Improving penicillin biosynthesis in *Penicillium chrysogenum* by glyoxalase overproduction

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**A R T I C L E   I N F O**

**Article history:**
Received 14 August 2012
Received in revised form 2 April 2013
Accepted 3 April 2013
Available online 19 April 2013

**Keywords:**
Glyoxalase
Glycation
Isopenicillin N synthase
Isopenicillin N acyltransferase
Methylglyoxal
2-Oxoaldehyde

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

Genetic engineering of fungal cell factories mainly focuses on manipulating enzymes of the product pathway or primary metabolism. However, despite the use of strong promoters or strains containing the genes of interest in multiple copies, the desired strongly enhanced enzyme levels are often not obtained. Here we present a novel strategy to improve penicillin biosynthesis by *Penicillium chrysogenum* by reducing reactive and toxic metabolic by-products, 2-oxoaldehydes. This was achieved by overexpressing the genes encoding glyoxalase I and II, which resulted in a 10% increase in penicillin titers relative to the control strain.

The protein levels of two key enzymes of penicillin biosynthesis, isopenicillin N synthase and isopenicillin N acyltransferase, were increased in the glyoxalase transformants, whereas their transcript levels remained unaltered. These results suggest that directed intracellular reduction of 2-oxoaldehydes prolongs the functional lifetime of these enzymes.

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1. Introduction

β-lactam antibiotics represent a class of important pharmaceuticals of major clinical value. Their economic significance is stressed by the fact that these antibiotics contribute to over 40% of the total antibiotic market (Kresse et al., 2007). The β-lactam antibiotic penicillin (PEN) is industrially produced by the filamentous fungus *Penicillium chrysogenum*. The PEN biosynthetic machinery is compartmentalized in *P. chrysogenum* in the cytosol and microbodies (peroxisomes) (Evers et al., 2004; Müller et al., 1991; Turner, 1992). The process starts with the condensation of three amino acids into the tripeptide α-aminoadipoyl-cysteinyl-valine (ACV). ACV is subsequently converted by cytosolic isopenicillin N synthase (IPNS) to isopenicillin N (IPN), which contains the characteristic β-lactam backbone. The third enzyme of PEN biosynthesis, isopenicillin N acyltransferase (IAT) is localized in peroxisomes (Müller et al., 1992). IAT catalyzes the incorporation of a novel side chain, using phenylacetly CoA or phenoxyacetly CoA as a substrate. The enzyme phenylacetly CoA ligase (PCL) is known as the major phenyl acetic acid activating enzyme (Lamas-Maceiras et al., 2006). PCL is also a peroxisomal enzyme, thus stressing the importance of this organelle in efficient PEN production in *P. chrysogenum* (Kiel et al., 2009; Meijer et al., 2010).

Since the isolation of the first PEN producing strain in 1943, random mutagenesis and selection procedures have been successful in generating a strain lineage that shows enhanced PEN production levels. More recently, also genetic engineering was introduced in strain improvement programs. These efforts have primarily focused on increasing the levels of (or introducing new) enzymes in the product pathway and on engineering of primary metabolism.

At present, there is an urgent need for the development of novel strategies because of the limited success of further enhancing the biosynthesis performance of current production strains by random mutagenesis approaches and the exhaustion of targets for metabolic engineering. A new strategy in this regard is to increase the period over which biosynthetic enzymes of secondary metabolism are functional. This was accomplished in the present study by ‘boosting’ the ability of the fungal cell factory to detoxify reactive products of metabolism. An important example of such a compound is methylglyoxal which is formed as by-product of the triosephosphate isomerase reaction in glycolysis (Thorntalley, 2008). Catalyzed by glyoxalase I (GLO1, EC 4.4.1.5, lactoylglutathione lyase) and II (GLO2, EC 3.1.2.6, hydroxacylglutathione hydrodrolase), 2-oxoaldehydes are converted into their corresponding 2-hydroxy acids in two consecutive steps (Thorntalley, 2008). Stressing the importance of the glyoxalase system on fungal...
viability, it was shown that overexpression of glyoxalase I and II encoding genes leads to increased survival on media containing glucose (2% concentration) as a sole carbon source in the fungal aging model Podospora anserina (Scheckhuber et al., 2010).

Here we demonstrate that overexpression of the corresponding genes (Pglo1, Pglo2) in P. chrysogenum results in an increase in PEN production. Double Pglo1/Pglo2 overexpression strains (PcGLO1/2OEx) showed an increase of PEN titers up to 10%. These findings correlate with an increased content of IPNS and IAT protein in PcGLO1/2OEx strains.

2. Methods

2.1. Strains/cultivation

DS17690 (Harris et al., 2006) was used as a high PEN production strain of P. chrysogenum. Sporulation of mycelia was stimulated by growth on R agar (Bartoszewska et al., 2011) at 25 °C for 10–11 days. For production of PEN V, spores were inoculated in 50 ml PEN production medium (Nijland et al., 2010) +0.05% phenoxycetic acid for 1 h at RT before transfer to shake flasks. Cultures were subsequently incubated in an orbital shaker (200 rpm) for 10 d at 25 °C or 30 °C. For cloning purposes, Escherichia coli strain DH5α was used.

2.2. Construction of PcGlo1 and PcGlo2 overexpression plasmids

For the construction of the PcGlo1 overexpression vector pPcGlo1Ex1, the PcGlo1 cDNA was amplified by PCR from a P. chrysogenum cDNA library using oligonucleotides cglo1f1 and cglo1r1 (Table 1). The PCR product was cut with HindIII/EcoRV and cloned into the HindIII/SmaI site of vector pCRHII (Kiel et al., 2005) containing the IPNS promoter and AT terminator sequences from P. chrysogenum. The strategy for the construction of the PcGlo2 overexpression vector pPcGlo2Ex1 is similar but uses oligonucleotides cglo2f1 and cglo2r1 (Table 1). Both constructs were verified by sequencing. The glyoxalase I/II overexpression cassettes were isolated from pPcGlo1Ex1 and pPcGlo2Ex1 by NotI restriction and subsequently purified using the Nucleospin kit (Machery Nagel, Düren, Germany).

2.3. Transformation and selection of P. chrysogenum

Protoplasts of P. chrysogenum high PEN production strain DS17690 were prepared and subsequently co-transformed with the linear PcGlo1/PcGlo2 expression cassettes and the amdS (acetamide synthase) selection marker from plasmid pSU15 (lab collection) according to a previously published protocol (Cantoral et al., 1988). Transformants were selected on plates containing acetamide as the sole nitrogen source and further analyzed by colony PCR (Bartoszewska et al., 2011) using oligonucleotides cglo1f1/cglo1r1 or cglo2f1/cglo2r2 (Table 1). Positive candidates were transferred to R agar to induce sporulation of the mycelium. Spores were streaked out on acetamide plates to receive single colonies, which were transferred to fresh acetamide medium and tested again using colony PCRs for the presence of the PcGlo1/PcGlo2 expression cassettes.

2.4. Preparation of crude cell extracts

Protease inhibitor cocktail (final concentrations in the medium: 10 μM 4-(2-aminoethyl)-benzenesulfonylfluoride, 0.14 μM E-64, 0.22 μM pepstatin A and 50 μM 1,10-phenanthroline in dimethyl-sulfoxide) (Sigma Aldrich, Zwijndrecht, The Netherlands) was added to the culture. Three ml of the culture was filled into a French Press chamber and exposed to high pressure. Protein concentration of the cell extracts was determined by using a kit from BioRad (Munich, Germany). Trichloroacetic acid (TCA) extracts of whole cells were prepared as described previously (Kiel et al., 2009).

2.5. Determination of glyoxalase I and glyoxalase II activity

Glyoxalase I activity, modified after Basu et al. (1988): assay buffer containing 3 mM methylglyoxal, 1 mM reduced glutathione, 16 mM MgSO4 and 33.3 mM potassium phosphate (pH 7.0) was freshly prepared and kept at RT for 1 h so that the glyoxalase I substrate, hemithioacetal, could be formed. Upon addition of cell extract, the increase of absorbance at 240 nm (i.e., formation of S-D-lactoylglutathione [SDLGSH]) was measured in a Lambda 35 UV/VIS spectrophotometer (Perkin-Elmer, Waltham, MA, USA) for 3 min. Enzymatic activity was calculated by using the molar coefficient of extinction for the formed product, SDLGSH (3100 M⁻¹ cm⁻¹ at 240 nm).

Glyoxalase II activity, modified after Maiti et al. (1997): protein was added to assay buffer (300 μM SDLGSH in 33.3 mM potassium phosphate [pH 7.0]) and the decrease in absorbance was measured at 240 nm for 3 min.

2.6. qPCR analysis of IPNS and IAT transcript levels

Quantitative PCRs for the determination of IPNS and IAT transcript levels were determined according to a previously published protocol (Nijland et al., 2010).

2.7. Western blot analysis

Western blotting was performed by established techniques. After blocking, the blots were incubated with primary antibodies against IPNS (1/10,000) or IAT (1/5000) (supplied by DSM, Delft, The Netherlands). As loading control, antibodies against translation elongation factor 1α (eF1α) from Hansenula polymorpha (1/5000) (lab collection) were utilized. Blots were decorated using α-lgG coupled with alkaline phosphatase, 1/10,000 (Sigma Aldrich, Zwijndrecht, The Netherlands).

2.8. HPLC determination of PEN V

Levels of PEN V in spent media were determined by high pressure liquid chromatography (HPLC) using an isocratic flow of acetoni-trile (350 g/l), KH2PO4 (640 mg/l) and H3PO4 (340 mg/l). The peaks were separated on a Platinum E5 5 μm C18 column (Grace, Deerfield, IL, USA) at a flow rate of 1 ml/min. The detection wavelength was set to 254 nm.

2.9. Protein identification in Coomassie stained gels

Proteins bands of interest were cut from an SDS-PAGE gel containing separated TCA extracts from the strains of interest and analyzed by
mass spectrometry (MALDI-TOF peptide mass fingerprint and MALDI-TOF/TOF peptide sequencing) by Alphalyse A/S, Odense, Denmark.

2.10. Assay to determine methylglyoxal sensitivity

50 ml of PEN V production medium was inoculated with sporulating mycelium grown on 15–20 rice grains for 1 h at room temperature with occasional mixing. 5 ml of a 1/10 dilution in sterile water was spotted onto R agar plates supplemented with increasing concentrations (0%–0.15%) methylglyoxal (40% stock solution, Sigma Aldrich, Zwijndrecht, The Netherlands). The plates were incubated at 25 °C for six days.

2.11. Detection of methylglyoxal-modified proteins

The levels of methylglyoxal-modified proteins were determined using the OxiSelect™ Methylglyoxal (MG) ELISA kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's instructions.
Methylglyoxal–protein adducts were probed with specific monoclonal antibodies. Subsequently the samples were washed and treated with horseradish peroxidase conjugated secondary antibodies and a substrate solution. Substrate turnover leads to the formation of a product that can be measured in a photometer at a wavelength of 450 nm. These data are compared to a standard curve prepared from methylglyoxal–bovine serum albumin (supplied with the kit).

2.12. Glyoxalase homology analyses

Homology between related proteins was determined using the BLOSUM62 algorithm implemented in ‘Clone Manager Suite 6’ (Scientific and Educational Software, Cary, NC, USA).

3. Results

3.1. Identification of PcGLO1 and PcGLO2 in the P. chrysogenum genome

The amino acid sequences of P. anserina GLO1 (UniProt accession number B2AQW8) and GLO2 (UniProt accession number B2B554) were used to search the genomic sequence of P. chrysogenum (van den Berg et al., 2008) for the corresponding homologs via the BlastP algorithm (Altschul and Lipman, 1990). For each protein, one homolog, Pc12g09820 (PcGLO1) and Pc21g08590 (PcGLO2) was identified (Fig. 1). The sequences of PcGLO1 and PcGLO2 are strongly conserved relative to their P. anserina counterparts, displaying 62% and 65% sequence identity, respectively (Fig. 1). It should be noted that both deposited sequences were corrected regarding the translation start of the proteins. The annotated sequence for Pc12g09820 starts at amino acid position +14 while the annotated sequence for Pc21g08590 starts at amino acid position −23. This correction was possible due to homology analysis including glyoxalase I and II sequences from various filamentous fungi (i.e., Aspergillus fumigatus, Aspergillus niger, P. anserina, Neurospora crassa and Sordaria macrospora) (Fig. 1).

3.2. Generation of glyoxalase I/II overexpression strains

We analyzed the impact of the glyoxalase system on PEN production by analyzing double (PcGlo1/PcGlo2) overexpression strains. Two independent glyoxalase I/II double overexpression strains (PcGLO1/2OEx) were analyzed for enzyme activities (Table 2). The data revealed that in both strains the levels of glyoxalase I and II activity were strongly enhanced (Table 2) relative to those of the parental strain DS17690 (hereafter designated as control). The highest activities were obtained in strain #7.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth temperature (°C)</th>
<th>PEN V [g/l]</th>
<th>% PEN V</th>
<th>PEN V [mg/g dry weight]</th>
<th>% Ysp [mg/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25</td>
<td>1.13</td>
<td>100</td>
<td>60.1</td>
<td>100</td>
</tr>
<tr>
<td>PcGLO1/2OEx #7</td>
<td>25</td>
<td>1.19*</td>
<td>105</td>
<td>96.0</td>
<td>160</td>
</tr>
<tr>
<td>PcGLO1/2OEx #12</td>
<td>25</td>
<td>1.18*</td>
<td>104</td>
<td>86.8</td>
<td>144</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>0.86</td>
<td>100</td>
<td>48.9</td>
<td>100</td>
</tr>
<tr>
<td>PcGLO1/2OEx #7</td>
<td>30</td>
<td>0.95**</td>
<td>110</td>
<td>66.0</td>
<td>135</td>
</tr>
<tr>
<td>PcGLO1/2OEx #12</td>
<td>30</td>
<td>0.94**</td>
<td>109</td>
<td>58.8</td>
<td>120</td>
</tr>
</tbody>
</table>

Ysp: mg PEN per gram carbon source.

* P < 0.05 (control strain vs. PcGLO1/GLO2OEx; Student’s t-test, two-tailed).

** P < 0.01 (control strain vs. PcGLO1/GLO2OEx; Student’s t-test, two-tailed).

![Fig. 2](image-url). Transformsants PcGLO1/2OEx #7 and PcGLO1/2OEx #12 show enhanced resistance to methylglyoxal: suspensions of germinating spores were spotted on agar plates containing increasing concentrations of methylglyoxal. The plates were incubated for six days at 25 °C. PcGLO1/2OEx #7 and PcGLO1/2OEx #12 are able to tolerate higher methylglyoxal levels compared to the control strain (C, DS17690).
which showed a 48-fold increased PcGLO1 activity together with a 108-fold increased PcGLO2 activity.

We next determined whether PcGLO1/2OEx strains show increased resistance against externally added methylglyoxal. As shown in Fig. 2, both transformants (PcGLO1/2OEx #7 and PcGLO1/2OEx #12) are able to tolerate enhanced methylglyoxal levels relative to the control strain. For example, the control strain hardly grows on plates supplemented with 0.15% methylglyoxal while both mutants are able to do so (Fig. 2, bottom row). These results indicate that increased levels of enzymatically active glyoxalase I and II are present in both transformants.

Taken together, our data indicated that strains with enhanced glyoxalase I and II activities were obtained.

3.3. PEN levels are enhanced in glyoxalase overproducing strains

Subsequently, PEN production of the glyoxalase I/II double overexpression strains was determined. To this purpose cells were grown for 10 days in PEN V production media at 25°C. As shown in Table 3, both strains showed a significant increase in PEN production (both in g PEN/l and in g PEN/g dry weight).

1D SDS-PAGE was performed to detect differences in protein patterns between the control and the PcGLO1/2OEx strain #7 (Fig. 3A). Coomassie brilliant blue staining led to the identification of three enhanced protein bands that were present in PcGLO1/2OEx #7 relative to the control. Mass spectrometry revealed that two of these bands represented PcGLO1 and PcGLO2, as expected. Interestingly, however, the third band was identified as IPNS (Fig. 3A, B), a finding that was confirmed in Western blots decorated with antibodies against IPNS (Fig. 4A). This positive effect on protein abundance was not confined to IPNS alone as also IAT appeared to be present at elevated levels (Fig. 4B).

Cooling costs are an important economic factor in large scale industrial PEN fermentations. Hence, performing P. chrysogenum fermentations at higher growth temperatures would be very cost effective. However, it has been demonstrated that IPNS is instable at enhanced growth temperatures (Gidijala et al., 2008). Growth of the control strain at 30°C is accompanied by a drop in PEN titers of 24% (Table 3), whereas the PEN titers produced by the mutants show a decrease of approximately 20% when the growth temperature was enhanced to 30°C. Moreover, when normalizing the PEN production to biomass, PEN productivities (g/g dry weight) in the PcGLO1/2OEx strains (66.0 mg/g dry weight [PcGLO1/2OEx #7] and 58.8 mg/g dry weight [PcGLO1/2OEx #12]) were similar to the productivity of the control strain at 25°C (60.1 mg/g dry weight [control]). Also at 30°C protein levels of IPNS and IAT are substantially increased in PcGLO1/2OEx mutants (Figs. 3 and 4).

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**Table 4**

Determination of relative IPNS and IAT transcript levels in control and PcGLO1/2OEx overexpression strains.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PcGLO1/2OEx #7</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPNS</td>
<td>207</td>
<td>204</td>
</tr>
<tr>
<td>IAT</td>
<td>0.67</td>
<td>0.63</td>
</tr>
</tbody>
</table>

γ-actin transcript levels was used as reference. X fold changes in transcript quantity relative to γ-actin (transcript level set to 1) are given in the table.
3.4. qPCR analysis of IPNS and IAT transcript levels

Increased protein levels of IPNS and IAT can be related to either elevated gene transcription or improved protein stability. Quantitative PCR (qPCR) using oligonucleotides directed against IPNS, IAT and the reference gene γ-actin was performed to determine the relative transcription levels of these genes (Table 4). IPNS transcript is present in higher amounts in both samples (control strain DS17690 and glyoxalase transformant PcGLO1/2OEx #7) than either IAT or γ-actin transcript (Table 4). This finding is consistent with the fact that IPNS but not IAT is readily visible in Coomassie stained gels (Fig. 3A). Between the control and the glyoxalase transformant there are no significant differences in IPNS and IAT transcript levels (Table 4). These results suggest that not an induction of gene expression but increased stability of IPNS and IAT leads to the elevated levels of these proteins in glyoxalase transformants.

3.5. PcGLO1/2OEx transformants show reduced methylglyoxal-mediated protein modifications

To test our hypothesis that the overexpression of PcGlo1 and PcGlo2 leads to decreased levels of intracellular methylglyoxal, we determined the extent of methylglyoxal-mediated protein modifications. As shown in Fig. 5, both transformants show a reduction in methylglyoxal-mediated protein modification relative to the parental strain.

3.6. Analysis of mycelial deterioration

Mycelial autolysis and fragmentation is a known factor that limits the biosynthetic capacity of fungal cell factories (Paul and Thomas, 1996). Overproduction of both glyoxylase I and II increases the replicative lifespan of P. anserina (Scheckhuber et al., 2010). Hence, a possible additional reason for the improved performance of the glyoxalase mutants could be related to a delay in mycelial fragmentation/autolysis. However, we observed no differences in this regard when mycelium samples from the control and a transformant were analyzed by light microscopy at different time points (Fig. 6). Therefore, overexpression of glyoxalase-coding genes has no effect on time-dependent mycelium deterioration.

4. Discussion

Here we demonstrate the positive effect of decreasing toxification by 2-oxoaldehydes by boosting the endogenous glyoxalase system of an important fungal cell factory, P. chrysogenum.

The reaction of 2-oxoaldehydes with proteins leads to the formation of advanced glycation end-products (AGEs) (Grillo and Colombatto, 2008). Molecular mechanisms capable of repairing proteins that are modified in this way are not known, so the only remaining option is proteolysis (e.g. by the proteasome) and the costly synthesis of new protein.

We show that both at standard and elevated growth temperatures, double glyoxalase I/II overexpression strains of P. chrysogenum (PcGLO1/2OEx) are able to produce enhanced levels of PEN relative to the control. This correlates with increased levels/stability of two enzymes of the PEN biosynthetic pathway, namely IPNS and IAT. As IPNS is very susceptible to inactivation by post-translational modifications (Dubus et al., 2000; Perry et al., 1988), enhanced protection against modification by 2-oxoaldehydes results in less functional impairment and subsequent degradation of the protein. The beneficial effect on IAT protein levels in the glyoxalase transformants is most likely a significant factor in the observed increase in PEN production, because IAT activity was recently reported to be a limiting factor in PEN biosynthesis in high-production strains like DS17690 (Nijland et al., 2010).

In a previous study it was shown that overexpression of both genes encoding the two enzymes of the glyoxalase system in P. anserina leads to an increased replicative lifespan when strains are grown on media containing elevated amounts of glucose (Scheckhuber et al., 2010). P. chrysogenum does not replicatively age but subject to autolysis and fragmentation during cultivation in liquid media. Due to the fact that microscopic analyses regarding the occurrence of autolysis in control and glyoxalase over-expressors revealed no differences it can be ruled out that increased PEN levels in PcGLO1/2OEx are due to increased ‘stability’ of the mycelium. The factors influencing replicative ageing in P. anserina and autolysis in P. chrysogenum are probably distinct.

We previously increased PEN production in P. chrysogenum by enhancing peroxisome numbers or inhibiting of autophagy. An increase in peroxisome numbers concomitant with increased PEN titers was obtained by overexpression of PEX11 (Kiel et al., 2005) or PEX14–17 (Opalinski et al., 2010), which both resulted in an approximately 2-fold increase. Inhibition of autophagy by deletion of ATG1 resulted in a smaller increase (PEN titers increased to 125% relative to the parental strain) (Bartoszewska et al., 2011), in the same range as our current observations (increase to 110% by PcGlo1 and PcGlo2 overexpression). It must be noted that the last
Fig. 6. Analysis of hyphae fragmentation by light microscopy: samples from the control strain (DS17690) and the glyoxalase I/II transformant PcGLO1/2OEx #7 were taken at the indicated time points from cultures (grown in PEN V production media). Mycelial fragmentation starts to occur after seven days of growth. No differences in hyphal fragmentation between the control strain and the transformant are detected.
two studies were performed with the relatively high producing strain, DS17690, whereas the earlier studies were performed with low producing strains (NRR1951 for PEX14-17 overexpression and Wisconsin 54-1255 for PEX11 overexpression). This may explain why a smaller difference is observed in relative PEN titers. In addition, however, different cultivation conditions were used, which makes these studies difficult to compare. The titer of the low producing NRR1951 strain is in the range of milligrams per liter, whereas current industrial strains produce 30–55 g/l PEN (van den Berg et al., 2008). The parental strain (DS17690) used in our current study produces approximately 1 g/l. Whether an increase in PEN levels can also be achieved by overproduction of PcGlo1 and PcGlo2 in current industrial strains is unknown as these strains are not freely available.

5. Conclusions

The *P. chrysogenum* genome contains one gene for glyoxalase I and one gene for glyoxalase II. Co-overexpression of both genes leads to increased levels of isopenicillin N synthase and isopenicillin N acyltransferase protein levels and enhanced PEN production. At elevated fermentation temperatures (30 °C) PcGlo1/2OEx strains produce up to 10% higher PEN titers relative to the parental strain.

Genetic engineering strategies to improve fungal cell factories by overexpression of genes involved in the product pathway have only a limited effect if the proteins are unstable. Our current results offer a novel approach for efforts to stabilize such proteins.

Acknowledgments

The authors would like to thank Jan A.K.W. Kiel, Łukasz Opalinski and Magdalena Bartoszewska for helpful comments, Stefan S. Weber for the determination of PEN V via HPLC and Susan Fekken for assistance with the qPCR analyses. CQS is supported by a fellowship from the Deutsche Forschungsgemeinschaft (SCHE 1686/2-1).

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