NADPH-dependent glutamate dehydrogenase in *Penicillium chrysogenum* is involved in regulation of β -lactam production

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The interactions between the ammonium assimilatory pathways and β -lactam production were investigated by disruption of the NADPH-dependent glutamate dehydrogenase gene (*gdhA*) in two industrial β -lactam-producing strains of *Penicillium chrysogenum*. The strains used were an adipoyl-7-ADCA- and a penicillin-producing strain. The *gdhA* gene disruption caused a decrease in maximum specific growth rate of 26 % and 35 % for the adipoyl-7-ADCA-producing strain and the penicillin-producing strain, respectively, compared to the corresponding reference strains. Interestingly, no β -lactam production was detected in either of the Δ *gdhA* strains.

Supplementation with glutamate restored growth but no β -lactam production was detected for the constructed strains. Cultures with high ammonium concentrations (repressing conditions) and with proline as nitrogen source (de-repressed conditions) showed continued β -lactam production for the reference strains whereas the Δ *gdhA* strains remained non-productive under all conditions. By overexpressing the NAD-dependent glutamate dehydrogenase, the specific growth rate could be restored, but still no β -lactam production was detected. The results indicate that the NADPH-dependent glutamate dehydrogenase may be directly or indirectly involved in the regulation of β -lactam production in industrial strains of *P. chrysogenum*.

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INTRODUCTION

β -Lactams have been produced industrially for more than 60 years, and the developments in this production process have been outstanding. An important element in strain improvement programmes is a thorough understanding of the physiology of the strains, as this may point to ways of improving the cultivation conditions or of genetically modifying the strain with the aim of improving the production, the latter often referred to as metabolic engineering. Therefore, microbial physiology constitutes an essential part of metabolic engineering (Nielsen & Olsson, 2002; Thykaer & Nielsen, 2003).

Several research groups have been working specifically on optimization of the β -lactam production of *Penicillium chrysogenum*, e.g. via gene dosage studies (Veenstra *et al.*, 1991; Theilgaard *et al.*, 2001; Skatrud & Queener, 1989; Mathison *et al.*, 1993; Gutierrez *et al.*, 1997). It is, however, of equal importance to gain insight into other aspects of the

metabolism to establish a general overview in order to apply metabolic engineering for further improvement of the production strains. It is essential that the redox metabolism is considered when characterizing the metabolism of cells. It functions as a tightly controlled connection between the different parts of the central metabolism and provides an important link between the primary and the secondary metabolism. A major player in redox metabolism in *P. chrysogenum* is ammonium assimilation (Fig. 1).

Ammonium is utilized by its incorporation into glutamate and glutamine in *P. chrysogenum*. This is achieved mainly by the activity of the NADPH-dependent glutamate dehydrogenase (GDHA) or through the reaction catalysed by glutamine synthetase (GS). GDHA catalyses the reductive amination of 2-oxoglutarate, resulting in glutamate, and this pathway is thermodynamically and energetically favoured for ammonium assimilation. The catabolic reaction from 2-oxoglutarate is catalysed by a related glutamate dehydrogenase, GDHB. However, the essential difference is that GDHB has a requirement for NAD⁺. Thus, both types of co-factors are used in the ammonium-assimilatory pathways (Fig. 1), and it is therefore possible to shift the co-factor

Abbreviations: ADCA, aminodeacetoxycephalosporanic acid; APA, aminopenicillanic acid; GDHA, glutamate dehydrogenase, NADPH-dependent; GDHB, glutamate dehydrogenase, NAD⁺-dependent.

Fig. 1. The ammonium assimilatory pathways in *P. chrysogenum*. GDHA, glutamate dehydrogenase, NADPH-dependent; GDHB, glutamate dehydrogenase, NAD⁺-dependent; GOGAT, glutamate synthase; GS, glutamine synthetase.

requirements through genetic engineering. This has been demonstrated previously, and the co-factor switch successfully led to increased bioethanol production by *Saccharomyces cerevisiae* (Nissen *et al.*, 2000).

In this work, a study of the interactions between the redox metabolism, the central carbon and nitrogen metabolism and the secondary metabolism was undertaken. Especially the role of the NADPH-dependent glutamate dehydrogenase was investigated. This was achieved by disrupting the *gdhA* gene encoding NADPH-dependent glutamate dehydrogenase in both penicillin- and cephalosporin-producing strains of *P. chrysogenum*. Targeted gene deletion or disruption is very commonly used in *Aspergillus* species, especially *Aspergillus nidulans*, and has been carried out by numerous research groups. Gene disruption and deletion has also been reported for several *Penicillium* strains (Laich *et al.*, 2003; Naranjo *et al.*, 2004; Baba *et al.*, 2006), but in these studies only wild-type or academic strains were exploited. In the present study gene disruption was carried out in high-yielding β -lactam-producing strains and this is to our knowledge the first report on targeted gene disruption in industrial strains of *P. chrysogenum*. In addition, strains overexpressing *gdhB* were constructed to compensate for the loss of GDHA. The constructed strains, together with the reference strains, were characterized in both batch and chemostat cultures, focusing on cellular performance, i.e. maximum specific growth rate, biomass yield and productivity.

METHODS

Strains. The penicillin-producing recipient strain was a high-producing strain of *Penicillium chrysogenum* designated DS12975. The other recipient strain was an adipoyl-7-ADCA-producing strain, originating from DS12975, where the expandase gene from *Streptomyces clavuligerus* had been inserted into the genome (Crawford *et al.*, 1995). Both reference strains were kindly donated by DSM Anti-Infectives (Delft, the Netherlands).

Plasmids and DNA fragments. The plasmids pAN7-1 and pAN8-1 were kindly donated by Dr Peter Punt, TNO, Quality of Life, the Netherlands.

Unless specifically stated all DNA manipulations were carried out as described by Sambrook *et al.* (1989). The DNA fragments and plasmids constructed for the fungal transformations are listed in Table 1.

PCR products were purified from a 1% agarose gel using the GFX PCR purification kit (Amersham Biosciences).

PCR primers and probes

Cloning and disruption of *gdhA*. A 433 bp probe amplified by PCR was used to screen an EMBL4 phage library of *P. chrysogenum* for the *gdhA* gene. The following primers were used to amplify the probe: 5'-GTCGTCTACTACGTCGAGCACATGATCCAGCACGCCTCCG-3' and 5'-AGCCCTCAGCGATGAACCTG-3'.

A 5.5 kb fragment containing the *gdhA* gene was identified and subcloned into a pBluescript vector, resulting in the plasmid pgdhAH1 (Table 1). The *gdhA* gene was disrupted by replacing 0.8 kb of the C-terminus of the gene sequence with the 4.1 kb *Bgl*II-*Xba*I hygromycin B resistance cassette (HygR) through construction of the plasmid pdgdhAH13 (Table 1, Fig. 2).

Cloning of *gdhB*. The following primers were designed for amplification of the *A. nidulans gdhB* gene (accession number AY223544): PgdhB-F (5'-CATGCCATGGTGTCTCCCGCATC-3') and PgdhB-R (5'-CCGGAAGATCTTTACAAGTTGAAGAAAGAG-3'). An *Nco*I cloning site was introduced into the forward primer and a *Bgl*II site included in the reverse primer (both are underlined in the primer sequences). A PCR product with the expected size (3.4 kb) was first completely digested with *Nco*I and was then partially digested with *Bgl*II as there is another *Bgl*II site in the *A. nidulans gdhB* gene. In parallel, plasmid pAlcR was constructed from the plasmid pAN7-1 (Bautista *et al.*, 2000) by replacing a 3.3 kb *Eco*RI-*Bam*HI fragment containing the *gpdA* promoter and *hph* gene of pAN7-1 with a 2.9 kb *Eco*RI-*Bam*HI fragment containing the *A. nidulans gpdA* promoter together with a small part of the *A. nidulans alcR* gene from pMH-B (Bautista *et al.*, 2000) (Fig. 3). The *gdhB* expression vector, pGdhB, was constructed by replacing a 0.6 kb *Nco*I-*Bam*HI fragment of *alcR* with the 3.4 kb *Nco*I-*Bgl*II *gdhB* fragment (Fig. 3).

Construction of strains. The fungal transformations were performed according to the procedure described by Cantoral *et al.* (1987) except that Glucanex (15 mg ml⁻¹) was used instead of Novozym 234 for protoplast formation. In the construction of the *gdhA*-disrupted strains, transformants were selected by their ability to grow through an agar overlay containing 1 mg hygromycin B ml⁻¹ (Punt & van den Hondel, 1992).

The *P. chrysogenum* strains were transformed with an 8.8 kb linear DNA fragment containing the *gdhA* gene disrupted with the HygR cassette purified from pAgdhAH13 (Fig. 2, Table 1). The fragment included 2.7 kb and 1.5 kb of homologous flanking regions upstream and downstream of the disrupted *gdhA* gene, respectively, to ensure integration by homologous recombination with the genomic DNA. Several transformants were selected and it was checked by PCR whether the HygR cassette had been integrated in the *P. chrysogenum* genome (data not shown). One transformant carrying the HygR cassette from each of the two different strain lines, designated AD12 and PD414, were chosen for the further experiments.

Strains overexpressing NAD-dependent glutamate dehydrogenase (*gdhB*) were constructed by co-transformation of pAN8-1 and pGdhB in the ratio 1:10 (μ g) using the phleomycin-resistance marker system (Punt & van den Hondel, 1992). As selection pressure 10 μ g phleomycin ml⁻¹ was used and resistant colonies were isolated by streaking on plates containing 50 μ g phleomycin ml⁻¹.

Table 1. Plasmids and DNA fragments used in this study

DNA type	Gene(s)	DNA ID	Feature	Digest/size (kb)	Reference
Phage library	Genome	EMBL4	EMBL4 phage library of <i>P. chrysogenum</i>		
Phage	<i>gdhA</i>	Phage D	Isolated phage containing the <i>gdhA</i> gene	<i>Sau3A</i> /16	This study
ORF	<i>gdhA</i>	<i>gdhA</i> gene	Complete DNA sequence of the <i>P. chrysogenum</i> <i>gdhA</i> gene	1.6	Diez <i>et al.</i> (1999)
ORF	<i>gdhB</i>	<i>gdhB</i> gene	Complete DNA sequence of the <i>A. nidulans</i> <i>gdhB</i> gene	3.4	PubMed AY223544
Plasmid	<i>hph</i>	pAN7-1	<i>E. coli</i> hygromycin B resistance gene under control of the <i>A. nidulans</i> <i>gpdA</i> promoter	6.5	Punt <i>et al.</i> (1987)
Plasmid	<i>ble</i>	pAN8-1	<i>E. coli</i> bleomycin resistance gene under control of the <i>A. nidulans</i> <i>gpdA</i> promoter	5.9	Punt & van den Hondel (1992)
Plasmid	<i>PgpdA-alcR</i>	pMH-B	<i>gpdA</i> promoter followed by a small <i>alcR</i> fragment separated by an <i>NcoI</i> digestion site	<i>EcoRI</i> / <i>BamHI</i> 3.3	Bautista <i>et al.</i> (2000)
Plasmid	<i>gdhA</i>	pgdhAH1	<i>gdhA</i> gene with approx. 1.5 kb flanking regions on each side subcloned into pBluescript	<i>HindIII</i> /5.5	This study
Plasmid	Δ <i>gdhA-hph</i>	p Δ gdhAH13	<i>gdhA</i> gene disrupted with the HygR cassette	<i>HindIII</i> /8.8	This study
Plasmid	<i>gdhB</i>	pGdhB	<i>gdhB</i> gene under control of the <i>A. nidulans</i> <i>gpdA</i> promoter	9.1	This study

Cultivation medium. All batch cultivations were carried out using defined medium containing, per litre: 25 g sucrose, 3.5 g $(\text{NH}_4)_2\text{SO}_4$, 0.8 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g Na_2EDTA , 0.04 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 ml silicone antifoam agent (Sigma) and 5 ml trace element solution. The trace element solution contained, per litre: 1.0 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 4 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 4 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$. In the cultivations with the adipoyl-7-ADCA-producing strains 12.5 g adipic acid (AA) l^{-1} was used as side chain precursor, dissolved with KOH in the ratio AA:KOH 2:1 (g g^{-1}). In the cultivations with the penicillin-producing strains, 6.5 g phenoxyacetic acid l^{-1} was included in the medium as side-chain precursor to produce penicillin V. During each cultivation, 14 samples were taken throughout the experiment.

For the chemostat cultivations 5 g glucose l^{-1} was used as carbon source in both the batch and the chemostat phase. The side-chain precursors were added at a concentration of 5 g l^{-1} and 6.5 g l^{-1} for adipic acid and phenoxyacetic acid, respectively.

A series of batch cultivations using either 135 mM NH_4^+ or 30 mM proline as nitrogen source were also conducted. Apart from the

nitrogen source, the medium used was the same as described above. However, the sampling frequency in these experiments was reduced to five samples during each cultivation.

Cultivation conditions. All cultivations were carried out in 5 litre bioreactors with an initial working volume of 4 litres. The temperature was maintained constant at 25 °C and pH was kept at 6.5 through addition of 2 M NaOH and 1 M HCl. The initial aeration rate and agitation were 0.2 vvm and 200 r.p.m., respectively. These were increased after germination (approx. 20 h) to 0.5 vvm and 300 r.p.m., and finally after observing mycelial development the aeration rate was increased to 1 vvm and agitation was increased to 500 r.p.m.; these parameters were then kept constant throughout the rest of the cultivation. All cultivations were inoculated with spores to an initial concentration of 2×10^9 spores kg^{-1} . For the chemostat cultivations, the continuous phase was initiated by feed addition when all the glucose in the batch phase was exhausted.

Sampling. Biomass samples were taken manually from the reactor. For dry weight measurements, the samples were filtered, washed with

Fig. 2. Experimental strategy used for disrupting the *gdhA* gene of *P. chrysogenum*. The *gdhA* gene was disrupted by replacing 0.8 kb of the C-terminus of the gene with the hygromycin B resistance cassette (HygR). In the upper right corner, the result of the Southern analysis is shown verifying the correct insertion of HygR: DS12975, penicillin-producing reference strain; PD414, *gdhA*-disrupted strain of the penicillin-producing strain line; DS36079, adipoyl-7-ADCA-producing reference strain, AD12, *gdhA*-disrupted strain of the adipoyl-7-ADCA-producing strain line.

Fig. 3. Construction of the *gdhB* overexpression plasmid. pAlcR was constructed from pAN7-1 by replacing a 3.3 kb *EcoRI*–*BamHI* fragment of pAN7-1 with a 2.9 kb *EcoRI*–*BamHI* fragment obtained from pMH-B, thereby introducing an *NcoI* site. pGdhB was constructed by replacing a 0.6 kb *NcoI*–*BamHI* *alcR* fragment of pAlcR with a 3.4 kb *NcoI*–*BglII* fragment of the *gdhB* gene.

water and dried at 105 °C to constant weight. The filtrate was kept at –80 °C and analysed for content of sugar and products.

Southern analysis. Biomass samples from the exponential growth phase of batch cultivations were harvested and total DNA was isolated with reference to standard procedures for filamentous fungi (Sambrook *et al.*, 1989).

The DNA fragment used as probe for the Southern blotting was 961 bp in length and was amplified from total DNA by PCR using the following gene-specific primers: 5'-ACCTTCCCTTCGAGCCTGAG-3' and 5'-AGAGGGAGATGACGGAGCCA-3'.

The probe was labelled with [α -³²P]dCTP by random priming using Rediprime II kit (Amersham Biosciences). Hybridization of the blotted DNA was performed in rotating incubators (Hybridization Oven/Shaker, Amersham), according to the ECL Hybridization kit protocol (Amersham Pharmacia Biotech).

Measurements of sugars. Sucrose, glucose and fructose were separated at room temperature by high-performance anion-exchange chromatography using a Carbpac PA-10 column (Dionex). The column was eluted at 1.2 ml min⁻¹ with carbonate-free NaOH. For the first 10 min the NaOH concentration in the eluent was 20 mM. A linear NaOH gradient from 20 to 30 mM was applied from 10 to 15 min after injection, and another linear gradient from 30 to 200 mM was applied from 15 to 30 min after injection. The sugars were quantified by pulsed amperometric detection using an ED 40 detector from Dionex.

Measurements of adipic acid and β -lactams. Adipate and the fermentation products of cultivations with the adipoyl-7-ADCA-producing strains were analysed using an isocratic HPLC method described by Robin *et al.* (2003b). Pure adipoyl-6-APA and adipoyl-7-ADCA standards were kindly donated by DSM Anti-Infectives (Delft, the Netherlands).

Penicillin V produced in the cultivations with the penicillin-producing strains was analysed according to the HPLC method of Christensen *et al.* (1994).

RESULTS

Strain construction

The *gdhA* gene encoding the NADPH-dependent glutamate dehydrogenase of *P. chrysogenum* has previously been sequenced and characterized (Diez *et al.*, 1999). Based on this sequence, the Δ *gdhA* strains were constructed by targeted gene disruption using the hygromycin B resistance marker system (Fig. 2) (Punt *et al.*, 1987; Punt & van den Hondel, 1992). The reason for choosing a dominant selectable marker was that no auxotrophic markers were available for the industrial strains exploited in this study. To verify that the *gdhA* gene had been disrupted by the HygR cassette, Southern blotting was carried out. From the Southern blot (Fig. 2), only a fragment of 5.5 kb was found in the reference strains, whereas an 8.8 kb fragment was found in the transformant strains, corresponding to the *HindIII* fragment containing the native *gdhA* gene and the *HindIII* fragment with the disrupted *gdhA* gene, respectively. Thus, the HygR cassette had been integrated at the correct position in the genomes of both the *P. chrysogenum* strains.

To compensate for the loss of *gdhA*, strains overexpressing the NADH-dependent glutamate dehydrogenase (*gdhB*) were constructed. Since the penicillin- and cephalosporin-producing Δ *gdhA* strains behaved similarly, strains

overexpressing *gdhB* were only constructed for the adipoyl-7-ADCA-producing strain line. Thus, only the AD12 strain was used as recipient strain for *gdhB* overexpression.

The *gdhB* gene was amplified by PCR from genomic DNA of *A. nidulans*. The PCR primers were designed to introduce *Nco*I and *Bgl*II restriction sites upstream and downstream of the *gdhB* coding region, respectively, and subsequently cloned into plasmid pAlcR, resulting in pGdhB (Fig. 3). The *gdhB* overexpression strains were constructed by co-transformation with pAN8-1 and pGdhB, using the dominant phleomycin-resistance marker for selection (Punt & van den Hondel, 1992). Two strains were selected and the presence of additional *A. nidulans* *gdhB* copies was verified by PCR (data not shown).

Sequencing the *gdhA* downstream region

As shown in Fig. 2, the *gdhA* gene was disrupted by replacing 0.8 kb of the C-terminus of the gene with the HygR cassette. Apart from the C-terminus of the gene being deleted, 300 bp of the downstream region was also deleted in this construct. The sequence of this part of the genome is not known. Therefore, to verify that no other essential gene or promoter region was deleted in the construction of the *gdhA*-disrupted strain, this 300 bp fragment was sequenced by MWG, Germany (data not shown). The sequence showed an A-rich segment 30 bp

upstream from the *Xba*I cloning site. This polyadenylation site indicated the termination of the *gdhA* transcript and since no start or stop codons were found in the last part of the sequence it was assumed that no other gene was disrupted during the construction of the Δ *gdhA* strain. This conclusion was supported by BLAST searches of the sequenced 300 bp fragment, which did not show alignments to any known genes.

Physiological characterization

The recipient strains and the constructed strains were all cultivated in batch cultures. Two different cultivations were performed, one with plain medium and one where glutamate was added to a final concentration of 10 mM. A typical time profile of the biomass concentration, sugar concentration and product formation during batch cultivation is shown in Fig. 4. The specific production rate r_p was estimated by dividing the volumetric productivity q_p , estimated from Fig. 4(c), by the average biomass concentration of the stationary phase (indicated by the rectangle in Fig. 4a). The physiological fermentation data from the batch cultures are summarized in Table 2.

Table 2 shows that disruption of *gdhA* had a radical impact on the maximum specific growth rates of the constructed strains. For the adipoyl-7-ADCA-producing strain $\mu_{\max}$ decreased by 27% compared to the reference strain,

Fig. 4. (a) A typical time profile during a batch cultivation showing the sugar concentration, product formation and biomass formation as a function of time. (b) Estimation of the biomass yield coefficient Y_{sx} . The biomass concentration was plotted as a function of the total C-mol carbon consumed for biomass. The biomass yield coefficient was estimated from the slope of the resulting curve. (c) Penicillin formation curve. The penicillin V titre was plotted against time and the penicillin V production rate was estimated from the slope of the curve.

Table 2. Overview of the cultivations performed and the resulting physiological data

Strain	Glu concn (mM)	$\mu_{\max}$ (h ⁻¹)	Y_{sx} [C-mol (C-mol) ⁻¹]	q_p ($\mu\text{mol l}^{-1} \text{h}^{-1}$)	Biomass* (g l ⁻¹)	r_p [$\mu\text{mol (g dry wt)}^{-1} \text{h}^{-1}$]
Ad-7-ADCA ref. strain	–	0.15	0.53	7.8	12.1 (0.3)	0.6
	10	0.14	0.52	8.6	15.7 (0.7)	0.5
Ad-7-ADCA $\Delta gdhA$ strain	–	0.11	0.49	0.0	8.7 (0.3)	0.0
	10	0.14	0.56	0.0	7.7 (0.5)	0.0
Ad-7-ADCA $\Delta gdhA + gdhB$ strain	–	0.15	0.54	0.0	11.3 (0.7)	0.0
	–	0.15	0.53	0.0	10.5 (0.5)	0.0
Penicillin ref. strain	–	0.15	0.55	16.1	8.5 (0.5)	2.0
	10	0.14	0.54	26.3	11.0 (0.5)	2.4
Penicillin $\Delta gdhA$ strain	–	0.10	0.56	0.0	5.4 (0.1)	0.0
	10	0.13	0.55	0.0	6.1 (0.8)	0.0

*Average biomass concentration for the production phase. The estimated biomass concentrations are based on a minimum of four measurements. The standard deviations are given in parentheses.

whereas a decrease of 32 % was observed for the penicillin-producing strain. Supplementing glutamate to the medium could however restore the specific growth rates of the transformants. Furthermore, overexpression of *gdhB* also had the expected effect of restoring the specific growth rate, as $\mu_{\max}$ in both cultivations with the $\Delta gdhA + gdhB$ strains was increased from 0.11 h⁻¹ in the $\Delta gdhA$ strain to 0.15 h⁻¹ in the cultivations with the *gdhB* overexpression strains.

The most remarkable result of the batch cultivations with the *gdhA*-disrupted strains was that no production of β -lactams was measured in any of the cultivations. Moreover, neither when glutamate was added to the medium nor with overexpression of *gdhB* was it possible to restore β -lactam production, although the strains were growing with the same $\mu_{\max}$ as the recipient strains.

To ensure that the lack of production in the constructed strains was not a result of the physiological conditions during the batch cultivations, a series of chemostat cultivations with the different strains at dilution rates of 0.065 h⁻¹ were carried out. For all cultivations, including the ones with the constructed strains, steady states with regard to biomass were achieved (data not shown) and the biomass yields were comparable with yields found in previous studies (van Gulik *et al.*, 2000; Robin *et al.*, 2003c). The specific productivities of the reference strains

were estimated to be 17(±2) $\mu\text{mol } \beta\text{-lactam (g dry wt)}^{-1} \text{h}^{-1}$, but as with the batch cultivations no production of β -lactam was detected in any of the constructed strains during carbon-limited chemostat cultures.

Influence of nitrogen source on β -lactam production

The results described above indicated that the lack of production in both batch and chemostat cultivations was caused by ammonium repression of the β -lactam genes (see discussion below). To test this hypothesis additional batch cultivations were carried out using different nitrogen sources (Table 3). Two main results were found from this study: it was not possible to eliminate β -lactam production in the high-producing industrial strains (recipient strains) by increasing the ammonium concentration fivefold in the medium; and the $\Delta gdhA$ strains remained non-productive during growth with proline as non-repressing nitrogen source (Table 3).

DISCUSSION

The genetic modification of the redox metabolism in *P. chrysogenum* by disruption of the NADPH-dependent glutamate dehydrogenase resulted, as expected, in fundamental changes in the cellular metabolism. A significant

Table 3. Influence of nitrogen source on the β -lactam production during batch cultivations

Strain	N-source	Concn (mM)	β -Lactam production
Ad-7-ADCA ref. strain	NH ₄ ⁺	27	Yes
Ad-7-ADCA ref. strain	NH ₄ ⁺	135	Yes
Ad-7-ADCA $\Delta gdhA$ strain	Proline	30	No
Penicillin ref. strain	NH ₄ ⁺	27	Yes
Penicillin ref. strain	NH ₄ ⁺	135	Yes
Penicillin $\Delta gdhA$ strain	Proline	30	No

decrease of about 30 % was observed in the maximum specific growth rate of the constructed $\Delta gdhA$ strains, which was caused by the lower capacity of these strains to assimilate ammonium. These findings are in accordance with similar studies in *S. cerevisiae*, where decreases of 22 % or 46 % were found during aerobic and anaerobic conditions, respectively (Moreira dos Santos *et al.*, 2004; Nissen *et al.*, 2000). As expected, the $\mu_{\max}$ of the $gdhA$ -disrupted strains could be restored by supplementing glutamate to the medium and by overexpression of $gdhB$.

The specific productivities in batch cultivations of the reference strains were on average estimated to be $0.6 \mu\text{mol (g dry wt)}^{-1} \text{h}^{-1}$ for the adipoyl-7-ADCA-producing strain and $2.2 \mu\text{mol (g dry wt)}^{-1} \text{h}^{-1}$ for the penicillin-producing strain. These results correlated with specific productivities described previously (Robin *et al.*, 2001; Theilgaard *et al.*, 2001). The difference in the specific productivity of the two recipient strains was explained by degradation of the side-chain precursor, adipate, during adipoyl-7-ADCA production (Thykaer *et al.*, 2002; Robin *et al.*, 2003a).

The complete loss of β -lactam production was not anticipated when this set of experiments was initiated. The dramatic effect the gene disruption had on the secondary metabolism led to speculation whether some other essential gene had been deleted, as the sequence of 300 bp downstream of the $gdhA$ gene was unknown. However, based on the results of the BLAST searches and the identification of the polyadenylation site in the deleted part of the downstream sequence, it was concluded that the $gdhA$ gene was the only essential gene element that had been deleted.

Ammonium has been shown to have a direct effect on the expression of the β -lactam biosynthetic genes similar to glucose repression. Ammonium concentrations higher than 100 mM strongly interfered with cephalosporin production in *Acremonium chrysogenum* and parallel studies in *P. chrysogenum* showed repression of both $pcbAB$ and $pcbC$, encoding ACVS and IPNS, respectively, by addition of 40 mM NH_4Cl (Shen *et al.*, 1984; Feng *et al.*, 1994). In the present study, the nitrogen source consisted of 27 mM ammonium, which did not cause repressing conditions in the recipient strains, as illustrated by significant production of β -lactams. For the $gdhA$ -disrupted strains it is argued that the intracellular concentration of glutamate is lower than in the recipient strains due to reduced utilization of ammonium, which results in a higher intracellular ammonium concentration in these strains. This is supported by metabolite profiling of *S. cerevisiae* carrying a deletion of the $gdh1$ gene, encoding NADPH-dependent glutamate dehydrogenase, and simultaneous overexpression of $gdh2$, encoding NADH-dependent glutamate dehydrogenase (Villas-Bôas *et al.*, 2005). Hypothetically, this higher concentration of ammonium could cause repression of the β -lactam biosynthetic genes, resulting in loss of β -lactam production. Supplementing the medium with glutamate increases the intracellular pools of both

glutamate and glutamine, whereas the ammonium concentration remains unchanged, thereby maintaining ammonium repression conditions. Therefore, supplementing glutamate to cultures of the $gdhA$ -disrupted strains restored the maximum specific growth rates, but not the β -lactam production.

The ammonium repression hypothesis was tested by two different approaches. First, it was tested whether it was possible to eliminate production of β -lactam in the industrial strains simply by increasing the ammonium concentration to 135 mM, which according to the literature should repress the β -lactam production significantly (Shen *et al.*, 1984; Feng *et al.*, 1994). Unexpectedly, both the high-producing industrial strains remained productive. The other approach focused on utilization of a non-repressive nitrogen source, in this case proline. If ammonium repression was causing the constructed $\Delta gdhA$ strains to be non-productive, cultivating the strains with proline would relieve the repression and production would occur. Unfortunately, as in the other cultivations with the $gdhA$ -disrupted strains, no β -lactam was produced. Therefore, it was concluded that the lack of production in the $gdhA$ -disrupted strains could not be caused by ammonium repression.

Considering the results and discussion above it may be argued that the GDHA enzyme is involved in protein-protein interactions with regulators of the β -lactam biosynthetic pathway, e.g. NRE. The *nre* gene is homologous to the major nitrogen regulatory gene *areA* from *A. nidulans* (Haas *et al.*, 1995). It has been demonstrated that NRE binds to fragments containing at least two GATA core sequences, among these the intergenic region between *acvA* and *pcbC*, encoding ACVS and IPNS, respectively (Haas & Marzluf, 1995). NRE has therefore been suggested to mediate both nitrogen metabolite regulation and β -lactam regulation in *P. chrysogenum* (Brakhage *et al.*, 2004). Studies in *A. nidulans* have shown that a GDHA loss-of-function mutant has very high AreA activity (Caddick, 1994). In these mutants, the GDHA protein is still present and only the glutamate dehydrogenase activity is lost (Kingham & Pateman, 1975). Following this line of argument it was expected that the activity of NRE would be elevated in the $gdhA$ -disrupted strains and the production of β -lactam should be increased if NRE was acting as a positive regulator. However, the lack of production shows that this is not the case and it is therefore speculated that NRE needs to be activated to induce β -lactam production and this activation could be achieved through binding with GDHA. Assuming that the GDHA protein is not synthesized in the $gdhA$ -disrupted strains, the NRE is not being activated and the production is not induced.

An alternative explanation of the observed results could be that 2-oxoglutarate acts as a repressor molecule for NRE. Organic acids are known to act as effector molecules of the FadR-binding proteins which are involved in regulation of

fatty acid biosynthesis and degradation in *Escherichia coli* (Pagliarulo *et al.*, 2004). As outlined above, the 2-oxoglutarate concentration is highly elevated in a $\Delta gdhA$ strain, which is a requirement for the thermodynamically unfavoured reaction, catalysed by GDHB, to take over the production of glutamate. The concentration of 2-oxoglutarate would therefore remain high independent of the nitrogen source, since it is required for biosynthesis of glutamate. It is therefore hypothesized that 2-oxoglutarate binds to NRE and prevents this protein from binding to the β -lactam promoter region, resulting in lack of production.

From the results described above it is concluded that besides being the key enzyme in ammonium assimilation, the NADPH-dependent glutamate dehydrogenase appears to be involved in regulation of β -lactam production in industrial strains of *P. chrysogenum*. Furthermore, this study clearly emphasizes the complex interactions between the central and secondary metabolism and the redox metabolism.

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