L-Glutamine as a nitrogen source for *Corynebacterium glutamicum*: derepression of the AmtR regulon and implications for nitrogen sensing

Nadine Rehm,^{1†} Tobias Georgi,^{2†} Eva Hiery,¹ Ursula Degner,² Alfred Schmiedl,³ Andreas Burkovski¹ and Michael Bott²

Correspondence

Michael Bott
m.bott@fz-juelich.de

¹Lehrstuhl für Mikrobiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, D-91058 Erlangen, Germany

²Institut für Biotechnologie 1, Forschungszentrum Jülich, D-52425 Jülich, Germany

³Lehrstuhl für Biochemie, Friedrich-Alexander-Universität Erlangen-Nürnberg, D-91058 Erlangen, Germany

Corynebacterium glutamicum, a Gram-positive soil bacterium employed in the industrial production of various amino acids, is able to use a number of different nitrogen sources, such as ammonium, urea or creatinine. This study shows that L-glutamine serves as an excellent nitrogen source for *C. glutamicum* and allows similar growth rates in glucose minimal medium to those in ammonium. A transcriptome comparison revealed that the nitrogen starvation response was elicited when glutamine served as the sole nitrogen source, meaning that the target genes of the global nitrogen regulator AmtR were derepressed. Subsequent growth experiments with a variety of mutants defective in nitrogen metabolism showed that glutamate synthase is crucial for glutamine utilization, while a putative glutaminase is dispensable under the experimental conditions used. The *gltBD* operon encoding the glutamate synthase is a member of the AmtR regulon. The observation that the nitrogen starvation response was elicited at high intracellular L-glutamine levels has implications for nitrogen sensing. In contrast with other Gram-positive and Gram-negative bacteria such as *Bacillus subtilis*, *Salmonella enterica* serovar Typhimurium and *Klebsiella pneumoniae*, a drop in glutamine concentration obviously does not serve as a nitrogen starvation signal in *C. glutamicum*.

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INTRODUCTION

Corynebacterium glutamicum is a Gram-positive soil bacterium which was isolated by Kinoshita and co-workers in a screening programme for L-glutamate-producing bacteria (Kinoshita *et al.*, 2004; Uda, 1960) and subsequently used for the industrial production of amino acids. Today, large amounts of L-glutamate (more than 1 524 000 tonnes per year) and L-lysine (more than 812 800 tonnes per year) are produced by using *C. glutamicum* strains (Hermann, 2003; Leuchtenberger *et al.*, 2005). The genome sequence of this species became available in 2003 (Ikeda & Nakagawa,

2003; Kalinowski *et al.*, 2003) and provided the basis for global analyses such as transcriptomics (Hüser *et al.*, 2003; Polen & Wendisch, 2004; Wendisch, 2003) or proteomics (Bendt *et al.*, 2003; Schaffer *et al.*, 2001) and to establish *C. glutamicum* as a model organism for high-GC Gram-positive bacteria.

A focus of our previous studies was a detailed understanding of nitrogen metabolism and its control in *C. glutamicum*. Ammonium assimilation involves three key reactions (Fig. 1): the reductive amination of 2-oxoglutarate to L-glutamate by glutamate dehydrogenase (GDH), the ATP-dependent conversion of L-glutamate and ammonia to L-glutamine by glutamine synthetase (GS), and the conversion of L-glutamine and 2-oxoglutarate to two molecules of L-glutamate by glutamate:2-oxoglutarate aminotransferase (also known as glutamate synthase; GOGAT). Inspection of the genome revealed another putative enzyme that might be of importance for the conversion of L-glutamine to L-glutamate, namely glutaminase (GlsK). However, the function of this protein has not yet been analysed experimentally.

†These authors contributed equally to this work.

Abbreviations: μ , growth rate; FBI-GS, feedback-inhibited glutamine synthetase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate:2-oxoglutarate aminotransferase (also known as glutamate synthase); GlSK, glutaminase; MRM, multiple reaction monitoring mode.

Microarray data and experimental details have been deposited in the Gene Expression Omnibus (GEO) database with the accession number GSE19779.

Fig. 1. Overview on enzymes, genes and reactions relevant for nitrogen assimilation in *C. glutamicum*. GDH, glutamate dehydrogenase; GOGAT, glutamate : 2-oxoglutarate aminotransferase (also glutamate synthase); GlS, putative glutaminase; GS, glutamine synthetase.

The regulation of nitrogen metabolism in *C. glutamicum* has been intensively studied. Expression of nitrogen-controlled genes is regulated by the TetR-family protein AmtR (Jakoby *et al.*, 2000), which blocks transcription of various genes during growth in nitrogen-rich medium. At least 35 genes are directly controlled by the AmtR repressor (Beckers *et al.*, 2005; Buchinger *et al.*, 2009) and these encode transporters and enzymes for ammonium assimilation (*amtA*, *amtB*, *glnA*, *gltBD*, *dapA*), creatinine (*codA*, *crnT*) and urea (*urtABCDE*, *ureABCEFGD*) metabolism, a number of biochemically uncharacterized enzymes and transport systems, as well as signal transduction proteins (*glnD*, *glnK*). In contrast with most TetR-type regulators, the dissociation of AmtR from its target promoters is not triggered by the binding of a low-molecular mass ligand, but by complex formation with the PII-type signal transduction protein GlnK (Beckers *et al.*, 2005). This interaction requires that the trimeric GlnK protein is adenylylated at tyrosine residue 51. Adenylation is catalysed by the adenylyltransferase GlnD in response to nitrogen deprivation, but it is not yet clear how this is sensed by the enzyme. Global analyses on nitrogen metabolism focused on either AmtR (Buchinger *et al.*, 2009) or the cell's response to ammonium limitation or starvation (Silberbach *et al.*, 2005a, b).

The work presented here analyses the use of glutamine as a nitrogen source for *C. glutamicum*. Glutamine is not only a key metabolite for nitrogen assimilation and transferase reactions but also a marker metabolite for nitrogen supply in different Gram-negative and Gram-positive bacteria

such as *Salmonella enterica* serovar Typhimurium (Ikeda *et al.*, 1996), *Klebsiella pneumoniae* (Schmitz, 2000) and *Bacillus subtilis* (Hu *et al.*, 1999). In enterobacteria, the NtrBC two-component signal transduction system activates transcription of σ^{54} -dependent promoters in response to nitrogen limitation (Weiss *et al.*, 2002). Phosphorylation of the response regulator NtrC by the NtrB kinase is controlled by the nitrogen regulatory protein PII (GlnB) mainly depending on the cellular glutamine concentration (via uridylylation/deuridylylation of PII by the uridylyl-transferase GlnD). In *B. subtilis*, GlnR, TnrA and GltC (Schreier *et al.*, 1989; Wray *et al.*, 1996, 1998; Picossi *et al.*, 2007) co-operate to regulate nitrogen metabolism (for review, see Fisher, 1999; Sonenshein, 2007; Amon *et al.*, 2010). GlnR is active during growth with excess nitrogen, where it represses the expression of *glnRA*, *ureABC* and *tnrA* (for review, see Sonenshein, 2007; Amon *et al.*, 2010), whereas TnrA is active during nitrogen-limited growth, where it activates and represses the expression of genes involved in the transport and metabolism of nitrogen compounds. The *glnA* gene encodes GS, which is feedback-inhibited by glutamine. The activities of TnrA and GlnR are controlled through direct protein-protein interactions by feedback-inhibited GS (FBI-GS), which is only present under nitrogen excess. FBI-GS activates the DNA-binding activity of GlnR through a transient association where FBI-GS acts as a chaperone that stabilizes GlnR-DNA complexes (Fisher & Wray, 2008, 2009). In contrast, TnrA is inactivated by FBI-GS through the formation of a stable complex which effectively blocks the DNA-binding ability of TnrA (Wray & Fisher, 2008).

The results presented in this paper indicate that glutamine is an excellent nitrogen source for *C. glutamicum* and that its metabolism is dependent on GOGAT. Furthermore, the data suggest that it is unlikely that glutamine serves as an indicator of the cellular nitrogen status.

METHODS

Bacterial strains, media and growth conditions. All strains and plasmids used in this work are listed in Table 1. The *C. glutamicum* type strain ATCC 13032 (Abe *et al.*, 1967) was used as wild-type. For growth experiments and DNA microarray experiments, cells from a fresh LB agar plate (Sambrook *et al.*, 2001) were inoculated into 70 ml LB medium (Sambrook *et al.*, 2001) in a 500 ml baffled shake flask and this first preculture was incubated overnight at 30 °C and 120 r.p.m. For the second preculture and the main culture, a modified CGXII medium (Keilhauer *et al.*, 1993) was used which contained 70 mM glucose as a carbon source and either 70 mM L-glutamine or 150 mM ammonium sulfate as a sole nitrogen source. The medium was supplemented with 0.03 g 3,4-dihydroxybenzoate l⁻¹ as iron chelator and 0.2 mg biotin l⁻¹. Before inoculation of the second preculture to OD₆₀₀ 0.1 and of the main cultures to OD₆₀₀ 0.5 (or 1.0 for growth experiments), cells were washed with culture medium without any carbon or nitrogen source. The second preculture was incubated overnight and the main cultures were incubated for the indicated times at 30 °C and 120 r.p.m. For the transcriptome comparison, the main cultures were grown to OD₆₀₀ 4.1 ± 0.2 before cells were harvested and used for RNA preparation.

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant genotype or description	Reference
Strains		
<i>C. glutamicum</i>		
ATCC 13032	Wild-type	Abe <i>et al.</i> (1967)
LNΔ <i>gdh</i>	ATCC 13032 Δ <i>gdh</i> , strain lacks glutamate dehydrogenase (GDH)	Müller <i>et al.</i> (2006)
LNΔ <i>gltBD</i>	ATCC 13032 Δ <i>gltBD</i> , strain lacks GOGAT	L. Nolden, personal communication
NR-2	ATCC 13032 Δ <i>glsK</i> , strain lacks putative GlcK	This study
NR-3	ATCC 13032 Δ <i>gdh</i> Δ <i>glsK</i> , strain lacks GDH and GlcK	This study
NR-4	ATCC 13032 Δ <i>gltBD</i> Δ <i>glsK</i> , strain lacks GOGAT and GlcK	This study
NR-5	ATCC 13032 Δ <i>gltBD</i> Δ <i>gdh</i> , strain lacks GOGAT and GDH	This study
NR-6	ATCC 13032 Δ <i>gltBD</i> Δ <i>gdh</i> Δ <i>glsK</i> , strain lacks GOGAT, GDH and GlcK	This study
<i>E. coli</i>		
DH5αMCR	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>)	Grant <i>et al.</i> (1990)
Plasmids		
pK18Δ <i>gdh</i>	pK18mobsacB derivative carrying <i>gdh</i> flanking DNA fragments	Müller <i>et al.</i> (2006)
pK19mobsacB	Vector for generation of chromosomal deletions in <i>C. glutamicum</i> , <i>ori</i> _{E.c.} , <i>sacB</i> , Kan ^R	Schäfer <i>et al.</i> (1994)
pK19mobsacB-Δ <i>glsK</i>	pK19mobsacB carrying DNA fragments of the <i>glsK</i> upstream and downstream region	This study
pEKEx2	<i>C. glutamicum</i> / <i>E. coli</i> shuttle vector for regulated gene expression, <i>ori</i> _{V.C.g.} , pUC18 <i>ori</i> _{V.E.c.} , P _{tac} , <i>lacI</i> ^R , pBL1, Kan ^R	Eikmanns <i>et al.</i> (1991)
pEKEx- <i>gltBD</i>	pEKEx2 derivative containing the glutamate synthase genes <i>gltBD</i> from <i>C. glutamicum</i>	This study

For all cloning purposes, *Escherichia coli* DH5αMCR (Grant *et al.*, 1990) was used as host and cells were cultivated aerobically at 37 °C.

Global gene expression analysis. Preparation of RNA and synthesis of fluorescently labelled cDNA were carried out as described previously (Lange *et al.*, 2003; Möker *et al.*, 2004). Custom-made whole-genome DNA microarrays for *C. glutamicum* ATCC 13032 printed with 70-mer oligonucleotides were obtained from Operon (Cologne) and are based on the genome sequence with GenBank accession no. NC_006958 (Kalinowski *et al.*, 2003). Hybridization and stringent washing of the microarrays were performed according to the supplier's instructions. Hybridization was carried out for 16–18 h at 42 °C using a MAUI hybridization system (BioMicro Systems). After washing, the microarrays were dried by centrifugation (5 min, 1600 g) and fluorescence was determined at 532 nm (Cy3-dUTP) and 635 nm (Cy5-dUTP) with 10 μm resolution using an Axon GenePix 6000 laser scanner (Axon Instruments). Quantitative image analysis was carried out using GenePix image analysis software and results were saved as a GPR file (GenePix Pro 6.0, Axon Instruments). For data normalization, GPR files were processed using the Bio-Conductor/R-packages limma and marray (www.bioconductor.org). Processed and normalized data as well as experimental details [these conformed to the MIAME guidelines (Brazma *et al.*, 2001)] were stored in the in-house microarray database for further analysis (Polen & Wendisch, 2004) and in the Gene Expression Omnibus (GEO) repository under the accession number GSE19779.

Using DNA microarray technology, the mRNA concentrations of *C. glutamicum* wild-type grown with glutamine as a sole nitrogen source were compared with those of wild-type cells grown with ammonium sulfate as a sole nitrogen source. As outlined above, the strains were cultivated in CGXII minimal medium with 70 mM glucose as carbon source and either 70 mM L-glutamine or 150 mM ammonium sulfate as sole nitrogen source. RNA used for the synthesis of labelled cDNA was prepared from cells in the exponential growth phase at OD₆₀₀ 4.1 ± 0.2. The comparison was performed three times, starting from independent cultures. To filter for differentially expressed genes and reliable signal detection in each of the three comparisons, the

following quality filter was applied: (i) flags ≤ 0 (GenePix Pro 6.0), (ii) signal/noise ≥ 3 for Cy5 (F635Median/B635Median, GenePix Pro 6.0) or Cy3 (F532Median/B532Median, GenePix Pro 6.0), (iii) ≥ twofold change on average in the comparison of wild-type glutamine/wild-type ammonium sulfate, (iv) significant change (*P* < 0.05) by Student's *t*-test.

RNA hybridization. RNA probes for the analysis of transcription of individual genes were generated by PCR and subsequent labelling with DIG RNA-labelling mix and T7 RNA polymerase (Roche Diagnostics). The oligonucleotides used for the *gdh* probe were *gdh*-for (5'-CCCGGGTGACATCGGAGTTGGTGG) and *gdh*-rev (5'-GGGCCCTAATACGACTCACTATAGGGGAGCTTAGCCACGTCAAC), for the *glnA* probe, *glnA*-for (5'-CCAAACCGTTGACGTGCG) and *glnA*-rev (5'-GGGCCCTAATACGACTCACTATAGGGGGATCCGGTGATTGGGATAC), for the *gltB* probe, *gltB*-for (5'-CCACTGTGTGCTGAAGGTGATGTCC) and *gltB*-rev (5'-GGGCCCTAAT-ACGACTCACTATAGGGGAAACGCTTC), and for the *gltD* probe, *gltD*-for (5'-GGGCCCCCGCGAAGGCTGGGTGCAACC) and *gltD*-rev (5'-GGGCCCTAATACGACTCACTATAGGGGTGAGGTAAT). The underlined sequences represent the T7 promoter sequence. All oligonucleotides were used at a final concentration of 0.5 μM. Chromosomal DNA as template and the following PCR programme were used: 3 min at 94 °C, 30 cycles of 15 s at 94 °C followed by 15 s at 60 °C and 1 min at 72 °C, then 10 min at 72 °C followed by cooling to 4 °C. The RNA isolated from cells was spotted directly onto nylon membranes using a Schleicher & Schuell Minifold I dot blotter. Hybridization of DIG-labelled RNA probes was detected with Kodak X-OMAT X-ray films using alkaline phosphatase-conjugated anti-DIG Fab fragments and CSPD as a light-emitting substrate as recommended by the supplier (Roche Diagnostics).

General molecular biology techniques. For plasmid isolation, transformation and cloning, standard techniques were used (Ausubel *et al.*, 1987; Sambrook *et al.*, 2001). *E. coli* strain DH5αMCR was used as the cloning host. Competent *C. glutamicum* cells were prepared according to the method described by van der Rest *et al.* (1999). Chromosomal *C. glutamicum* DNA was isolated as described by

Eikmanns *et al.* (1994). DNA sequence analyses were carried out using an ABI sequencer and the freely available software Chromas Lite.

Construction of deletion mutants. Unmarked deletions were introduced into the genome of *C. glutamicum* according to the protocol described by Schäfer *et al.* (1994) and Niebisch & Bott (2001). All deletions were verified by PCR and the flanking regions in the genome were sequenced as a control (data not shown). For construction of the *glsK* deletion plasmid, 0.7 kb DNA fragments covering the upstream and the downstream region of *glsK* were amplified and fused by overlap-extension PCR using chromosomal DNA from ATCC 13032 as a template. The oligonucleotide primers were designed in such a way that an *Xba*I restriction site (flanking the upstream fragment) or an *Sph*I restriction site (flanking the downstream fragment) were introduced (5'-CGCTCTAGAGCGAATCCACCGAAATCGTCTTCATCT-3', 5'-GCCCATAAAGCTACCGGAGAATCAAGACTAGTGAT-3', 5'-ATCACTAGTCTTGATTCTCCGGTAGCTTTATGGGC-3', 5'-CGCGCATGCTTTAAGCTGCGAGGCCTTCTTCGAGTTT-3', restriction sites shown in bold). The resulting 1.4 kb DNA fragment was ligated to *Xba*I/*Sph*I-restricted vector pK19mobsacB, leading to plasmid pK19mobsacB-Δ*glsK*. This plasmid was then applied to introduce a chromosomal deletion of *glsK* as described previously (Schäfer *et al.*, 1994) into strains ATCC 13032, LNA*gdh* and LNA*gltBD* leading to strains NR-2, NR-3 and NR-4, respectively. In order to generate a chromosomal *gdh* deletion in the *C. glutamicum* mutant strains LNA*gltBD* and NR-4, plasmid pK18Δ*gdh* was used (Müller *et al.*, 2006).

Construction of a *gltBD* overexpression plasmid. A plasmid for overexpression of *gltBD*, designated pEKEx-*gltBD*, was generated as follows. First, a 2.7 kb fragment of the 5'-region of *gltB* (*gltB'*) was amplified by PCR using chromosomal DNA as template and the oligonucleotides 5'-GGGCCCCGGATCCATGAAACCACAAGGACTCTAC-3' and 5'-GGGCCCCGGTACCGGCTTCAGCAGAAATCGAGCC-3'. The *gltB'* fragment was ligated into pEKEx2 using the *Bam*HI and *Acc*65I sites introduced by the oligonucleotides (shown in bold), leading to the plasmid pEKEx-*gltB'*. The second fragment of 3.5 kb, harbouring the 3'-region of *gltB* and the entire *gltD* gene (*gltBD''*), was amplified by PCR using chromosomal DNA as template and the oligonucleotides 5'-GGGCCCCGGACCGCAAGCCAATTTTCGGTG-3' and 5'-GGGCCCCGGTACCCTAGACAGCCAGCGGCACGTC-3'. The *gltBD''* fragment was cloned into pEKEx-*gltB'* using the *Rsr*II restriction site, which naturally occurs in the downstream region of *gltB*, and the *Acc*65I restriction site, introduced by PCR (shown in bold). The resulting plasmid, pEKEx-*gltBD*, was sequenced as a control.

Enzyme activity measurements and protein determination. GS activity was determined by using the protocol described by Shapiro & Stadtman (1970). The protein content of cell extracts was determined using a Bradford assay (Roth).

Western blot analyses. For the preparation of *C. glutamicum* cell extracts, 2 ml aliquots were transferred from the culture to tubes containing 300 mg glass beads (0.25 mm, Sigma) and immediately frozen in liquid nitrogen. Samples thawed on ice were disrupted using a FastPrep FP120 instrument (Q-BIOgene). Subsequently, glass beads and cell debris were removed by centrifugation. Protein concentrations were determined as described above. SDS-PAGE was carried out using 10 % acrylamide gels and a Tricine buffer system as described by Schägger & von Jagow (1987). After electrophoresis, the gel-separated proteins were transferred onto a PVDF membrane by electroblotting and incubated with a rabbit polyclonal antiserum against *C. glutamicum* GS. Antibody binding was visualized by appropriate secondary antibodies coupled to alkaline phosphatase and the BCIP/NBT alkaline phosphatase substrate (Sigma-Aldrich).

Measurement of intracellular L-glutamine concentrations. Cells were grown as described above in minimal medium and 2–4 ml culture was harvested in the exponential growth phase by centrifugation (2 min, 8000 g, 25 °C). Cell pellets were washed once with 4 ml PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, adjusted with HCl to pH 7.4). Metabolites were extracted from the cell pellets by adding 4 ml 80 % (v/v) ethanol, previously heated to 80 °C, to the cell pellets. The extracts were then incubated under shaking for 30 min at 80 °C. After centrifugation, the supernatant containing the extracted metabolites was kept and the disrupted cells were again treated with ethanol as described above. The second supernatant was mixed with the first and an appropriate amount was dried using a vacuum centrifuge. Metabolites were dissolved in double distilled water (1 ml) and the solution was filtered through a 10 kDa exclusion membrane in order to remove proteins and other small cell particles. The samples were then used for metabolite quantification by liquid chromatography coupled to mass spectrometry. The LC-MS system used consisted of a liquid chromatograph DIONEX ICS-3000 equipped with an Autosampler AS-50, a column ACCLAIM-OA 5 μm, 4 × 250 mm, and a precolumn ACCLAIM-OA 5 μm, 4.3 × 10 mm (all from Dionex). The chromatograph was operated by the software Chromatography MS Link 2.0.0.2315 (DC/MS-Link 2.0; Dionex) which is a plug-in module of the MS software ANALYST 1.4.2 (Applied Biosystems). MDS SCIEX TurboV (MDS Analytical Technologies) was used as an ion source. Samples were kept at 8 °C in the autosampler before injection. The system was equilibrated with buffer (2.5 mM ammonium acetate, pH 3.5). A flow rate of 0.25 ml min⁻¹ and a column temperature of 30 °C were used. The run time of each sample took about 50 min. A gradient of 50 mM ammonium acetate, rising from 5 to 30 % (v/v), then decreasing to 5 % (v/v) again, was used, followed by increasing the acetonitrile content from 0 to 90 % (v/v) to elute hydrophobic

Fig. 2. Growth of *C. glutamicum* wild-type in carbon- and nitrogen-free CGXII medium supplemented with 70 mM glucose and 150 mM ammonium sulfate (■), 70 mM glucose and 70 mM L-glutamine (□), 70 mM glutamine and 150 mM ammonium sulfate (▲), or 70 mM glutamine (△). Representative results of at least three independent experiments are shown.

Table 2. Genes whose mean mRNA ratio was altered $\geq$ twofold when *C. glutamicum* was grown in glucose minimal medium with L-glutamine instead of ammonium as the sole nitrogen source

The ratios represent mean values from three DNA microarray experiments starting from independent cultures.

Group	Locus tag	Gene name	Annotation	mRNA ratio Gln : NH ₄
A: genes belonging to the AmtR regulon				
	cg0103	<i>crnT</i>	Creatinine permease	664.3
	cg0104	<i>codA</i>	Creatinine deaminase	39.4
	cg0113	<i>ureA</i>	Urease, γ subunit	24.7
	cg0114	<i>ureB</i>	Urease, β subunit	19.4
	cg0115	<i>ureC</i>	Urease, α subunit	27.3
	cg0116	<i>ureE</i>	Urease, accessory protein	22.5
	cg0117	<i>ureF</i>	Urease accessory protein	27.0
	cg0118	<i>ureG</i>	Urease, accessory protein	24.3
	cg0119	<i>ureD</i>	Urease, accessory protein	18.1
	cg0229	<i>gltB</i>	GOGAT, large subunit	92.5
	cg0230	<i>gltD</i>	GOGAT, small subunit	230.7
	cg1061	<i>urtA</i>	ABC-type urea uptake system, substrate binding protein	229.2
	cg1062	<i>urtB</i>	ABC-type urea uptake system, permease subunit	56.9
	cg1064	<i>urtC</i>	ABC-type urea uptake system, permease subunit	375.4
	cg1065	<i>urtD</i>	ABC-type urea uptake system, ATP-binding protein	27.5
	cg1066	<i>urtE</i>	ABC-type urea uptake system, ATP-binding protein	38.5
	cg1256	<i>dapD</i>	Tetrahydrodipicolinate succinylase	6.64
	cg1295		Predicted hydrolase or acyltransferase	14.1
	cg1783	<i>soxA</i>	Sarcosine oxidase	143.3
	cg1784	<i>ocd</i>	Ornithine cyclodeaminase	750.3
	cg1785	<i>amtA</i>	High-affinity ammonia permease	1826.4
	cg2258	<i>glnD</i>	PII adenylyltransferase	20.8
	cg2260	<i>glnK</i>	Nitrogen regulatory protein PII	23.2
	cg2261	<i>amtB</i>	Low affinity ammonium uptake protein	140.6
	cg2429	<i>glnA</i>	GS I	9.5
	cg3335	<i>malE</i>	Malic enzyme	2.08
B: genes belonging to the DtxR regulon				
	cg0160		Hypothetical protein Cg0160	0.50
	cg0466		Conserved secreted protein	0.46
	cg0589		Siderophore ABC transporter, permease protein	0.40
	cg0590		Siderophore ABC transporter, permease protein	0.32
	cg0591		Siderophore ABC transporter, ATP-binding protein	0.29
	cg0770		Siderophore ABC transporter, permease protein	0.45
	cg0921		Cytoplasmic siderophore-interacting protein	0.29
	cg0922		Secreted siderophore-binding protein	0.27
	cg0924		Secreted siderophore-binding lipoprotein	0.30
	cg0926		Siderophore ABC transporter, permease protein	0.38
	cg0927		Siderophore ABC transporter, permease protein	0.48
	cg1120	<i>ripA</i>	Transcriptional regulator of iron proteins, AraC family	0.32
	cg1418		Secreted siderophore-binding lipoprotein	0.45
	cg3404		Secreted siderophore-binding lipoprotein	0.41
C: genes not belonging to the AmtR or DtxR regulons				
	cg0233		Hypothetical protein Cg0233	0.50
	cg0464	<i>ctpA</i>	Copper-transporting ATPase	0.39
	cg1129	<i>aroF</i>	3-deoxy-7-phosphoheptulonate synthase	0.42
	cg1606	<i>pyrG</i>	CTP synthetase	6.69
	cg1607		NTP pyrophosphohydrolase including oxidative damage repair enzyme	2.52
	cg1608	<i>xerD</i>	Tyrosine recombinase	2.02
	cg1660		Hypothetical protein Cg1660	0.48
	cg1832		ABC-type cobalamin transport system, permease	9.14
	cg1833		ABC-type cobalamin transport system, secreted binding lipoprotein	14.63

Table 2. cont.

Group	Locus tag	Gene name	Annotation	mRNA ratio Gln:NH ₄
	cg1922		Hypothetical protein Cg1922	2.01
	cg2283		Hypothetical protein Cg2283	0.38
	cg2560	<i>aceA</i>	Isocitrate lyase	0.24
	cg3082		Bacterial regulatory proteins, ArsR family	0.22
	cg3083		Predicted Co/Zn/Cd cation transporter	0.36
	cg3084		Predicted flavoprotein involved in K ⁺ transport	0.43
	cg3096		Aldehyde dehydrogenase	0.30
	cg3107	<i>adhA</i>	Zn-dependent alcohol dehydrogenase	0.33
	cg3138	<i>ppmA</i>	Putative membrane-bound protease modulator	0.02
	cg3139		Hypothetical protein Cg3139	0.02
	cg3140	<i>tagA1</i>	Putative DNA-3-methyladenine glycosylase I protein	0.04
	cg3141	<i>hmp</i>	Flavo-haemoprotein	0.48
	cg3169	<i>pck</i>	Phosphoenolpyruvate carboxykinase (GTP)	2.12
	cg3195		Flavin-containing monooxygenase (FMO)	0.40
	cg3280		Putative secreted protein	0.48
	cg3282		Cation transport ATPase	0.46

amino acids and wash the column. Glutamine eluted at a retention time of 9.8 min. Analysis was carried out by a mass spectrometer 3200 QTRAP (Applied Biosystems) using a multiple reaction monitoring mode (MRM) with a dwell time of 50 ms per MRM. The detection parameters for glutamine were as follows: ion-source-specific parameters CUR, 10 p.s.i.; IS, 5500 V; GS1, 40 p.s.i.; GS2, 45 p.s.i.; TEM, 500 °C. The compound-specific parameters in MRM mode were as follows: MRM transition, 147 to 130; DP, 23.5 V; EP, 5.5 V; CEP, 14 V; CE, 14 V; CXP, 2.5 V. The data obtained were analysed by the ANALYST 1.4.2 software. Internal glutamine concentrations of *C. glutamicum* cells were then calculated from the data using the following correlations: OD₆₀₀=1 corresponds to 0.3 mg cell dry weight ml⁻¹ and 1 mg cell dry weight corresponds to a cytoplasmic volume of 1.6 µl (Botzenhardt *et al.*, 2004).

RESULTS

Growth of *C. glutamicum* on glutamine as the carbon and/or nitrogen source

In order to determine the growth parameters of *C. glutamicum* with L-glutamine as the sole nitrogen and/or carbon source, wild-type cells were cultivated in CGXII minimal medium containing different carbon and nitrogen sources. As shown in Fig. 2, *C. glutamicum* wild-type ATCC 13032 was able to grow with 70 mM L-glutamine as the sole carbon and nitrogen source to a final OD₆₀₀ ~10 ± 1.2, but with a very slow growth rate (μ) between 0.02 and 0.03 h⁻¹. The additional presence of 150 mM ammonium sulfate had no influence on glutamine-dependent growth. In contrast, cells cultivated with 70 mM glucose and 70 mM L-glutamine as the sole nitrogen source showed a similar growth rate (μ =0.34 ± 0.01 h⁻¹) to cells grown with 70 mM glucose and 150 mM ammonium sulfate as the sole nitrogen source (μ =0.33 ± 0.01 h⁻¹). The final OD₆₀₀ was 28.7 ± 3.8 for growth on glucose and L-glutamine and 18.4 ± 0.9 for growth on glucose and ammonium, indicating that L-glutamine is incorporated into biomass to the same extent as

during growth on L-glutamine as the sole carbon and nitrogen source. In summary, these data show that L-glutamine is metabolized slowly as a carbon source but serves as an excellent nitrogen source, allowing the same growth rates as allowed by ammonium.

Comparison of global gene expression profiles of *C. glutamicum* grown on L-glutamine or ammonium as a sole nitrogen source using DNA microarrays

To analyse how L-glutamine influences global gene expression when used as a sole nitrogen source instead of ammonium, DNA microarray analyses were performed. For this purpose, RNA was isolated from exponentially growing cells (OD₆₀₀ 4.1 ± 0.2) cultivated in CGXII medium containing glucose as the carbon source and either L-glutamine (70 mM) or ammonium sulfate (150 mM) as the nitrogen source. As shown above, the growth rates of *C. glutamicum* in these two media are very similar and therefore growth-rate-dependent effects on transcription were expected to be negligible. In fact, transcription of genes whose expression is typically growth-rate-dependent, such as the *atpABCDEF* operon or ribosomal genes, was not altered. Overall, 65 genes showed significantly ($P < 0.05$) changed mRNA levels by a factor of two or more (average of three biological replicates). These genes can be subdivided into three groups (Table 2).

Group A comprises 26 genes which belong to the nitrogen starvation stimulon of *C. glutamicum* and are regulated by the transcriptional master regulator of nitrogen metabolism, AmtR (Jakoby *et al.*, 2000). All of these genes showed increased transcript abundance on L-glutamine as the nitrogen source (Table 2). Among these genes were *glnD* and *glnK*, encoding key proteins for nitrogen signal transduction (Meier-Wagner *et al.*, 2001), which showed 21- and 23-fold increased mRNA levels, respectively. The

glnA gene, which codes for GS, was 10-fold upregulated. The genes of the *gltBD* operon, encoding the large and the small subunit of GOGAT (Beckers *et al.*, 2001), showed 93- and 231-fold increased mRNA levels, respectively, and the genes for ammonium uptake, *amtA* and *amtB*, displayed 1826- and 141-fold increased mRNA levels, respectively. Furthermore, expression of several operons for uptake and utilization of alternative nitrogen sources was upregulated in cells growing with L-glutamine as a sole nitrogen source. These were the creatinine uptake and utilization genes *cg0103* and *cg0104* (664- and 39-fold increased mRNA levels, respectively) (Bendt *et al.*, 2004), the urease operon *ureABCEFGD* (Nolden *et al.*, 2000), whose genes were at least 18-fold upregulated, the urea uptake system *urtABCDE* (Beckers *et al.*, 2004), whose expression was at least 28-fold increased, and *soxA* (143-fold) and *ocd* (750-fold), which are supposed to be involved in the degradation of sarcosine and ornithine and are found in an operon together with *amtA* (Jakoby *et al.*, 1999). The gene *cg1295* showed a 14-fold increased mRNA level and is the only gene of this group whose function is presently unknown. Together, 24 known AmtR-regulated genes in *C. glutamicum* were strongly induced by at least a factor of 9.5 when cultivated with L-glutamine instead of ammonium as the sole nitrogen source. The five remaining genes of the AmtR regulon observed here either were not differentially expressed by a factor of at least two (*cg1646*, *gluD*, *gdh* and *mez*) or, in the case of *cg1296*, showed a strongly increased mRNA level, but exhibited too much variation to meet the *P*-value cut-off. The data clearly show that growth of *C. glutamicum* with L-glutamine as the sole nitrogen source causes derepression of the AmtR-regulated genes (Beckers *et al.*, 2005; Jakoby *et al.*, 2000).

Group B (Table 2) comprises 14 genes, which were previously shown to be repressed under excess iron conditions by DtxR, the master regulator of iron homeostasis in *C. glutamicum* (Brune *et al.*, 2006; Wennerhold & Bott, 2006). The mRNA level of these genes was lower by a factor of between two and four in cells grown with L-glutamine as the nitrogen source compared with cells grown with ammonium. Twelve of the 14 genes are involved in binding or uptake of iron: *cg0589*, *cg0590*, *cg0591*, *cg0770*, *cg0926* and *cg0927* encode components of siderophore ABC transporters; *cg0922*, *cg0924*, *cg1418* and *cg3404* code for secreted siderophore-binding lipoproteins; *cg0466* encodes a haem transport-associated protein; and *cg0921* encodes a cytoplasmic siderophore-interacting protein (Frunzke & Bott, 2008; Wennerhold & Bott, 2006). Besides the genes involved in iron uptake, the mRNA level of the *ripA* gene was decreased by a factor of three. The AraC-type regulator RipA represses a set of genes encoding prominent iron proteins, such as aconitase or succinate dehydrogenase, under iron starvation and in this way reduces the iron demand of the cell (Wennerhold *et al.*, 2005). The function of the protein encoded by *cg0160*, whose mRNA level was also twofold lower during growth on L-glutamine as the nitrogen source, is unknown. The

above results indicate that the iron starvation response is turned on earlier during growth with ammonium than during growth with L-glutamine as a nitrogen source.

Recently, evidence was provided that the iron starvation response is induced by acidic pH (Follmann *et al.*, 2009). Therefore, we followed the pH during growth of *C. glutamicum* with glutamine or ammonium as the nitrogen source. Indeed, a difference in the pH profiles was observed. With glutamine, a slight increase in the pH from 7.0 to 7.4 was observed within 33 h of cultivation, whereas with ammonium, the pH dropped from 7.0 to 6.1 in the same period. At the time when the cells were harvested for RNA isolation (~5 h after the start of cultivation), there was a pH difference of about 0.10–0.15 between the cultures (data not shown), which might at least partially be responsible for the differences in the expression of the DtxR target genes.

Group C (Table 2) comprises a functionally diverse set of 25 genes whose mRNA levels were changed at least twofold and which do not belong to the AmtR or DtxR regulons. It includes genes involved in carbon metabolism, transport, transcriptional regulation and other functions. Three genes involved in the utilization of ethanol showed three- to fourfold lower mRNA levels with L-glutamine as the nitrogen source, i.e. those encoding alcohol dehydrogenase (*adhA*), acetaldehyde dehydrogenase (*cg3096*) and isocitrate lyase (*aceA*). On the other hand, the mRNA level of the gluconeogenic *pck* gene encoding phosphoenolpyruvate carboxykinase was twofold increased in cells grown with L-glutamine. For most of the genes summarized in group C, an obvious relationship to the utilization of L-glutamine as a nitrogen source is missing (see also Discussion).

Growth of mutant strains with ammonium as the nitrogen source

The microarray data suggest that one or several AmtR-controlled genes have a role in the utilization of glutamine as the nitrogen source. This assumption was investigated further by mutant analyses. *C. glutamicum* has two primary pathways for the synthesis of L-glutamate and L-glutamine (Fig. 1), which are the major nitrogen donors for biosynthetic reactions (Jakoby *et al.*, 1997; Shiio & Ozaki, 1970; Tesch *et al.*, 1998). GDH assimilates ammonium by the reductive amination of 2-oxoglutarate to L-glutamate. Alternatively, ammonium is assimilated via the GS/GOGAT pathway. GS uses ammonium to amidate L-glutamate under consumption of ATP to form L-glutamine. The amide group is then transferred reductively to 2-oxoglutarate by GOGAT, resulting in the net conversion of 2-oxoglutarate to L-glutamate. L-Glutamine might be metabolized by a presently uncharacterized glutaminase, encoded by the *C. glutamicum* *glsK* gene (*cg2728*), to L-glutamate and ammonia. In order to characterize the function of these different enzymes in glutamine metabolism, a series of mutant strains was constructed (details in Table 1) and tested first for growth on ammonium as the nitrogen source.

During growth in CGXII minimal medium with glucose as the carbon source and ammonium sulfate as the nitrogen source, the wild-type had a doubling time of 115 ± 4 min. Growth was unaltered in the $\Delta glsK$ mutant NR-2 (112 ± 6 min), the $\Delta gltBD$ mutant LN $\Delta gltBD$ (113 ± 7 min) and the $\Delta gltBD\Delta glsK$ double mutant NR-4 (111 ± 6 min). In contrast, *gdh* deletions significantly affected growth with ammonium. The Δgdh mutant LN Δgdh showed a doubling time of 193 ± 23 min, which was essentially unaltered in combination with a *glsK* mutation in the $\Delta gdh\Delta glsK$ double deletion strain NR-3 (175 ± 16 min). In combination with a *gltBD* deletion, the negative impact of a *gdh* deletion on growth was even more pronounced. For the $\Delta gltBD\Delta gdh$ mutant NR-5, a doubling time of 229 ± 39 min was observed; for the $\Delta gltBD\Delta gdh\Delta glsK$ triple mutant NR-6 this was 286 ± 48 min. The results are summarized in Table 3.

As shown recently (Müller *et al.*, 2006), a *gdh* mutation elicits a partially deregulated nitrogen starvation response in *C. glutamicum*, as shown by partial adenylation of GlnK and the increased transcription of *glnA* and *gltBD*. Ammonium is then assimilated exclusively via the GS/GOGAT pathway under an extra consumption of 1 mol ATP per mol ammonium assimilated, which might be the reason for the impaired growth of strain LN Δgdh .

The $\Delta gltBD\Delta gdh$ double mutant showed a strongly decreased growth rate. As this strain can still assimilate ammonium via GS, we assume that glutamate synthesis is the main factor that contributes to decreased growth. In the absence of GDH and GOGAT, the synthesis of glutamate can be achieved by transamination of 2-oxoglutarate using another amino acid as the amino donor. However, this route requires an alternative ammonium assimilation reaction. One possibility for such a reaction is aspartate ammonia lyase (aspartase), which catalyses the reversible conversion of fumarate and ammonia to aspartate. This enzyme is used in industry for the production of aspartate. Aspartate can then serve as an amino donor for the transamination of 2-oxoglutarate to glutamate, presumably by the transaminase AspT (Marienhagen *et al.*, 2005). *C. glutamicum* possesses an aspartate ammonia lyase encoded by *cg1697* and thus could

make use of it for ammonia assimilation in a $\Delta gltBD\Delta gdh$ double mutant. Another reaction involved in ammonia assimilation is the one catalysed by diaminopimelate dehydrogenase. This enzyme is involved in L-lysine biosynthesis and catalyses the reductive amination of piperidine-2,6-dicarboxylate to DL-diaminopimelate, which is subsequently decarboxylated to L-lysine. However, in contrast with L-aspartate it is unknown whether DL-diaminopimelate can serve as an amino donor in a transaminase reaction. Furthermore, there are many genes of unknown function present in the genome of *C. glutamicum*, which might provide a further route to glutamate synthesis together with one of the different transaminase reactions, which have only been partially characterized to date (Marienhagen *et al.*, 2005; Marienhagen & Eggeling, 2008).

The triggering of the nitrogen starvation response in the different strains was verified on the level of gene expression, GS content and GS activity (Fig. 3a–c). In short, *glnA* and *gltBD* expression, GS protein level and GS activity were increased in all strains carrying a *gdh* deletion, confirming the results obtained by Müller *et al.* (2006) which showed that a partial nitrogen starvation response is elicited by a lack of GDH.

Growth of mutant strains with L-glutamine as the nitrogen source

As shown by the transcriptome analyses presented above, growth with L-glutamine as the sole nitrogen source elicited the nitrogen starvation response in *C. glutamicum* wild-type. The molecular basis of this phenomenon was investigated by growth experiments with the mutants described above (Table 3). With a doubling time of 130 ± 8 min, growth of the wild-type with L-glutamine was almost as fast as with ammonium. Also, the $\Delta glsK$ mutant NR-2 (126 ± 2 min), the Δgdh mutant LN Δgdh (125 ± 12 min) and the $\Delta gdh\Delta glsK$ double deletion strain NR-3 (127 ± 13 min) reached wild-type growth rates. In contrast, growth of all *gltBD* mutants was significantly impaired. The $\Delta gltBD$ mutant LN $\Delta gltBD$ had a doubling time of 303 ± 51 min, the $\Delta gltBD\Delta glsK$ double

Table 3. Growth parameters of *C. glutamicum* strains cultivated with different nitrogen and carbon sources

t_d , Doubling time; values are mean $\pm$ SD. –, Not determined.

Carbon/nitrogen source:	Glucose/ NH_4^+		Glucose/L-glutamine		L-Glutamine/ NH_4^+		L-Glutamine/L-glutamine	
Strain	t_d (min)	μ (h^{-1})	t_d (min)	μ (h^{-1})	t_d (min)	μ (h^{-1})	t_d (min)	μ (h^{-1})
Wild-type	115 ± 4	0.36	130 ± 8	0.32	1903 ± 373	0.02	1908 ± 215	0.02
NR-2 ($\Delta glsK$)	112 ± 6	0.37	126 ± 2	0.33	–	–	–	–
LN Δgdh (Δgdh)	193 ± 23	0.22	125 ± 12	0.33	–	–	–	–
NR-3 ($\Delta gdh\Delta glsK$)	175 ± 16	0.24	127 ± 13	0.33	–	–	–	–
LN $\Delta gltBD$ ($\Delta gltBD$)	113 ± 7	0.37	303 ± 51	0.14	–	–	–	–
NR-4 ($\Delta gltBD\Delta glsK$)	111 ± 6	0.37	376 ± 122	0.11	–	–	–	–
NR-5 ($\Delta gltBD\Delta gdh$)	229 ± 39	0.18	468 ± 8	0.09	–	–	–	–
NR-6 ($\Delta gltBD\Delta gdh\Delta glsK$)	286 ± 48	0.15	474 ± 58	0.09	–	–	–	–

Fig. 3. Expression analyses, GS protein levels and GS activities of mutant strains grown with ammonium (a–c) or glutamine (d–f) as the nitrogen source. (a, d) RNA hybridization using probes against *gdh*, *glnA*, *gltB* and *gltD*. Total RNA was prepared from the wild-type (bar 1) and from mutant strains NR-2 (ΔglsK ; bar 2), $\text{LN}\Delta\text{gdh}$ (Δgdh ; 3), NR-3 ($\Delta\text{gdh}\Delta\text{glsK}$; 4), $\text{LN}\Delta\text{gltBD}$ (ΔgltBD ; 5), NR-4 ($\Delta\text{gltBD}\Delta\text{glsK}$; 6), NR-5 ($\Delta\text{gltBD}\Delta\text{gdh}$; 7) and NR-6 ($\Delta\text{gltBD}\Delta\text{gdh}\Delta\text{glsK}$; 8). Two micrograms total RNA per sample was spotted. (b, e) GS protein levels. Cell extracts (10 μg protein per lane) were separated by using SDS-PAGE and were visualized by Western blotting with anti-GS antiserum. (c, f) Specific GS activity. The average activity of three independent measurements is shown for each strain. Error bars, SD.

mutant NR-4 of 376 ± 122 min, the $\Delta\text{gltBD}\Delta\text{gdh}$ strain NR-5 showed a doubling time of 468 ± 8 min, and for the $\Delta\text{gltBD}\Delta\text{gdh}\Delta\text{glsK}$ triple mutant NR-6 a doubling time of 474 ± 58 min was observed. These results indicate that GOGAT is the main glutamine-metabolizing enzyme in *C. glutamicum* and provide an explanation for the derepression of the AmtR-regulated genes, since the GOGAT-encoding *gltBD* operon is under strict AmtR control in *C. glutamicum* (Beckers *et al.*, 2001).

The triggering of the nitrogen starvation response in the different mutants was tested again using RNA hybridization experiments, Western blotting and GS activity measurements (Fig. 3d–f). As expected from the DNA microarray data, the mRNA levels of *glnA*, *gltB*, and *gltD* were high, as were the GS protein levels. GS activity was high in most strains [$2\text{--}3 \text{ U (mg protein)}^{-1}$] but was significantly lower in strains NR-4 ($\Delta\text{gltBD}\Delta\text{glsK}$) and NR-6 ($\Delta\text{gdh}\Delta\text{gltBD}\Delta\text{glsK}$). Since the GS protein level was also high in these strains, the reduced activity seems to be due to activity regulation by adenylation via the adenylyl-transferase GlnE (Nolden *et al.*, 2001a).

Effect of plasmid-encoded *gltBD*

In light of the idea that GOGAT is primarily responsible for glutamine metabolism, it should be possible to rescue a

chromosomal *gltBD* deletion by plasmid-encoded *gltBD*. For this complementation assay, growth of the wild-type and the ΔgltBD mutant carrying either pEKEx2 or pEKEx2-*gltBD* was tested in the presence of 1 mM IPTG. In fact, growth of the ΔgltBD mutant in CGXII medium with glucose as the carbon source and L-glutamine as the nitrogen source was improved when it carried the *gltBD* expression plasmid, but it did not reach wild-type levels (Fig. 4). Doubling times observed were 108 min for the wild-type, 230 min for strain $\text{LN}\Delta\text{gltBD}$, 248 min for strain $\text{LN}\Delta\text{gltBD}$ carrying the control plasmid pEKEx2 and 164 min for strain $\text{LN}\Delta\text{gltBD}$ carrying pEKEx2-*gltBD*. This supports the proposed function of GOGAT in L-glutamine metabolism. The reason for the observed incomplete complementation of strain $\text{LN}\Delta\text{gltBD}$ by the multi-copy plasmid pEKEx2-*gltBD* remains unclear. Transcription of plasmid-borne and chromosomally expressed *gltB* and *gltD* was tested by RNA hybridization experiments and similar transcript levels were found (data not shown).

Glutamine as a sensor molecule for nitrogen control

As described above, GOGAT is important for efficient utilization of glutamine as a nitrogen source. Since the corresponding *gltBD* operon is under strict nitrogen

Fig. 4. Growth complementation of *C. glutamicum* LNΔ*gltBD*. The *C. glutamicum* wild-type (■) and strains LNΔ*gltBD* (□), LNΔ*gltBD*/pEKEx2 (▲) and LNΔ*gltBD*/pEKEx-*gltBD* (△) were grown in nitrogen-free CGXII glucose minimal medium supplemented with 70 mM L-glutamine and 1 mM IPTG. Representative results of five independent experiments are shown.

regulation, derepression of transcription is a prerequisite for fast growth with glutamine as a sole nitrogen source. Previous work showed that growth on glutamine as a sole carbon and nitrogen source results in relatively high intracellular glutamine levels in the range of 100 mM (Niebisch *et al.*, 2006), which indicates that *C. glutamicum* does not sense nitrogen starvation as a drop in glutamine concentration. This assumption was further investigated here.

When L-glutamine was measured in the wild-type grown with ammonium as the nitrogen source, a cytoplasmic glutamine concentration of 15 ± 1 mM was determined, which is in the range of previously published data (Müller *et al.*, 2006; Nolden *et al.*, 2001b). About twofold higher concentrations were found when the cells were grown with 70 mM L-glutamine as the nitrogen source with a cytoplasmic L-glutamine concentration of 31 ± 7 mM for the wild-type. These data clearly indicate that a drop in the cytoplasmic glutamine concentration does not serve as a signal for nitrogen starvation in *C. glutamicum*. When both ammonium and glutamine were used as nitrogen sources, an intracellular concentration of 35 ± 5 mM L-glutamine was determined, indicating that the presence of ammonium did not inhibit glutamine uptake. It was previously suggested that ammonium might be a sensor metabolite for the nitrogen status of the cell (Müller *et al.*, 2006; Nolden

Fig. 5. Suppression of the nitrogen starvation response by ammonia as tested by RNA hybridization using a *gltB* probe. Two micrograms total RNA per sample was spotted. *C. glutamicum* wild-type was grown in minimal medium with 70 mM glucose, 150 mM ammonium sulfate and 70 mM L-glutamine (a), or with 70 mM glucose and 70 mM glutamine (b), and samples for RNA isolation were taken in the early exponential growth phase at OD₆₀₀ ~4. A pulse of 150 mM ammonium sulfate was given to culture (b) and samples for RNA isolation were taken from this immediately after ammonium addition (c), or after 5 (d), 15 (e), 30 (f), 60 (g) or 120 (h) min.

et al., 2001b). In fact, when an ammonium pulse was given to glutamine-grown cells with derepressed AmtR target genes, *gltB* expression ceased within minutes in these cells (Fig. 5), making it even more likely that ammonium is an effector molecule.

DISCUSSION

In this work, we showed that L-glutamine serves as a good nitrogen source for *C. glutamicum* and allows similar growth rates to ammonium. DNA microarray analysis revealed that the target genes of the master regulator of nitrogen metabolism, AmtR, are derepressed in cells cultivated with L-glutamine as a sole nitrogen source (Table 2). As discussed in more detail below, this response is due to the requirement of GOGAT for glutamine utilization, whose structural genes *gltBD* are repressed by AmtR. Besides the members of the AmtR regulon, more than a dozen genes of the DtxR regulon showed a lower mRNA level in glutamine-grown cells compared with ammonium-grown cells (Table 2). DtxR directly senses the intracellular concentration of Fe²⁺ and functions as a master regulator of iron homeostasis in *C. glutamicum*: it represses or activates more than 60 genes under iron-sufficient conditions (Brune *et al.*, 2006; Frunzke & Bott, 2008; Wennerhold & Bott, 2006). Under iron limitation, bound Fe²⁺ dissociates from DtxR and the protein dissociates from DNA, causing derepression or deactivation of its target genes. The majority of the repressed genes encode iron uptake systems, but some also encode transcriptional regulators, such as RipA, which is responsible for reducing the cellular iron demand under iron limitation (Wennerhold *et al.*, 2005). A possible explanation for the higher mRNA level of DtxR target genes in ammonium-grown cells could be the lower pH (0.1–0.15 pH units) of these cultures compared with the glutamine-grown cultures at the time when the cells were harvested for RNA isolation. It was recently shown that the iron starvation response is induced by acidic pH (Follmann *et al.*, 2009).

Besides the targets of AmtR and DtxR, 25 other genes of highly diverse functions also showed more than twofold-altered mRNA levels in glutamine-grown cells (Table 2, group C). For most of these genes, such as cg1832, cg1833, cg3138, cg3139 and cg3140, we do not have an explanation for their differential expression. The 0.3- to 0.4-fold decreased expression of the genes encoding alcohol dehydrogenase (*adhA*), aldehyde dehydrogenase (cg3096) and isocitrate lyase (*aceA*) in glutamine-grown cells might be due to an altered activity of the transcriptional regulator RamA, which is known to positively regulate these genes (Arndt & Eikmanns, 2007; Auchter *et al.*, 2009; Cramer *et al.*, 2006). The gene *pyrG* (cg1606) presumably encodes CTP synthetase (EC 6.3.4.2), an enzyme catalysing the ATP-dependent amination of UTP to CTP with either ammonia or L-glutamine as the source of nitrogen. Its sevenfold increased expression in glutamine-grown cells might indicate that its activity with glutamine is lower than with ammonia. The *pyrG* gene could be a novel member of the AmtR regulon, as its expression was also threefold increased in a Δ *amtR* mutant compared with the wild-type (Buchinger *et al.*, 2009).

Analysis of mutant strains lacking the genes for a putative GlsK (*glsK*), GDH (*gdh*) or GOGAT (*gltBD*), either alone or in combination, revealed that the latter enzyme is crucial for glutamine metabolism as its absence leads to an almost threefold increase in the doubling time. The fact that the *gltBD* operon is part of the AmtR regulon (Beckers *et al.*, 2001, 2005; Schulz *et al.*, 2001) provides an explanation for the triggering of the nitrogen starvation response. GDH and GlsK are not relevant for glutamine utilization as long as GOGAT is present, as the doubling times of the Δ *gdh* and Δ *glsK* mutants were similar to the wild-type. In the absence of GOGAT, however, both GlsK and GDH appear to contribute to glutamine metabolism, as indicated by the significantly prolonged doubling times of the Δ *gltBD* Δ *glsK* and Δ *gltBD* Δ *gdh* double mutants compared with the Δ *gltBD* single mutant (Table 3). In order to better understand the role of the GlsK protein, a biochemical characterization of its properties will be required. In other bacteria such as *Rhizobium etli* (Durán *et al.*, 1995, 1996), GlsK was shown to have a significant role in the metabolism of L-glutamine, especially when glutamine is provided as a carbon source.

A prerequisite for the utilization of L-glutamine is its transport into the cell. *C. glutamicum* was previously reported to possess a secondary Na⁺-dependent glutamine uptake system which uses both the membrane potential and the sodium ion gradient as driving forces (Siewe *et al.*, 1995). Glutamine uptake showed Michaelis–Menten kinetics, with a K_m of 36 μ M and a V_{max} of 12.5 nmol min⁻¹ (mg dry weight)⁻¹ at pH 7. The K_m value for the co-transported sodium ion was reported to be 1.4 mM. The gene(s) encoding this glutamine uptake system has not been identified yet. In the transcriptome comparison performed in our work, none of the genes with an increased mRNA level during growth with L-glutamine as

the nitrogen source was annotated as a putative transporter for this or a related amino acid. This might suggest that expression of the gene encoding the secondary L-glutamine transporter is constitutive and not induced by glutamine. In *E. coli*, an ABC transport system encoded by the *glnHPQ* operon is responsible for glutamine uptake (Nohn *et al.*, 1986). *C. glutamicum* contains a gene that has been annotated as *glnH* (cg3045) and may encode a glutamine-binding lipoprotein. However, *glnH* is not associated with genes characteristic for ABC transporters, but with a gene encoding a membrane protein of unknown function (*glnX*, cg3044) and the serine/threonine protein kinase gene *pknG* (Niebisch *et al.*, 2006). Interestingly, mutants lacking *glnX*, *glnH* or *pknG* showed a growth defect on agar plates containing L-glutamine as the sole carbon and nitrogen source. Evidence was provided that this phenotype is not caused by a defect in glutamine uptake, but by an inhibition of the 2-oxoglutarate dehydrogenase complex (Bott, 2007; Niebisch *et al.*, 2006).

Growth on glutamine obviously led to a change in the concentration of a metabolite indicating the cellular nitrogen status. In enterobacteria, nitrogen supply in the cells is perceived by measuring two metabolites of the central nitrogen cycle. The NtrBC two-component signal transduction system activates transcription of σ^{54} -dependent promoters in response to nitrogen limitation (Weiss *et al.*, 2002). Phosphorylation of the response regulator NtrC by the NtrB kinase is controlled by the nitrogen regulatory protein PII (GlnB) depending on the cellular glutamine concentration (via uridylylation/deuridylylation of PII by the uridylyltransferase GlnD; Zhang *et al.*, 2010) and on the cellular 2-oxoglutarate concentration as well as the adenylate energy charge (Jiang & Ninfa, 2009a, b). At low glutamine and high 2-oxoglutarate concentrations, the activation of the phosphatase activity of NtrB by PII is inhibited and the inhibition of the autophosphorylation activity of NtrB by PII is relieved. As a consequence, NtrC is phosphorylated and NtrBC-regulated genes are expressed.

In *C. glutamicum*, high 2-oxoglutarate concentrations favour modification of the PII-type protein GlnK and transcription of AmtR-regulated genes (Müller *et al.*, 2006). However, these results obtained with a *gdh* deletion strain also suggest that there is a second marker metabolite, since only a partially deregulated nitrogen control was observed, as indicated by a partially, not fully, adenylated GlnK protein. Due to its central position in nitrogen metabolism and the situation in enterobacteria and *B. subtilis*, L-glutamine would be a possible candidate for such a marker metabolite. However, as the AmtR regulon was derepressed in L-glutamine-grown cells although the intracellular L-glutamine concentration was higher than in ammonium-grown cells, L-glutamine is obviously not involved in nitrogen sensing in *C. glutamicum*.

The data obtained here are in accordance with previous suggestions, which favour ammonium instead of glutamine

as the sensor of the cellular nitrogen supply (Müller *et al.*, 2006; Nolden *et al.*, 2001b). Further work is necessary to strengthen this model.

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