may represent a significant problem in programmes aimed at developing *P. chrysogenum* for the production of other products. One example is the successful metabolic engineering of *P. chrysogenum* for industrial production of 7-aminodeacetoxycephalosporanic acid (7-ADCA), a key synthon for the production of clinically important cephalosporins. Recently, production of the cephamycin ad7-ACCCA by an engineered *P. chrysogenum* strain was reported. In both cases, adipic acid is used as a side chain precursor in fermentation. In contrast to PAA, adipic acid does not repress oxalate production

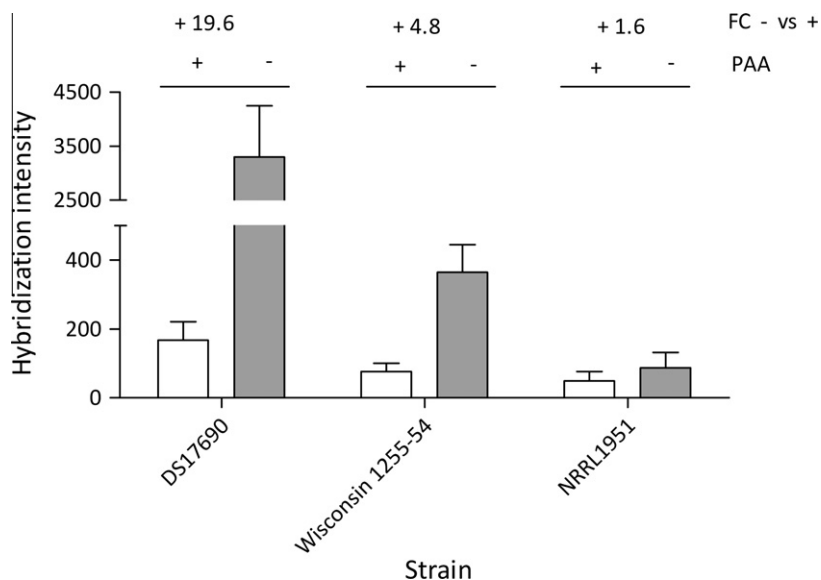


Fig. 4. Expression of the gene Pc22g24830/*PcoahA* in several strains of the *P. chrysogenum* strain lineage, DS17690, Wisconsin 1255-54 and NRRL1951. RNA samples were obtained from the three strains grown in presence and absence of phenylacetic acid in independent glucose-chemostat cultures at $D = 0.03 \text{ h}^{-1}$ and hybridized to Affymetrix Genechip microarrays. The average and the standard deviation were derived from triplicate experiments.

(Table 3). Indeed, significant concentrations of oxalate are found in ad7ACCA-producing cultures (Fig. 2). Therefore, in this context, elimination of oxalate production is an industrially relevant target.

Previous studies have sought to eliminate oxalate formation in *A. niger* by either classical mutagenesis (Ruijter et al., 1999; van den Hombergh et al., 1995; Wenzel et al., 2004) or targeted gene deletion (Pedersen et al., 2000b,c). Whilst, in *A. niger*, targeted deletion of *oahA* successfully eliminated oxalate production and did not affect citric acid production, it did lead to a significant reduction of α -amylase production (Pedersen et al., 2000c). In *P. chrysogenum*, deletion of *PcoahA* did not reveal any negative impact on β -lactam product formation (Table 3) suggesting that it can be rapidly implemented in strain improvement processes.

The identification of Pc22g24830/*PcoahA* as the sole relevant oxaloacetase gene in *P. chrysogenum* was greatly facilitated by the availability of the full genome sequence of the fungus (van den Berg et al., 2008), the availability of chemostat-based transcriptome data and functional analysis in *S. cerevisiae*. Heterologous expression of the *PcoahA* gene in yeast provides an unequivocal demonstration of the sufficiency of the *oah* gene for oxaloacetase function.

Although oxaloacetase activity in the yeast strain expressing *PcoahA* were similar to those in *P. chrysogenum*, extracellular oxalate levels was much lower in the yeast cultures. This may either reflect different intracellular conditions or the absence of an efficient oxalate export mechanism in *S. cerevisiae*. Availability of the artificial yeast system offers an attractive method for further studies on the physiological role and regulation of oxalate production.

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