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Regulation of GlnK activity: modification, membrane sequestration and proteolysis as regulatory principles in the network of nitrogen control in *Corynebacterium glutamicum*

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Summary

P_{II}-type signal transduction proteins play a central role in nitrogen regulation in many bacteria. In response to the intracellular nitrogen status, these proteins are rendered in their function and interaction with other proteins by modification/demodification events, e.g. by phosphorylation or uridylylation. In this study, we show that GlnK, the only P_{II}-type protein in *Corynebacterium glutamicum*, is adenylylated in response to nitrogen starvation and deadenylylated when the nitrogen supply improves again. Both processes depend on the GlnD protein. As shown by mutant analyses, the modifying activity of this enzyme is located in the N-terminal part of the enzyme, while demodification depends on its C-terminal domain. Besides its modification status, the GlnK protein changes its intracellular localization in response to changes of the cellular nitrogen supply. While it is present in the cytoplasm during nitrogen starvation, the GlnK protein is sequestered to the cytoplasmic membrane in response to an ammonium pulse following a nitrogen starvation period. About 2–5% of the GlnK pool is located at the cytoplasmic membrane after ammonium addition. GlnK binding to the cytoplasmic membrane depends on the ammonium transporter AmtB, which is encoded in the same transcriptional unit as GlnK and GlnD, the *amtB-glnK-glnD* operon. In contrast, the structurally related methylammonium/ammonium permease AmtA does not bind GlnK. The membrane-bound GlnK protein is stable, most likely to inactivate AmtB-dependent ammonium transport in order to prevent a detrimental futile cycle under post-starvation ammonium-rich conditions, while the majority of GlnK is degraded within 2–4 min. Proteolysis in the transition period from nitrogen starvation to nitrogen-rich growth seems to be specific for GlnK; other proteins of the nitrogen metabolism, such as glutamine synthetase, or proteins unrelated to ammonium assimilation, such as enolase and ATP synthase subunit F_1 β, are stable under these conditions. Our analyses of different mutant strains have shown that at least three different proteases influence the degradation of GlnK, namely FtsH, the ClpCP and the ClpXP protease complex.

Introduction

Nitrogen is an essential component of almost all macromolecules in a bacterial cell such as cell wall polymers, nucleic acids and proteins. Accordingly, most prokaryotes have developed sophisticated control mechanisms to provide an optimal nitrogen supply for their metabolism and to cope with situations of changes in the external concentration of nitrogen sources. During the last decades, the assimilation of ammonium and the underlying global regulatory network of nitrogen control have been intensively studied in Gram-negative bacteria like *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae*, leading to the paradigm of nitrogen control. However, with the detailed investigation of the low G + C content Gram-positive bacterium *Bacillus subtilis*, it became obvious that in addition other regulatory mechanisms exist besides the enterobacterial model (Fisher, 1999).

The regulation of nitrogen metabolism in the high G + C content Gram-positive actinomycetes was mainly studied in the last years (for review, see Burkovski, 2003a,b). This group of bacteria includes producers of antibiotics, e.g. various *Streptomyces* species, pathogens such as *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Corynebacterium diphtheriae*, as well as amino acid and nucleotide producers like *Corynebacterium efficiens* and *Corynebacterium glutamicum.*

For *C. glutamicum*, detailed information of transport and assimilation of nitrogen sources is available on the molecular level. Uptake systems for ammonium and glutamate were studied (Kronemeyer *et al*., 1995; Siewe *et al*., 1996; Meier-Wagner *et al*., 2001; Trötschel *et al*., 2003) and assimilatory enzymes and pathways have been investigated (Börmann *et al*., 1992; Jakoby *et al*., 1997; 1999; 2000; Siewe *et al*., 1998; Tesch *et al*., 1999; Beckers *et al*., 2001; Nolden *et al*., 2001a). Additionally, the key components of nitrogen control were identified, namely, AmtR, the master regulator of nitrogen control in *C. glutamicum*, GlnK, the only P_{II}-type signal transduction protein in this organism, and two modifying enzymes, a putative adenylyltransferase (Nolden *et al*., 2001a) and a putative uridylyltransferase (Jakoby *et al*., 1999). The AmtR protein represses transcription of various genes during nitrogen surplus (for review, see Burkovski, 2003a,b). Deletion of the *amtR* gene unblocks expression control and leads to a deregulated transcription of AmtR-regulated genes (Jakoby *et al*., 2000). In the wild type, AmtR is controlled by the *glnD* gene product, a putative uridylyltransferase, and by the GlnK protein, which functions as the central signal transduction protein for nitrogen regulation in *C. glutamicum*. It has been shown that GlnK is essential for the adaptation of *C. glutamicum* to nitrogen starvation. In response to nitrogen deprivation, the protein is synthesized and immediately modified by the *glnD* gene product at a specific amino acid residue, tyrosine 51. An exchange of tyrosine 51 to phenylalanine by site-directed mutagenesis of the *glnK* gene inhibits the cellular response to nitrogen limitation (Nolden *et al*., 2001b). Furthermore, a *glnD* deletion strain lacking uridylyltransferase is impaired in its response to nitrogen shortage. This mutant has a decreased growth rate in the presence of limiting amounts of ammonium or urea and proteome analyses by [^{35}S]-methionine *in vivo* labelling and two-dimensional gel electrophoresis followed by autoradiography revealed that the response to nitrogen starvation is impaired at the protein level (Nolden *et al*., 2001b).

In this study, the modification of GlnK, which was assumed to be a uridylylation (Jakoby *et al*., 1999; Nolden *et al*., 2001b), was reinvestigated, because in the actinomycete *Streptomyces coelicolor* adenylylation of GlnK was shown (Hesketh *et al*., 2002). In addition, we studied the response of *C. glutamicum* to an improving supply of nitrogen, i.e. a situation in which nitrogen limitation is followed by an ammonium pulse, which leads to a cessation of the cellular nitrogen starvation response.

Results

Immunoprecipitation and mass spectrometric analysis of C. glutamicum *GlnK*

The GlnK protein was isolated from nitrogen-starved cells and cells that were subjected to an ammonium pulse after a starvation period. For this purpose, a GlnK-specific polyclonal anti-serum generated in guinea pigs was coupled to protein A-coated magnetic beads and the latter were used for immunoprecipitation of GlnK. After elution of the antibody-bound protein, the samples were separated either by SDS-PAGE or two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). From the gels protein bands or spots were excised and subjected to tryptic in-gel digestion. When peptide mass fingerprints generated by MALDI-TOF-MS were analysed (the theoretically expected fragments are given in Fig. 1A), the mass of the GlnK fragment carrying tyrosine residue 51, the site of modification by the *glnD* gene product uridylyltransferase (Jakoby *et al*., 1999; Nolden *et al*., 2001b), was shifted 329 Da when GlnK was modified in response to nitrogen starvation compared to the unmodified, native GlnK isolated from the post-starvation culture (Fig. 1B). This result is consistent with an AMP residue as the nitrogen starvation-dependent modification of GlnK at tyrosine 51 instead of an UMP as assumed earlier, which would result in a shift of 306 Da. In response to an ammonium pulse, the AMP group is removed and within minutes after the addition of ammonium the unmodified Tyr51-carrying fragment can be observed in the MALDI mass spectrum (Fig. 1B) and a pI shift of the corresponding protein can be observed by 2-D PAGE (data not shown).

Additional modifications of GlnK, such as the proteolytic cleavage of the first three N-terminal amino acid residues as reported for the *S. coelicolor* protein (Hesketh *et al*., 2002), were not observed by MALDI-TOF-MS and fingerprint analyses of *C. glutamicum* GlnK. The 1673.03 Da trypsin fragment starting with leucine 3 was found independently of the cellular nitrogen supply (Fig. 1B), which excludes cleavage after amino acid residue 3. To exclude that only the first two N-terminal amino acids are cleaved off in response to an ammonium pulse, GlnK was isolated from nitrogen-starved cells and post-starvation cells of the wild type and of strain JS-1, which allows the isolation of higher protein amounts, and treated with chymotrypsin. Independent of the nitrogen supply, a GlnK fragment corresponding to amino acid residues 1–11 was observed in the two strains (data not shown), which indicates that the N-terminal region of *C. glutamicum* GlnK is in contrast to the *S. coelicolor* protein, not prone to proteolytic modification.

Function of the glnD *gene product*

As shown by MALDI-TOF-MS, the modification of GlnK is compatible with an adenylyl residue. This implies that the *C. glutamicum glnD* gene product functions as an adenylyltransferase rather than a uridylyltransferase. This observation imposes the question whether GlnD differs in other

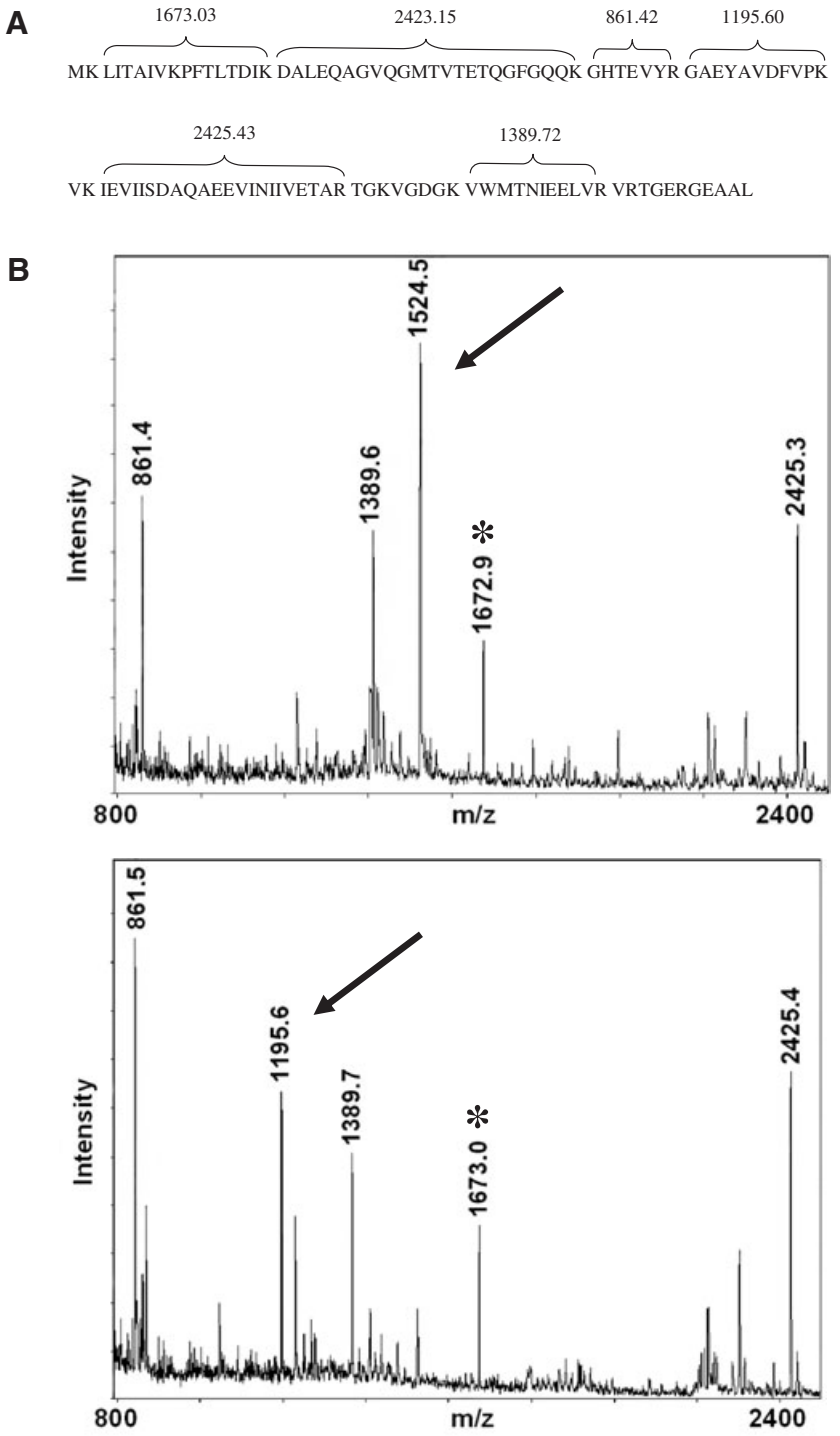

Fig. 1. Mass spectrometric analysis of GlnK.
A. Theoretical peptide masses of a tryptic GlnK digest. Fragments identified by MALDI-TOF-MS fingerprint analyses (see B) and their corresponding masses are indicated.
B. MALDI-TOF peptide mass fingerprints of GlnK. Protein spots were excised from 2-D gels, subjected to a tryptic in-gel digest and analysed by MALDI-TOF-MS (top: GlnK from nitrogen-deprived culture; bottom: GlnK isolated from cells 30 min after the addition of 100 mM $(NH_4)_2SO_4$ to a nitrogen-deprived culture). Fragment 5 which carries the putative modification is indicated by an arrow. The 329 Da shift observed for GlnK isolated from nitrogen-starved cells is consistent with an adenylylation of the protein. Fragment 2, which starts with lysine 3, and which is proteolytically modified in *S. coelicolor* in response to improving nitrogen supply (for details, see text), is found under all conditions (marked by an asterisk).

aspects from the corresponding *E. coli* protein, which is bifunctional and works as a uridylyltransferase and as uridylyl-removing enzyme.

A *glnD* mutant allele was constructed which codes for a truncated GlnD protein lacking the C-terminal half of the enzyme. A similar mutation of the corresponding *S. coelicolor* gene resulted in an enzyme capable to modify but unable to demodify GlnK (Hesketh *et al.*, 2002). In *C. glutamicum*, the first phenotype observed for a strain carrying the truncated *glnD′* allele was a severe growth defect. The doubling time of the control strain LN-D pZ8-1 was with 3.1 ± 00.25 h significantly faster than for LN-D pZglnD′ (11.6 ± 0.3 h). Obviously, the plasmid-encoded mutant GlnD protein is detrimental for the cells, while deletion of the complete *glnD* gene had not such a severe effect (Nolden *et al.*, 2001b). When Western blot analyses

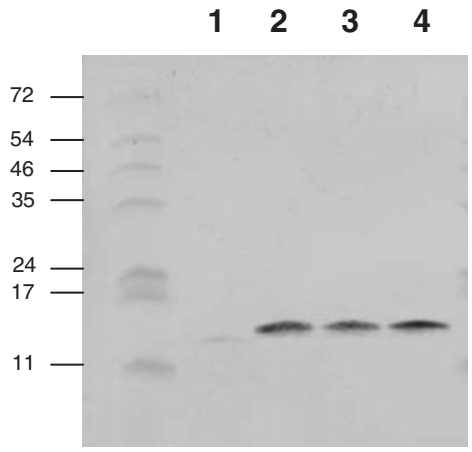

Fig. 2. GlnK modification by GlnD. Proteins were prepared from *glnD* deletion strain LN-D pZglnD′ grown in nitrogen-rich minimal medium (1), incubated for 2 h in nitrogen-free minimal medium (2), and from nitrogen-starved cells 15 and 30 min after addition of 100 mM $(NH_4)_2SO_4$. The cell extracts were fractionated and the cytoplasmic proteins were subjected to SDS-PAGE (50 μg protein per lane) and Western blotting. Masses of marker proteins (in kDa) are indicated.

were carried out, low amounts of GlnK could be observed in cells grown in nitrogen-rich minimal medium. When these cells were starved for nitrogen, GlnK was synthesized in higher amounts and immediately adenylylated (Fig. 2). Native gel electrophoresis experiments verified that GlnK synthesized in strains LN-D pZglnD′ formed trimers as in the wild type (data not shown). In response to the addition of ammonium to nitrogen-starved cells, GlnK was not demodified in strain LN-D pZglnD′ (Fig. 2). Obviously, C-terminal truncation of GlnD prevents the removal of the adenylyl residue from modified GlnK upon ammonium addition. This indicates that the *C. glutamicum* GlnD protein is bifunctional, with its modifying activity located at the N-terminal part and demodification activity and/or its regulation located at the C-terminal domain of the enzyme.

Degradation of GlnK in response to ammonium pulse

In a previous study, it was shown that in the wild type GlnK is mainly synthesized in response to nitrogen deprivation and only an extremely low basal concentration of this protein is present during growth in nitrogen-rich minimal medium (Nolden *et al*., 2001b). To investigate the appearance of GlnK in more detail, cells were grown under different nitrogen supply and subjected to SDS-PAGE and Western blotting. In nitrogen-rich minimal medium, GlnK is only detectable in Western blotting experiments, when high amounts of cell extracts were used and after prolonged development of the blots (Nolden *et al*., 2001b). When blots were not overstained, GlnK was not detected in cells grown in nitrogen-rich minimal medium, while the protein was synthesized in response to nitrogen limitation. After 2 h of starvation, 100 mM $(NH_4)_2SO_4$ was added to the culture. This concentration is sufficient to meet the cellular ammonium demand for several hours independent of the presence of specific transport systems. Upon ammonium addition to nitrogen-deprived cells, GlnK disappeared from the cell extract within 30 min (Fig. 3A). To investigate whether degradation of GlnK is protein-specific or typical for all proteins involved in nitrogen metabolism, or is a general phenomenon after an ammonium pulse, three other proteins, glutamine synthetase, enolase and the ATP synthase F_1 β subunit, were studied by Western blot analyses as well. As expected, the amount of glutamine synthetase increased in response to nitrogen starvation (Fig. 3A), because enzyme activity (Jakoby *et al*., 1997) and the expression of the corresponding gene, *glnA* (Nolden *et al*., 2001a), was found to be nitrogen-controlled and moderately enhanced in response to nitrogen limitation. In contrast to GlnK, the amount of glutamine synthetase did not decrease after an ammonium pulse. The intracellular enolase concentration did not change at all in response to alterations in the nitrogen supply, while the amount of ATP synthase F_1 β subunit, which was tested as a control for other membrane-associated proteins, was only slightly reduced under the experimental conditions and the protein was still present 4 h after the addition of ammonium to nitrogen-starved cells (Fig. 3A). These results indicate that fast proteolysis in response to improving nitrogen supply is a GlnK-specific regulation mechanism in the global network of nitrogen control in *C. glutamicum*.

To analyse degradation of GlnK in more detail, Western blot experiments were carried out with protein extracts prepared from nitrogen-starved wild-type cells within minutes after the addition of ammonium (Fig. 3B). In response to an ammonium pulse, the apparent molecular weight of GlnK was lowered because of the deadenylylation of the protein and the amount of this protein in the cell extract decreased dramatically within minutes after ammonium addition, while a basal level of GlnK was preserved for more than 20 min (Fig. 3B). Quantification of the Western blot signals revealed that 1 min after ammonium addition to nitrogen-starved cells about one-third of GlnK is degraded. After 4 min only one-third of GlnK is still present in the cell extract of the wild type, after 8 min its amount is diminished to 13 ± 10%, and after 20 min to 11 ± 5% (Fig. 3B, for each condition three bands from independent experiments were analysed, the starvation sample was set to 100%).

Membrane sequestration of GlnK

To investigate the degradation of GlnK in more detail, the cell extract was separated by ultracentrifugation into cytoplasm and membrane fraction and both were examined

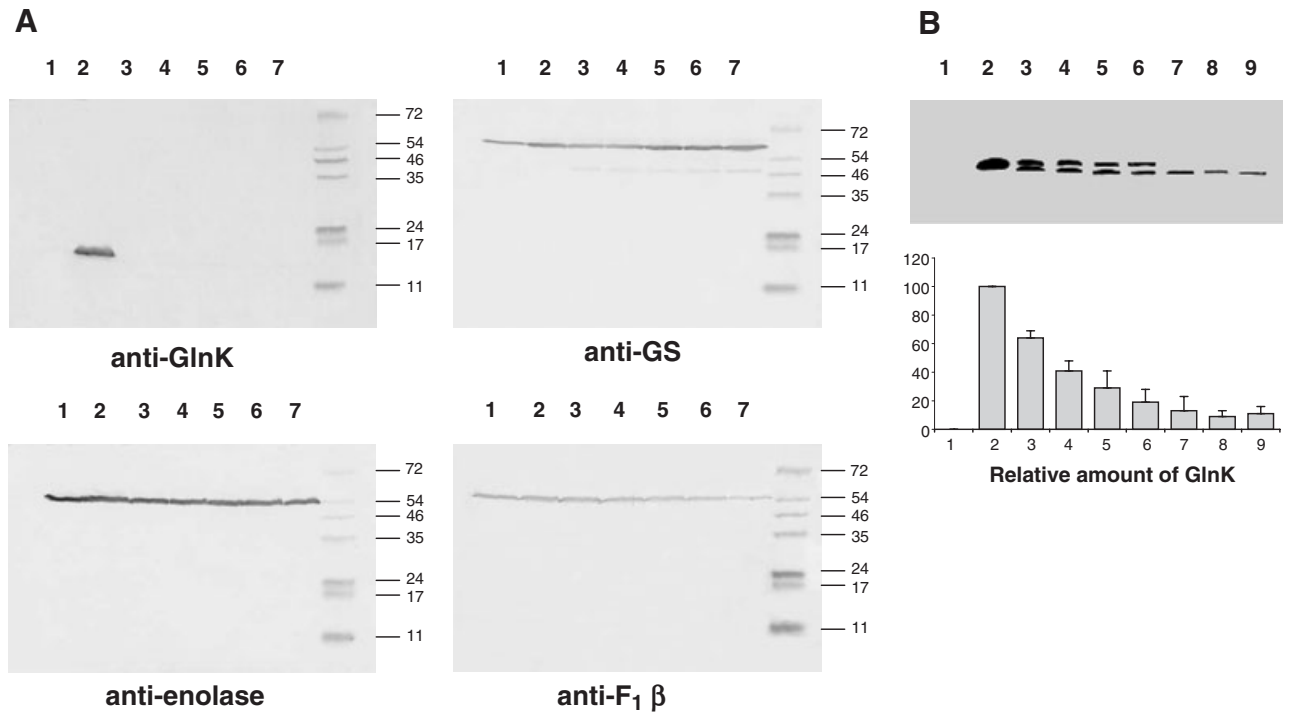

Fig. 3. Protein stability depending on the nitrogen supply.
A. Cell extracts (25 µg protein per lane) of ATCC 13032 cells grown under nitrogen surplus (1), after 2 h of nitrogen starvation (2) and 0.5, 1, 2, 3 and 4 h after the addition of 100 mM $(NH_4)_2SO_4$ to nitrogen-starved cells (3–7) were probed with anti-sera directed against GlnK, glutamine synthetase (GS), enolase and the F_1 β subunit of ATP synthase. Masses of marker proteins (in kDa) are indicated.
B. Degradation of GlnK was monitored with a GlnK-specific anti-serum in cell extracts of the wild type grown in nitrogen-rich minimal medium (1), incubated for 2 h in nitrogen-free medium (2) and 1, 2, 4, 6, 8, 10 and 20 min (3–9) after the addition of 100 mM $(NH_4)_2SO_4$ (top). The GlnK signal was quantified and the signal obtained in the extract of nitrogen-starved cells was set to 100% (bottom; experiments were carried out in triplicate and standard deviations are shown).

by Western blotting as well. The cytoplasm showed essentially the same pattern as the unfractionated cell extract, with a more pronounced degradation of GlnK after an ammonium pulse (Fig. 4), while distinct differences were found in the membrane fraction. During nitrogen starvation, GlnK was not detectable in this fraction even after prolonged development of the respective Western blots. Only after addition of 100 mM $(NH_4)_2SO_4$, GlnK was sequestered to the membrane (Fig. 4). This membrane-bound protein corresponds to the deadenylylated GlnK as indicated by its apparent molecular weight (Fig. 4) and by mass spectrometric analysis of GlnK protein isolated by immunoprecipitation from this cell fraction and subjected to trypsine digestion (data not shown). Adenylylated GlnK was not detected at the membrane. Native gel electrophoresis revealed that the membrane-associated GlnK protein forms trimers (data not shown). The Western blot experiments indicate that membrane-bound GlnK might be partially protected from proteolysis.

The amount of GlnK protein in the cytoplasm (during nitrogen starvation before ammonium pulse) and in the membrane fraction (after the addition of 100 mM $(NH_4)_2SO_4$) was determined by Western blot analyses using known amounts of purified GlnK as a standard. Based on these data, we calculated that roughly 2% and 5% of the total *C. glutamicum* GlnK protein is bound to the membrane when time intervals between 1 and 20 min were analysed (data not shown).

Dependence of GlnK localization on ammonium transporters

Recently, it was shown that *E. coli* GlnK binds to the ammonium permease AmtB (Coutts *et al*., 2002; Javelle *et al*., 2004) and binding of GlnK proteins to ammonium transporters was reported in other organisms such as *Azotobacter vinelandii* (Coutts *et al*., 2002) and *B. subtilis* (Detsch and Stülke, 2003) as well. In *C. glutamicum*, the situation is more complex because of the presence of two known ammonium transport proteins, which might serve as membrane binding sites of GlnK. The first *C. glutamicum* gene encoding an ammonium transporter was isolated by Siewe and coworkers (Siewe *et al*., 1996) and designated *amt*. Later, the *amtB* gene encoding a second ammonium uptake protein was identified and characterized (Jakoby *et al*., 1999; Meier-Wagner *et al*., 2001). For

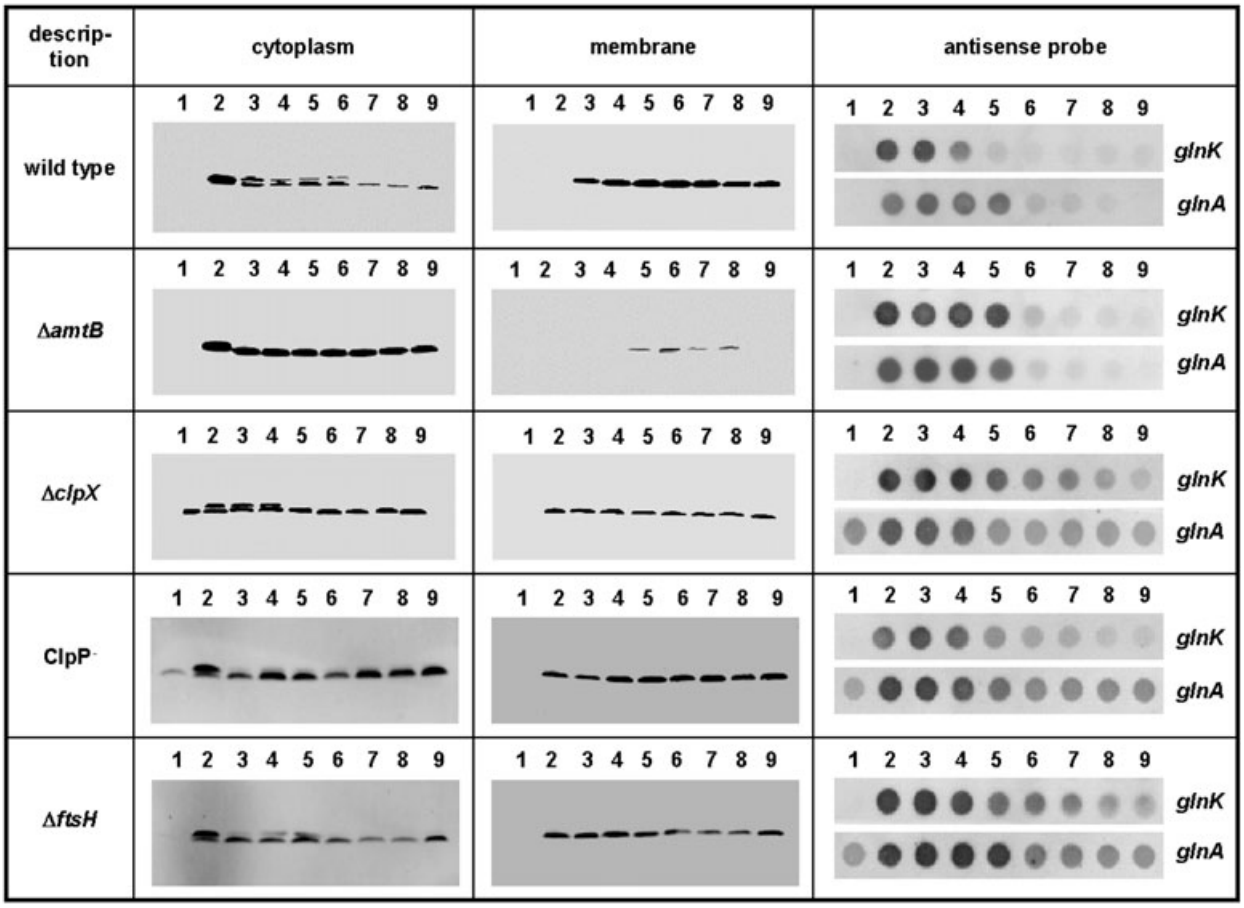

Fig. 4. Localization and covalent modification of GlnK in different *C. glutamicum* cells and influence on the transcription of nitrogen-controlled genes. Cell extract of *C. glutamicum* cells grown under nitrogen surplus (1), after 2 h of nitrogen starvation (2) and 1, 2, 4, 6, 8, 10 and 20 min after the addition of 100 mM $(NH_4)_2SO_4$ (3–9) were separated by ultracentrifugation in cytoplasm and membrane fraction, subjected to SDS-PAGE (25 μg protein per lane) and Western blotting, and subsequently probed with a GlnK-specific anti-serum. To study the influence of the different mutations on nitrogen control, transcription of the nitrogen-regulated *glnK* and *glnA* gene was investigated by RNA hybridization experiments (time points correspond to that in Western blot experiments).

consistency of nomenclature, the designation of *amt* introduced by Siewe and coworkers for the first characterized ammonium transporter (Siewe *et al.*, 1996) is changed to *amtA* in this communication and the term *amt* is only used for ammonium transporter-encoding genes in general.

To investigate a putative binding of GlnK to the ammonium transporters, single and double deletion strains of the corresponding genes, *amtA* and *amtB*, were studied. The deletion of the *amtA* gene had no effect on GlnK synthesis, localization and proteolysis (data not shown), while in the *amtB* mutant (Fig. 4) or *amtA-amtB* double mutant strain (data not shown) only minor amounts of GlnK were detectable at the membrane after prolonged incubation of the Western blot, which indicates that the AmtB protein is crucial for membrane sequestration of GlnK. In *amtB* mutant strains, proteolysis of GlnK after the addition of 100 mM $(NH_4)_2SO_4$ to nitrogen-starved cells was not detectable. Quantification of Western blot signals showed that 20 min after ammonium pulse 92 ± 11% of GlnK was still present in the cytoplasm of *amtB* deletion strain LN-1.1 as well as 88 ± 8% in the cytoplasm of *amtA-amtB* double mutant JS-1 (data not shown). These results indicate that membrane localization of GlnK is essential for its subsequent degradation.

The absence of AmtB also influenced the nitrogen-dependent modification of GlnK. Deadenylylation of the protein in response to an improved nitrogen supply was much faster in *amtB* mutant strain compared to the wild type (Fig. 4). In less than 1 min, the protein was completely in the deadenylylated state in these cells, while full demodification of GlnK in the wild type was reached only after 8 min.

The block of GlnK degradation in an amtB *deletion strain does not prevent repression of transcription of nitrogen-controlled genes upon ammonium addition*

In order to investigate a possible influence of GlnK pro-

teolysis on the expression of nitrogen-controlled genes, RNA hybridization experiments were carried out with total RNA isolated from wild type as well as *amtA* and *amtB* deletion strains, grown under different nitrogen supply. In these experiments, anti-sense probes for the *glnA* and *glnK* gene, coding for glutamine synthetase and GlnK, were used (Fig. 4), as well as probes for *amtA*, *amtB* and *gltB*, encoding the *C. glutamicum* ammonium permeases AmtA and AmtB and the large glutamate synthase (GOGAT) subunit (data not shown).

Wild-type samples revealed the typical regulation pattern for all genes tested as observed and reported previously (Jakoby *et al*., 2000; Beckers *et al*., 2001; Meier-Wagner *et al*., 2001; Nolden *et al*., 2001a,b). The strictly controlled *amtA*, *amtB*, *glnK* and *gltB* genes were not transcribed when cells were grown in nitrogen-rich medium, while the less stringently controlled *glnA* gene showed already a considerable transcription in cells grown in nitrogen-rich medium (for *glnK* and *glnA* transcription, see Fig. 4). Enhanced expression of all genes was detectable after 2 h of incubation in nitrogen-free medium; this transcription ceased in response to an improved nitrogen supply. Within 2–4 min after the addition of 100 mM $(NH_4)_2SO_4$, mRNA levels were significantly diminished. Obviously, the absence of AmtB – and/or AmtA (data not shown) – did not impair the cellular response to changes in the nitrogen supply. These results show that the stable presence of GlnK in *amtB* deletion strains after an ammonium pulse is not the result of a deregulated *glnK* expression. To verify the results by an independent approach, real-time polymerase chain reaction (PCR) experiments were carried out using RNA isolated from the wild type and the different *amt* gene single and double mutant strains. As an example for nitrogen starvation-controlled gene expression, transcription of the *glnK* gene was quantified. The transcription of *glnK* during logarithmic growth ($OD_{600} \approx 5$) in nitrogen-rich minimal medium was set to 1. Although the factors of upregulation differed significantly in the various strains because of the low basal level of *glnK* expression during nitrogen-rich growth, these experiments clearly revealed an intact regulation of the nitrogen starvation-controlled *glnK* gene during nitrogen surplus, nitrogen starvation, and after an ammonium pulse (Table 1).

Deletions of Clp protease complex-encoding genes affect GlnK degradation

A protease which is known to be responsible not only for the degradation of damaged or unfolded proteins, but also for the proteolysis of regulators in prokaryotes as well as eukaryotes, is the Clp protease complex, which is formed by an oligomer of proteolytic subunits, designated ClpP, together with associated accessory proteins, the Clp ATPases (Gottesman, 1999).

Deletion strains of the Clp ATPases-encoding genes *clpC* and *clpX* have been generated in *C. glutamicum* and examination of these mutants showed that the Clp protease complex is involved in GlnK degradation in response to an ammonium pulse. In the cell extract of *clpC* and *clpX* deletion strains, GlnK is already present during nitrogen-rich growth and can be found even after prolonged incubation following the addition of ammonium (data not shown, for data on cytoplasm, see below). To exclude that the presence of GlnK even during nitrogen-rich growth is the result of a disturbed nitrogen control rather than of an impaired proteolysis, the influence of *clp* deletions on nitrogen starvation-dependent gene expression was investigated. RNA hybridization experiments showed that nitrogen control is intact in the *clpX* mutant strain. Expression of *glnK* and *glnA* is enhanced in response to nitrogen starvation and ceases after the addition of 100 mM $(NH_4)_2SO_4$ to nitrogen-starved cells (Fig. 4). Similar results were obtained when *amtA* and *amtB* probes were used or when a *clpC* deletion strain was studied (data not shown). With these experiments, GlnK could be identified as the first substrate of Clp protease in *C. glutamicum* besides its own transcriptional regulator ClgR (Engels *et al*., 2004).

In order to investigate whether the lack of GlnK proteolysis influences the localization of GlnK, cells were grown under different nitrogen supply, harvested and separated into cytoplasm and membrane fraction. The proteins of these fractions were separated by SDS-PAGE, blotted

Table 1. Real-time PCR experiments.

	Nitrogen supply		
Strain	Nitrogen-rich minimal medium	After 2 h of nitrogen starvation	15 min after addition of 100 mM $(NH_4)_2SO_4$
ATCC 13032 (wild type)	1.0 ± 0.7	89.7 ± 0.7	2.2 ± 0.7
MJ2 38 (Δ*amtA*)	1.0 ± 0.2	33.4 ± 0.6	1.4 ± 1.3
LN-1.1 (Δ*amtB*)	1.0 ± 0.3	22.0 ± 0.5	0.5 ± 0.2
JS-1 (Δ*amtA*-Δ*amtB*)	1.0 ± 0.6	131.5 ± 1.3	1.3 ± 0.9

As an example for nitrogen starvation-controlled gene expression, transcription of the *glnK* gene was quantified. The basal level of *glnK* transcription during logarithmic growth ($OD_{600} \approx 5$) in nitrogen-rich minimal medium was set to 1. Independent experiments were carried out in triplicate.

and probed with a GlnK-specific anti-serum. In the *clpC* and *clpX* deletion strains, unmodified GlnK was present even during nitrogen-rich growth (for the *clpX* mutant, see Fig. 4, data not shown for *clpC* mutant, which revealed an identical behaviour). During starvation, the modified and the unmodified form of the protein was observed. The reason for this is unclear, but might reflect a secondary effect of the protease gene mutations. Furthermore, GlnK demodification in response to an ammonium pulse is faster in *clp* mutant strains compared to the wild type. This corresponds exactly to the modification pattern in the *amtB* mutant strain (Fig. 4) and seems to be caused by the lack of proteolysis. Also membrane sequestration of GlnK was changed in comparison to the wild type (Fig. 4). The unmodified GlnK protein present during nitrogen starvation was as well sequestered to the membrane under these conditions. This result indicates that deadenylylation might be important for binding of GlnK to the membrane.

Because the proteolytic part of the Clp protease complex formed by the *cplP1P2* operon is essential in *C. glutamicum* (Engels *et al*., 2004), a conditional mutant of *clpP1P2* was constructed and characterized with respect to GlnK-specific proteolysis. This strain was not able to degrade GlnK in response to a post-starvation ammonium pulse when the conditional mutation of *clpP1P2* was induced (Fig. 4). As a consequence of the ClpP deficiency, GlnK could be observed in the cytoplasm even under nitrogen-rich growth conditions. Compared to the GlnK amount present during nitrogen starvation (set to 100%), 85 ± 6% of GlnK can already be observed before the onset of starvation. One minute after ammonium pulse 98 ± 5% and 20 min after pulse 91 ± 3% of GlnK is still present. As expected, expression of plasmid-encoded *clpP* by anhydrotetracycline addition results in a GlnK pattern very similar to that in the wild type. In this strain, significant amounts of GlnK are not detectable during nitrogen-rich growth and the protein synthesized during starvation was degraded within minutes. After 1 min only 63 ± 7% of the GlnK amount present during starvation (which was set to 100%) can be observed, 4 min later 27 ± 6% can be found, and 20 min after pulse the protein is diminished to 11 ± 10% (data not shown).

Influence of FtsH

Although we had already identified the Clp protease complex to affect the degradation of GlnK, we suspected that another protease might be involved in proteolysis of GlnK at the membrane, because Clp proteases mainly degrade cytoplasmic proteins. While two insertions in genes coding for putative membrane-bound protease, namely in NCgl 1939 and NCgl2737, revealed no effect on GlnK proteolysis compared to the wild type (data not shown), a deletion of the *ftsH* gene led to an impaired GlnK degradation. The FtsH enzyme is located at the cytoplasmic membrane because of two membrane-spanning regions and it is involved in proteolysis of regulator proteins in other organisms (for review, see Gottesman, 1999). Western blot experiments revealed that the *ftsH* deletion strain has, in contrast to the ClpC-, ClpX- and ClpP-deficient mutants, no background level of GlnK even under nitrogen surplus (Fig. 4). In response to nitrogen deprivation, the GlnK amount in the cytoplasm increased and the protein was partially adenylylated, indicating that although significantly impaired by the *ftsH* deletion nitrogen control is in principle intact in this strain. This was verified by RNA hybridization experiments (Fig. 4). In response to an ammonium pulse, the GlnK protein is not degraded although it is sequestered to the membrane (Fig. 4). Obviously, FtsH is involved in GlnK proteolysis, which is the first reported function of this enzyme in *C. glutamicum*. Also in this strain, unmodified GlnK protein is found at the membrane during nitrogen starvation and GlnK demodification in response to ammonium addition to starved cells is faster than in the wild type as already observed in the *clp* mutant strains.

Addressing a putative sensory function of ammonium transporters in C. glutamicum

In order to address a putative sensory function of the *C. glutamicum* ammonium transport proteins as shown for *E. coli* AmtB recently (Javelle *et al*., 2004), nitrogen-starved cells were supplied with ammonium and the response of the wild type, *amtA* and *amtB* single mutant strains, as well as an *amtA-amtB* double deletion strain, was investigated. When a final concentration of 10 mM ammonium was added to cells with an optical density of 8 (≈ 3 μg ml^{-1} cell dry weight), within 10 min, the transcription of the nitrogen-controlled *gltB* gene was significantly diminished in the wild type. The signal was again detectable 50 min after ammonium pulse (Fig. 5). The same was true for the different *amt* mutant strains (for *amtB*, see Fig. 5, *amtA* single and *amtA-amtB* double mutant not shown). A similar result was obtained with a *glnK* probe (data not shown). In all strains, nitrogen control was obviously intact. Interestingly, *amtB* single or double mutant strains showed a faster response to ammonium addition after nitrogen starvation compared to the wild type (Fig. 5) or an *amtA* deletion strain (data not shown). This behaviour corresponds exactly to the extremely fast deadenylylation of GlnK observed in the *amtB* mutant (Fig. 4).

Additionally, Western blot experiments were carried out with nitrogen-starved wild type cells, supplied with low concentrations of ammonium (0.2 mM $(NH_4)_2SO_4$). No short-term sequestration of GlnK to the membrane was observed in these experiments (data not shown).

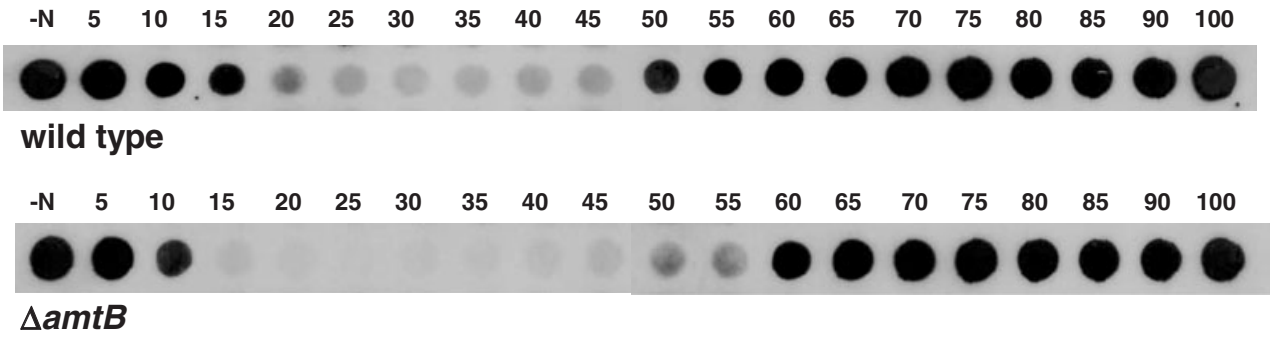

Fig. 5. Effect of AmtB on transcription of nitrogen-controlled genes. RNA was isolated from cells incubated in nitrogen-free minimal medium and from nitrogen-starved cells harvested at the indicated time points (min) after the addition of 5 mM $(NH_4)_2SO_4$. Transcription of the strictly nitrogen-controlled *gltB* gene, coding for the large glutamate synthase subunit (Beckers *et al*., 2001), was monitored with a *gltB* anti-sense probe. Cells of the wild type (top) and *amtB* mutant (bottom) strain LN-1.1 had an identical OD_{600} of 8 (corresponding to a cell dry weight of ≈ 3 μg ml^{-1}).

Discussion

Modification of GlnK in C. glutamicum

The GlnD-dependent modification of GlnK at tyrosine 51 is essential for signal transduction in nitrogen control in *C. glutamicum* (Nolden *et al*., 2001b). When MALDI-TOF-MS fingerprints of tryptic GlnK digests were analysed, it became evident that the *C. glutamicum* protein is adenylylated rather than uridylylated in response to nitrogen starvation. Although not different from a mechanistic point of view, the occurrence of adenylylation might be interesting from an evolutionary sight. Adenylylation of GlnK proteins has been reported until now exclusively for *S. coelicolor* (Hesketh *et al*., 2002) and *C. glutamicum*. Future work might show whether this modification is restricted to these two organisms or, more likely, is widely distributed among actinomycetes.

In *S. coelicolor*, a proteolytic modification of the N-terminal region of GlnK was shown (Hesketh *et al*., 2002). In response to an ammonium pulse, the N-terminal three amino acids, methionine, lysine and leucine, are cleaved off. While these amino acid residues are present at the N-terminus of *C. glutamicum* GlnK as well, cleavage was not observed in this organism when the GlnK protein was isolated by immunoprecipitation using a polyclonal GlnK anti-serum coupled to magnetic beads, digested with chymotrypsin and subjected to MALDI-TOF-MS. While a physiological relevance of this proteolytic modification in *S. coelicolor* has not been shown, the complete proteolytic degradation of GlnK observed in *C. glutamicum* necessarily leads to a block of the nitrogen starvation response.

Membrane sequestration of GlnK

In response to an ammonium pulse, GlnK is deadenylylated. This demodification might be important for membrane localization because adenylylated GlnK protein was never observed at the membrane. Only in Clp and FtsH protease mutant strains, where unmodified GlnK was observed in the cells even after 2 h of nitrogen starvation, the protein is located at the membrane and during starvation. Additionally, the AmtB protein is essential for the membrane localization of GlnK, as indicated by the analysis of *amtB* deletion strains. An direct interaction of the two proteins is assumed. AmtB is also crucial for GlnK degradation, because in a strain lacking AmtB, the amount of GlnK stays constant during post-starvation ammonium-rich growth.

It appears that the putative interaction of GlnK and AmtB has multiple purposes. First, membrane sequestration leads to the proteolytic degradation of the GlnK signal transduction protein shutting the nitrogen starvation signal cascade off. For this purpose, however, a total degradation of GlnK would be optimal, while in *C. glutamicum* about 2–5% of the GlnK protein is protected against degradation within 1 h and even in unstarved cells, a very low GlnK amount is detectable on Western blots when high amounts of protein were loaded onto the gels and chemiluminescence detection of the immunoreaction was prolonged (Nolden *et al*., 2001b). We propose that the proteolysis-protected protein stays bound to AmtB and might have another, second function, i.e. to stop AmtB transport activity in the case of an improved nitrogen supply. This might prevent an energy-costly futile cycle of ammonium, where ammonium is transported into the cell by AmtB and leaves the cell via diffusion of ammonia. A corresponding transport activity regulation was observed for *E. coli* GlnK and AmtB (Coutts *et al*., 2002).

As a third function of GlnK–AmtB interaction, besides the switch-off of nitrogen signalling and the regulation of ammonium transport, a putative additional sensory input via ammonium sensing by AmtB was discussed for *E. coli* (Coutts *et al*., 2002; Javelle *et al*., 2004). In our experiments, we found no indication of a sensory input of AmtB – or Amt – in *C. glutamicum*; however, because this is a

negative result, we cannot totally exclude a sensing function of AmtB.

GlnK proteolysis

Upon ammonium addition, the bulk of *C. glutamicum* GlnK is degraded. Proteolysis is GlnK-specific and cannot be observed for other proteins involved in nitrogen metabolism such as glutamine synthetase. This situation differs from that in *E. coli* (Coutts *et al*., 2002; Blauwkamp and Ninfa, 2003) or *S. coelicolor* (Hesketh *et al*., 2002), where GlnK seems to be stable for longer time periods. In *C. glutamicum*, only about 2–5% of the GlnK protein is protected from proteolysis by an unknown mechanism. Both proteolysis and protection are dependent on the presence of the ammonium permease AmtB, an observation that emphasizes the multiple functions of this protein.

Based on the data obtained, we suggest that binding of GlnK to the membrane in response to a post-starvation ammonium pulse leads to an interaction with the membrane-bound FtsH protease, which subsequently marks GlnK for complete degradation by the ClpCP and the ClpXP protease complex. Clp protease complexes of other organisms have already been shown to be involved in the conditional degradation of substrate proteins with a variety of functions, such as transcriptional regulators, metabolic key enzymes, a subunit of the error-prone polymerase and chemoreceptors, thereby controlling diverse physiological processes (for a recent review covering regulated proteolysis in bacteria including that performed by the Clp protease, see Jenal and Hengge-Aronis, 2003). It is also not unusual that more than one protease targets the same conditional substrate protein. For instance, *E. coli* σ^{32} is degraded by the ATP-dependent proteases FtsH, Lon, ClpAP and HslUV (Kanemori *et al*., 1997), while degradation of the first dedicated enzyme of methionine biosynthesis in *E. coli*, homoserine *trans*-succinylase, is performed by Lon, ClpP and HslUV (Biran *et al*., 2000). In *E. coli*, it was also shown that proteolysis of a number of different proteins depends on ClpAP as well as ClpXP (Weichart *et al*., 2003), a situation which is similar to the ClpCP- and ClpXP-dependent GlnK degradation in *C. glutamicum*. An explanation for the need of both accessory proteins, ClpC and ClpX, might be the formation of hetero-oligomeric complexes of ClpP, ClpC and ClpX. Although these were not shown *in vivo*, *in vitro* experiments with *E. coli* subunits revealed that ClpA and ClpX ATPases bind simultaneously to opposite ends of ClpP peptidase to form active hybrid complexes (Ortega *et al*., 2004).

The appearance of GlnK in Clp protease mutant strains even during growth in nitrogen-rich minimal medium remains unclear. A deregulated expression of *glnK* was excluded by RNA hybridization experiments. Therefore, the protein seems to accumulate in these strains based on a low basal level of transcription (as shown by real-time PCR) and a very high protein stability.

Regulation of GlnK demodification

When RNA hybridization experiments were carried out with *amtB* deletion strains, it became obvious that *C. glutamicum* is able to compensate for the lack of GlnK proteolysis by an extremely fast deadenylylation of GlnK in response to ammonium addition. The regulatory mechanism, which causes this enhanced demodification rate when AmtB is absent but prevents it in the *C. glutamicum* wild type, is unknown so far. Somehow the presence of AmtB has to be sensed by a component of the signal transduction cascade; a simple mechanism in this respect would be an interaction of AmtB and GlnD. This idea might be supported by the chromosomal organization of the corresponding genes, which form a transcriptional unit, the *amtB-glnK-glnD* operon. Similar gene clusters are found in various mycolic acid-containing actinomycetes (Burkovski, 2003b), and it was proposed that conservation of gene order might be a fingerprint of proteins that physically interact (Dandekar *et al*., 1998). However, experimental evidence for an interaction of AmtB and GlnD is lacking.

An opposite effect of AmtB was shown in *E. coli.* In this organism, the absence of AmtB slows down the deuridylylation of P_{II}~UMP upon ammonium addition (Blauwkamp and Ninfa, 2003). Strains lacking *amtB* do not completely deuridylylate P_{II} even after ammonium pulse, while in the wild type deuridylylation was completed within less than 15 min.

Model of GlnK function

For the function of GlnK in *C. glutamicum* the following model is proposed (Fig. 6): in response to nitrogen limitation the *C. glutamicum*, GlnK protein is synthesized and adenylylated by the *glnD* gene product at tyrosine 51 (Nolden *et al*., 2001b). In the modified state, the GlnK protein is located in the cytoplasm and is supposed to interact with AmtR (Nolden *et al*., 2001b; Burkovski, 2003a,b), leading to the release of the repressor from its target DNA and subsequently to the expression of nitrogen-controlled genes. An ammonium pulse, which improves the nitrogen supply of the starved cells, causes deadenylylation of GlnK. Mutant analyses indicated that deadenylylation is dependent on the C-terminal domain of the *glnD* gene product. This enzyme was first shown to be bifunctional in *E. coli* carrying out modification and demodification of P_{II}-type signal transduction proteins (Adler *et al*., 1975) and also the GlnD protein of *S. coelicolor* is able to adenylylate and deadenylylate GlnK

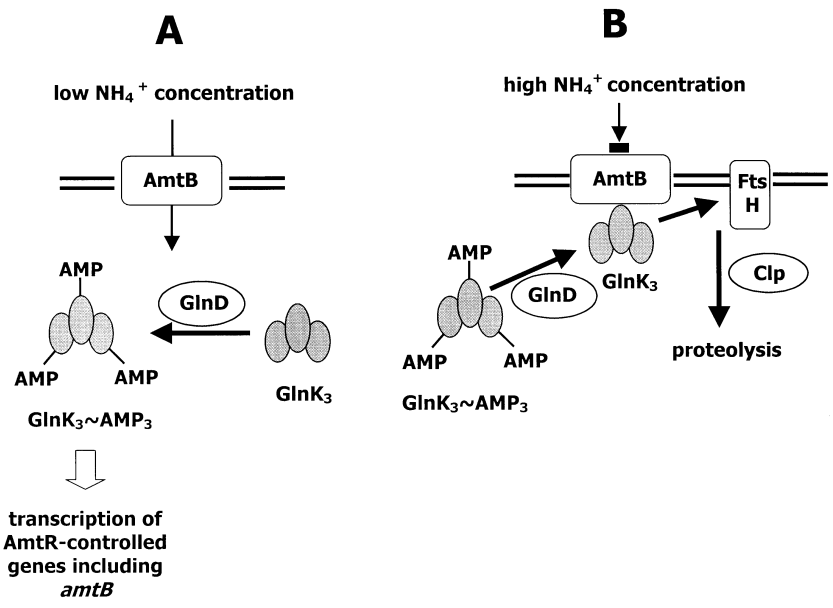

Fig. 6. Model of GlnK function.
A. The GlnK protein, which forms homotrimers in the cytoplasm of the cell (Jakoby *et al.*, 1999; Nolden *et al.*, 2001b), is synthesized in response to nitrogen starvation and subsequently adenylylated by GlnD. Adenylylated GlnK is supposed to interact with the master regulator of nitrogen-control AmtR, leading to the expression of nitrogen starvation-induced genes.
B. A post-starvation ammonium pulse results in the deadenylylation of GlnK by GlnD. The unmodified trimeric GlnK is unable to interact with AmtR, which represses transcription of nitrogen-controlled genes under these conditions (not shown). The proposed binding of unadenylylated GlnK to AmtB is assumed to block ammonium transport in order to prevent an energy-wasting futile cycle. Furthermore, the membrane localization of GlnK leads to the proteolysis of the majority of this protein depending on membrane-bound FtsH which is assumed to mark GlnK for complete degradation by the cytoplasmic Clp protease complex.

(Hesketh *et al.*, 2002). In *C. glutamicum*, the unmodified GlnK protein is unable to interact with AmtR, which now blocks transcription of nitrogen-controlled genes. Deadenylylation of GlnK during the transition from nitrogen-starvation to nitrogen-rich growth induces the binding of the protein to the ammonium permease AmtB. Interaction of GlnK and AmtB is, in analogy to the *E. coli* model (Coutts *et al.*, 2002), assumed to block ammonium transport in order to prevent an energy-wasting futile cycle during which ammonium is actively transported into the cell, is accumulating and is diffusing back into the medium. Furthermore, by an unknown mechanism the binding of GlnK to AmtB leads to the interaction with FtsH, earmarking GlnK for proteolytic degradation by the cytoplasmic Clp protease complex. This will irrevocably stop the cellular response to nitrogen starvation. The challenge of future experiments will be to identify and characterize the interacting domains of GlnK~AMP and AmtR, GlnK and AmtB, GlnK and FtsH, GlnK and ClpC, as well as GlnK and ClpX by biochemical and genetic approaches in order to validate the proposed model and to investigate the putative sensor function of AmtB in more detail.

Experimental procedures

Strains and growth conditions

Strains and plasmids used in this study are listed in Tables 2 and 3. Bacteria were routinely grown at 30°C (*C. glutamicum*) or 37°C (*E. coli*). If appropriate, antibiotics were added in standard concentrations (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). In order to study the effects of nitrogen starvation under highly comparable conditions, a standard inoculation scheme was applied. A fresh *C. glutamicum* culture in

Table 2. Strains used in this study.

Strains	Relevant genotype/description	Reference
C. glutamicum		
ATCC 13032	Wild type	Abe *et al.* (1967)
ATCC 13032Δ*clpC*	Δ*clpC*	Engels *et al.* (2004)
ATCC 13032Δ*clpX*	Δ*clpX*	This study
ATCC 13032Δ*ftsH*	Δ*ftsH*	This study
ATCC 13032 ptet-clpP-cJC1-pgap-tetR	Conditional *clpP* mutant strain, induction by anhydrotetracycline, Km^r	This study
JS-1	ATCC 13032 Δ*amtA*-Δ*amtB*	This study
LN-1.1	ATCC 13032 Δ*amtB*	Nolden *et al.* (2001b)
LN-D	ATCC 13032 Δ*glnD*	Nolden *et al.* (2001b)
MJ2-38	ATCC 13032 Δ*amtA*	Meier-Wagner *et al.* (2001)
E. coli		
DH5α*mcr*	*supE44 hsdR17 recA1 endA1 gyrA96 thi1 relA mcrA* Δ(*mrr-hsdRMS-mcrBC*)	Grant *et al.* (1990)

Table 3. Plasmids used in this study.

Plasmids	Description	Reference
pGEM3z	*E. coli* plasmid for *in vitro* transcription, Ap^r	Promega, Mannheim, Germany
pGEM16S	0.5 kb internal 16S rRNA gene fragment in pGEM3z	Nolden *et al*. (2001b)
pGEMamtA	0.5 kb internal *amtA* fragment in pGEM3z	Jakoby *et al*. (2000)
pGEMamtB2	0.6 kb internal *amtB* fragment in pGEM3z	This study
pGEMglnK	0.3 kb internal *glnK* fragment in pGEM3z	Jakoby *et al*. (2000)
pGEMglnA	0.6 kb internal *glnA* fragment in pGEM3z	Nolden *et al*. (2002)
pGEMgltB	0.8 kb internal *gltB* fragment in pGEM3z	Beckers *et al*. (2001)
pJC1	*C. glutamicum*/*E. coli* shuttle vector, Km^r, pBL1 $oriV_{C.\,g.}$, pACYC177 $oriV_{E.\,c.}$	Cremer *et al*. (1990)
pK19-Delta ftsH	pK19*mobsacB* derivative carrying *ftsH* deletion construct	This study
pK19-Delta clpC	pK19*mobsacB* derivative carrying *clpC* deletion construct	(Engels *et al*., 2004)
pK19-Delta clpX	pK19*mobsacB* derivative carrying *clpX* deletion construct	This study
pK19*mobsacB*	Vector for allelic exchange in *C. glutamicum*, pK18 $oriV_{E.\,c.}$, Km^r, *sacB*	Schäfer *et al*. (1994)
pK19*mobsacB*-del2	Vector for deletion of an internal *amtB* fragment	Meier-Wagner *et al*. (2001)
pK19-P_{tetA}-*clpP1P2*	pK19*mobsacB* derivative carrying the *tetA* regulatory region from transposon Tn*10* fused to the *clpP1* 5′ end for construction of a conditional *clpP1P2* mutant	This study
pWH853	pBR322 derivative carrying the *tetR* gene from transposon Tn*10*	Wissmann *et al*. (1991)
pWH1012	pCB302b derivative carrying *the tetA* regulatory region from transposon Tn*10*	Sizemore *et al*. (1990)
pZ8-1	*E. coli–C. glutamicum* shuttle vector, Km^r	Degussa AG, Halle-Künsebeck, Germany
pZglnD′	pZ8-1 carrying a *glnD* allele which encodes a C-terminally truncated GlnD protein	This study

BHI medium was used to inoculate minimal medium (per litre 42 g MOPS, 20 g $(NH_4)_2SO_4$, 5 g urea, 0.5 g $K_2HPO_4 \times 3\ H_2O$, 0.5 g KH_2PO_4, 0.25 g $MgSO_4 \times 7\ H_2O$, 0.01 g $CaCl_2$, 50 g glucose, 0.2 mg biotin, 10 mg $FeSO_4$, 10 mg $MnSO_4$, 1 mg $ZnSO_4$, 0.2 mg $CuSO_4$, 0.02 mg $NiCl_2 \times 6\ H_2O$, 0.09 mg H_3BO_3, 0.06 mg $CoCl_2 \times 6\ H_2O$, 0.009 mg $NaMoO_4 \times 2\ H_2O$; pH adjusted to pH 7.0 using NaOH; Keilhauer *et al*., 1993) for overnight growth. This culture, with an overnight OD_{600} of ≈ 25–30, was used to inoculate fresh minimal medium to an OD_{600} of ≈ 1, and cells were grown for 4–6 h until the exponential growth phase was reached (OD_{600} ≈ 4–5). To induce nitrogen starvation, cells were harvested by centrifugation and the pellet was suspended in and transferred to prewarmed minimal medium without nitrogen source. The nitrogen-deprived cells were incubated at 30°C under aeration. The conditional *clpP* mutant strain ATCC 13032 ptet-clpP-cJC1-pgap-tetR was grown in the presence of 0.2 μg ml^{-1} anhydroxytetracycline. To induce the *clpP* mutation, cells of this strain were grown for 6–7 h in anhydroxytetracycline-free medium.

Immunoprecipitation using magnetic beads

For the isolation of GlnK, magnetic beads were used (Dynal), which are coated with recombinant protein A, a bacterial cell wall protein with four Ig Fc binding sites. First, IgGs from a GlnK-specific anti-serum generated in guinea pigs were coupled to the magnetic beads via their Fc region as described by the supplier. Subsequently, binding of GlnK was achieved by incubation of cell extracts or cell fractions (cytoplasm or membrane fraction) with these beads at 22°C for about 1 h. The GlnK protein was eluted with 0.1 M glycine (pH 2.7).

Sample preparation, polyacrylamide gel electrophoresis and Western blotting

For the preparation of *C. glutamicum* cell extracts, aliquots were transferred from the culture to tubes containing glass beads and immediately frozen in liquid nitrogen. Samples thawed on ice were disrupted using a FastPrep FP120 instrument (Q-BIOgene) placed in a cooling chamber. Subsequently, glass beads and cell debris were removed by low speed centrifugation (4000 *g*, 4°C). For further fractionation, the cell extract was separated by ultracentrifugation into a cytoplasmic and a membrane fraction as described (Coutts *et al*., 2002). Protein concentrations were determined by the Amido Black method (Schaffner and Weissmann, 1973) using bovine serum albumin as a standard. Native PAGE was carried out with Tris-glycine buffered 10% acrylamide gels (Laemmli, 1970; modified, purchased from anamed Elektrophorese GmbH). SDS-PAGE was carried out using 12% acrylamide gels and a Tricine buffer system as described by Schägger and von Jagow (1987). Two-dimensional gel electrophoresis was carried out as described previously (Hermann *et al*., 1998; 2000; 2001). After electrophoresis, the proteins were stained with Coomassie brilliant blue. Alternatively, the gel-separated proteins were transferred onto a polyvinylidene difluoride membrane by electroblotting (PVDF, Roth) and incubated with GlnK-, enolase-, glutamine synthetase- or F_1 β-specific anti-sera generated in guinea pigs, rabbits or mice. Antibody binding was visualized by using appropriate anti-antibodies coupled to alkaline phosphatase or peroxidase (Sigma-Aldrich) and the BCIP/NBT alkaline phosphatase substrate (Sigma-Aldrich) or the Uptima (Interchim) detection solution. For quantification, the AIDA 2.11 software package was used (Raytest).

Mass spectrometry

MALDI-TOF-MS was carried out by the bioanalytics service unit at the Center for Molecular Medicine Cologne or in cooperation with the Department of Organic Chemistry (M. Schäfer, University of Cologne). MALDI-MS experiments were conducted in the reflectron mode (Resolution FWHM ≥ 10 000) on a Voyager-*DE STR* reflectron TOF Mass Spectrometer (Applied Biosystems) equipped with a N2-UV-laser (337 nm, 3 ns pulse length). Excised gel pieces were washed twice for 5 min with 500 µl 50 mM NH_4HCO_3 and once for 30 min with 500 µl 50 mM NH_4HCO_3, destained twice for 30 min with 50 mM NH_4HCO_3 in 50% acetonitrile, shrunken with 100 µl acetonitrile for 5 min, and dried under vacuum for 30 min (Concentrator 5301, Eppendorf). Tryptic in-gel digestion was started by rehydration of the gel matrix by the addition of 1–2 µl 25 mM NH_4HCO_3 containing 10 µg ml^{-1} trypsine (sequence grade; Promega). After 30 min, 25 mM NH_4HCO_3 was added to cover the sample and digestion was continued overnight at room temperature. Another 2 µl 25 mM NH_4HCO_3 and the following incubation for 90 min at room temperature were used for additional peptide extraction. This peptide solution (0.5 µl) was mixed with 0.5 µl 5 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid on a standard 100 spot stainless steel sample plate (Applied Biosystems). Data acquisition and subsequent analysis was performed by Voyager Instrument Control Panel software and Voyager data Explorer software (version V5.1; Applied Biosystems). External mass calibration was performed close to each sample spot using calibration mixtures 1 and 2 of the Sequazyme Peptide Mass Standard Kit (Applied Biosystems). Samples were analysed manually in the positive reflector mode with delayed extraction of ions (150 ns), 20 kV acceleration voltage and 66% acceleration grid voltage.

All database searches were performed using the GPMAW software Version 6.0 (Lighthouse Data). The resulting peptide mass lists were compared with a local database of *C. glutamicum* proteins provided by the Degussa AG. The search criteria were set to a mass accuracy of ≤100 ppm and preferably none, maximal one miss-cleaved peptide per protein. Proteins were considered as identified when more than 30% amino acid sequence was covered by the identified peptides and four or more peptides matched the search criteria with a deviation of mass accuracy based on an incorrect calibration equation.

General molecular biology techniques

For plasmid isolation, transformation and cloning standard techniques were used (Ausubel *et al*., 1987; Sambrook *et al*., 1989). DNA sequence determination was carried out using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 310 automated sequencer (PE Applied Biosystems). Oligonucleotides used are listed in Table 4.

Construction of a glnD *truncation*

A truncated *glnD* allele was amplified by PCR and ligated to *E. coli–C. glutamicum* shuttle vector pZ8-1 using *Eco*RI and *Bam*HI sites introduced into the primer sequences (see Table 4). The generated *glnD´* allele covers the first 1.1 kb of *glnD* and lacks the following 1.0 kb.

Table 4. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′→3′)
AmtB1	GCG CGC AAG CTT GCG CGT GGG TGA Ctt Tgg C (*Hin*dIII)
AmtB2	GCG CGC GAA TTC CAA CTA CGT CGA GGG AGT CC (*Eco*RI)
Delta clpX-1	TAT ATC TAG AAC CCA TTG CCA ATA TTG TTG GTT AG (*Xba*I)
Delta clpX-2	*CCC ATC CAC TAA ACT TAA ACA* TTG CTG GGT CCT GCC CGA TGA C
Delta clpX-3	*TGT TTA AGT TTA GTG GAT GGG* CTG GTG AAG TTT GGT CTC ATC CC
Delta clpX-4	TAT ACC CGG GTA CGC GGT CTT TTC TTC TGC GAC (*Xma*I)
Delta clpX-out-fw	ACA GGT AAA GCG CTA AGA TGG AAC
Delta clpX-out-rv	CCG GTG ATA ACT CCT GCC AAC
Delta ftsH-1	TAT ACC CGG GTG GTG CGC ATC CTC AAG GAC (*Xma*I)
Delta ftsH-2	*CCC ATC CAC TAA ACT TAA ACA* GTA TTT CTT GTT TTT CAT ACG CAG TGG
Delta ftsH-3	*TGT TTA AGT TTA GTG GAT GGG* TCA GCC CAT CGA TGG TGA TTC
Delta ftsH-4	TAT ATC TAG ACT TAG CAA ACA TAT CCG CTA AAC G (*Xba*I)
Delta ftsH-out-fw	CTT ACG GAA ACT CCA CCT CC
Delta ftsH-out-rv	GGA GGT CAA GCG CTC CTG AAC
gap-promoter-XbaI	TAT ATC TAG ACA TCA TGT TGT GTC TCC TCT AAA GAT TGT AGG (*Xba*I)
gap-promoter-BamHI	TAT AGG ATC CGA ACG ATT TCA GGT TCG TTC CC (*Bam*HI)
glnD	GCGCGCGAATTCTATGAATAATCCAGCCCAGCT (*Eco*RI)
glnD′	GCGCGCGGATCCTGCGGTCCCATTCTGGAAC (*Bam*HI)
Para-clpP1P2-1	TAT ACC CGG GGA AGA GTT CGA CAA GAA GAA CGT CG (*Xma*I)
Para-clpP1P2-2	*CCC ACT CGA GAA ACA TGC ATA* TTT AAA AAA GCA AAA AAA TAA CGC TTC TC (*Xho*I and *Nsi*I)
Para-clpP1P2-3	*TAT GCA TGT TTC TCG AGT GGG* GAT TTA AGA ATG TAG GAG TTG ACT G (*Nsi*I and *Xho*I)
Para-clpP1P2-4	TAT ATC TAG ATA TCA GCT GCG GTA CCA CCA ACA C (*Xba*I)
tet-4	TAT ACT CGA GTT TCT CTA TCA CTG ATA GGG AGT GG (*Xho*I)
tet-6	TAT AAT GCA TTT TTG TTG ACA CTC TAT CAT TGA TAG (*Nsi*I)

In some cases oligonucleotides were designed to introduce recognition sites for restriction endonucleases (recognition sites underlined, restriction endonucleases indicated in parentheses) or complementary 21mer sequences for generating cross-over PCR products (printed in italics).

Construction of an amtA-amtB *deletion mutant*

A chromosomal deletion of the *amtB* gene was generated in the *C. glutamicum* genome according to the protocol described by Schäfer *et al*. (1994). For this purpose, plasmid pK19*mobsacB*-del2 carrying an internal *amtB* deletion (Meier-Wagner *et al*., 2001) was used to generate an *amtA-amtB* double mutant strain based on *amtA* deletion strain MJ2-38. The unmarked deletion of *amtB* in the genome was verified by PCR (data not shown).

Construction of clpX *and* ftsH *deletion mutants*

In-frame deletion of the *clpX* and *ftsH* genes in *C. glutamicum* was essentially performed as described previously (Niebisch and Bott, 2001). The *clpX* up- and downstream regions were amplified using the oligonucleotide pairs Delta clpX-1/Delta clpX-2 and Delta clpX-3/Delta clpX-4 respectively. The corresponding regions of *ftsH* were amplified using the oligonucleotide pairs Delta ftsH-1/Delta ftsH-2 and Delta ftsH-3/Delta ftsH-4. The respective up- and downstream regions were joined using the two oligonucleotides introducing restriction sites (Delta clpX-1/Delta clpX-4 and Delta ftsH-1/Delta ftsH-4 respectively). The resulting cross-over PCR products were digested and cloned into, respectively, cut pK19*mobsacB* (Schäfer *et al*., 1994). Transformation of the pK19*mobsacB* derivatives into *C. glutamicum* and screening for the first and second recombination event were performed as described previously (Niebisch and Bott, 2001). For confirmation of the chromosomal deletion, putative in-frame deletion mutants were analysed via colony PCR using oligonucleotide pairs Delta clpX-out-fw/Delta clpX-out-rv and Delta ftsH-out-fw/Delta ftsH-out-rv annealing outside the chromosomal regions used for generating the deletion constructs and resulting in amplification products of sizes matching those expected in case of chromosomal deletion of *clpX* and *ftsH* respectively.

Construction of a conditional clpP1P2 *mutant*

As disruption of the *C. glutamicum clpP1* and *clpP2* genes proved to be impossible (S. Schaffer, unpubl.), we constructed a conditional *clpP1P2* mutant with the operon under control of the *tetA* promoter from transposon Tn*10*. To that end, the regions up- and downstream of the *clpP1P2* promoter (as mapped by Engels *et al*., 2004) were amplified using the oligonucleotide pairs Delta Para-clpP1P2-1/Delta Para-clpP1P2-2 and Delta Para-clpP1P2-3/Delta Para-clpP1P2-4. In a second step the amplification products were fused by cross-over PCR using Delta Para-clpP1P2-1 and Delta Para-clpP1P2-4 and introducing a 21 bp linker harbouring *Nsi*I and *Xho*I restriction sites between the $P_{clpP1P2}$ up- and downstream regions. The cross-over PCR product was digested with *Xma*I and *Xba*I and cloned into pK19*mobsacB* (Schäfer *et al*., 1994) cut with the same enzymes, resulting in pK19-clp. The *tetA* promoter was amplified with oligonucleotides tet-4 and tet-6 using plasmid pWH1012 as template. The PCR product (73 bp) digested with *Nsi*I and *Xho*I was ligated into, respectively, cut pK19-clp, yielding pK19-P_{tetA}-*clpP1P2*. This plasmid was used to replace the native *clpP1P2* promoter by P_{tetA} as described for the construction of in-frame deletion mutants. The replacement was confirmed by sequencing the corresponding genomic region and the new *C. glutamicum* strain named P_{tetA}-*clpP1P2*. In order to allow for tightly controlled anhydrotetracycline-dependent *clpP1P2* expression, the TetR protein encoded by the transposon Tn*10 tetR* gene was overproduced in *C. glutamicum* P_{tetA}-*clpP1P2* by introducing plasmid pJC1-P_{gap}-*tetR*. This plasmid was constructed by digesting plasmid pWH853 (Wissmann *et al*., 1991) with *Pst*I and *Bam*HI and ligating the 1606 bp fragment carrying *tetR* into, respectively, cut pJC1 (Cremer *et al*., 1990), resulting in pJC1-*tetR*. The promoter upstream of *tetR* results only in low-level expression of *tetR*. Therefore, the transcriptional and translational start signals of the *C. glutamicum gap* gene which encodes glyceraldehyde-3-phosphate dehydrogenase, one of the most abundant proteins in this organism, were amplified using oligonucleotides gap-promoter-XbaI and gap-promoter-BamHI. The PCR product (351 bp) was digested with *Xba*I and *Bam*HI and ligated into, respectively, cut pJC1-*tetR*, thereby fusing the *gap* transcriptional and translational start signals in frame to *tetR* and resulting in pJC1-P_{gap}-*tetR*.

RNA preparation and hybridization analyses

Total RNA was prepared after disruption of the *C. glutamicum* cells by glass beads using the NucleoSpin RNAII Kit as recommended by the supplier (Macherey-Nagel). The RNA was blotted onto positively charged nylon membranes (Bio-Bond Sigma) using a Schleicher and Schuell Minifold I Dot Blotter. Hybridization of digoxigenin-labelled RNA probes was detected with a Fuji luminescent image analyser LAS1000 (Raytest) or Kodak X-OMAT X-ray films (Sigma-Aldrich) using alkaline phosphatase conjugated anti-digoxigenin Fab fragments and CSPD as light-emitting substrate as recommended by the supplier (Roche Diagnostics). For the detection of *amtB* transcription, an internal *amtB* fragment was amplified via PCR using primers AmtB1 and AmtB2 and chromosomal DNA as template, the PCR product was ligated to pGEM3z DNA.

Real-time PCR

For real-time PCR, a TaqMan device (Applied Biosystems), the QuantiTect SYBR Green reverse transcriptase polymerase chain reaction (RT-PCR) kit (Qiagen) and 100 ng of template RNA were used. Reverse transcription was carried out at 50°C for 30 min, the reverse transcriptase was inactivated and the polymerase activated by 15 min incubation at 94°C, PCR was carried out by 40 cycles of the following programme: DNA denaturation for 15 s at 94°C, followed by primer annealing for 30 s at 60°C, and DNA polymerization for 30 s at 72°C.

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