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# Regulation of AmtR-controlled gene expression in *Corynebacterium glutamicum*: mechanism and characterization of the AmtR regulon

**Gabriele Beckers,[1] Julia Strösser,[1] Ulrich Hildebrandt,[2] Jörn Kalinowski,[3] Mike Farwick,[4] Reinhard Krämer[1] and Andreas Burkovski[1*†]**

[1]*Institut für Biochemie der Universität zu Köln, Zülpicher Str. 47, D-50674 Köln, Germany.*
[2]*Institut für Botanik der Universität zu Köln, Gyrhofstr. 15, D-50931 Köln, Germany.*
[3]*Institut für Genomforschung, Universität Bielefeld, Universitätsstr. 25, D-33615 Bielefeld, Germany.*
[4]*Degussa AG, Kantstr. 10, D-33788 Halle-Künsebeck, Germany.*

## Summary

**AmtR, the master regulator of nitrogen control in *Corynebacterium glutamicum*, represses transcription of a number of genes during nitrogen surplus. Repression is released by an interaction of AmtR with signal transduction protein GlnK. As shown by pull-down assays and gel retardation experiments, only adenylylated GlnK, which is present in the cells during nitrogen limitation, is able to bind to AmtR.**

**The AmtR regulon was characterized in this study by a combination of bioinformatics, transcriptome and proteome analyses. At least 33 genes are directly controlled by the repressor protein including those encoding transporters and enzymes for ammonium assimilation (*amtA*, *amtB*, *glnA*, *gltBD*), urea and creatinine metabolism (*urtABCDE*, *ureABCEFGD*, *crnT*, *codA*), a number of biochemically uncharacterized enzymes and transport systems (NCgl1099, NCgl1100, NCgl 1915–1918) as well as signal transduction proteins (*glnD*, *glnK*). For the AmtR regulon, an AmtR box has been defined which comprises the sequence tttCTATN$_6$AtAGat/aA. Furthermore, the transcriptional organization of AmtR-regulated genes and operons was characterized.**

Accepted 11 August, 2005. *For correspondence. E-mail aburkov@biologie.uni-erlangen.de; Tel. (+49) 9131 85 28086; Fax (+49) 9131 85 28082. †Present address: Lehrstuhl für Mikrobiologie, Universität Erlangen-Nürnberg, Staudtstr. 5, D-91058 Erlangen, Germany.



## Introduction

*Corynebacterium glutamicum* was isolated in 1957 by Kinoshita and coworkers in a screening program for L-glutamate-producing bacteria from a soil sample collected at Ueno Zoo in Tokyo and at that time designated as *Micrococcus glutamicus* (Kinoshita *et al*. 1957; Udaka, 1960; Eggeling and Sahm, 2001). Less than 50 years later, enormous amounts of L-glutamate (more than 1 400 000 t per year) and L-lysine (more than 560 000 t per year) are produced by use of different *C. glutamicum* strains, in addition to smaller amounts of some industrially less important amino acids (L-alanine, L-isoleucine and L-proline) and in addition to different nucleotides (Leuchtenberger, 1996; Hermann, 2003).

The regulation of nitrogen metabolism in *C. glutamicum* and related species was the subject of research mainly in the last years (for review, see Burkovski, 2003a,b). For *C. glutamicum*, detailed information on transport and assimilation of nitrogen sources as well as nitrogen regulation is available on a molecular level (for review, see Burkovski, 2003a,b; 2005). Uptake systems for ammonium, creatinine, glutamate and urea were studied and assimilatory enzymes and pathways were investigated. Additionally, the key components of nitrogen control were identified, namely, GlnK, the sole $P_{II}$-type signal transduction protein in this organism, two adenylyltransferases encoded by *glnE* and *glnD*, and AmtR, a TetR-type repressor protein. The expression of nitrogen starvation-induced genes in *C. glutamicum* is controlled by this transcriptional regulator. AmtR blocks transcription of various genes during nitrogen surplus. Consequently, deletion of the *amtR* gene leads to a deregulated transcription of AmtR-regulated genes (Jakoby *et al*., 2000). Gel-retardation experiments in which extracts from *amtR*-expressing *Escherichia coli* cells or purified AmtR protein were used, indicated that AmtR binds to the sequence ATCTATAGN$_4$ATAG upstream of the *amtA* gene and subsequent studies showed that AmtR not only regulates transcription of *amtA*, but also expression of the *amtB-glnK-glnD* operon encoding an ammonium carrier, GlnK and GlnD (Jakoby *et al*., 2000) as well as the *gltBD* operon encoding glutamate synthase (Beckers *et al*., 2001) and the *glnA* gene coding for glutamine synthetase (Nolden *et al*., 2001a). AmtR is

assumed to be controlled by a signal cascade including GlnD and GlnK, most likely by a direct interaction with the GlnK protein, which is adenylylated during nitrogen starvation (Strösser *et al*., 2004). Deletion of the *glnD* or *glnK* gene or replacement of the conserved tyrosine residue 51 by phenylalanine in GlnK interrupts the transfer of the nitrogen starvation signal to AmtR and leads to a permanent repression of AmtR-controlled genes (Nolden *et al*., 2001b).

This study was initiated to investigate AmtR-dependent regulation in detail and to define the *C. glutamicum* AmtR regulon. For this purpose, different global analysis techniques were applied, i.e. a bioinformatics approach for genome data mining, transcriptome analyses by DNA microarrays and characterization of cellular protein profiles by two-dimensional gel electrophoresis and peptide mass fingerprint analyses.

## Results

### *AmtR forms a complex with signal transduction protein GlnK*

In response to nitrogen limitation, AmtR it released from its target DNA and transcription of various genes is not longer blocked (Burkovski, 2003a,b; 2005). The signal for the release of AmtR from the DNA was unclear until now. However, an interaction of the repressor protein with GlnK was assumed based on site-directed mutagenesis experiments. Cells carrying a *glnK* allele which codes for a GlnK protein with an exchange of tyrosine 51 to phenylalanine (GlnKY51F) do not respond to nitrogen limitation on the level of transcription (Nolden *et al*., 2001b) even when depleted of all nitrogen sources for several hours.

As a first approach interaction of AmtR and GlnK was addressed by native gel electrophoresis. Strain ATCC 13032 pEKEXglnK was grown in the presence of 0.5 mM IPTG to induce *glnK* expression. Depending on the nitrogen supply, nitrogen-rich or nitrogen-free medium, GlnK was present in its unmodified or adenylylated form in these cells. To investigate protein–protein interactions, cytoplasmic protein fractions were subjected to native polyacrylamide gel electrophoresis, either in the absence or presence of purified AmtR~$His_6$ protein. After gel electrophoresis, proteins were blotted and subsequently probed with a GlnK-specific antiserum. While the addition of AmtR to cell extract containing unmodified GlnK had no effect on GlnK mobility, AmtR incubation with GlnK-AMP resulted in a strong retardation of the GlnK band indicating an AmtR/GlnK–AMP interaction (Fig. 1).

To verify the AmtR/GlnK–AMP interaction, an AmtR protein carrying a C-terminal hexahistidine tag (AmtR~$His_6$) was coupled to an affinity column. Cytoplasmic protein extracts from *C. glutamicum* cells grown in nitrogen-rich standard minimal medium and from nitrogen-starved cells were applied on the AmtR~$His_6$ column. After washing and elution, protein fractions were separated by SDS-PAGE and stained with Coomassie brilliant blue. All proteins eluted were excised and identified by MALDI-TOF-MS and peptide mass fingerprint analyses. In addition, presence or absence of GlnK in the elution fractions was tested by Western blot analyses using a GlnK-specific antiserum (Fig. 2). Four minor *E. coli* contaminations (DNA cytosine methylase, 50S ribosomal subunit L28, small ribosomal subunit pseudo uridinesynthase A, N-alanyl-γ-D-glutamyl-meso-diaminopimelate ligase) from the AmtR~His6-expressing strain were detectable in all elution fractions (data not shown). More interestingly, Western blot experiments as well as the mass spectrometric analyses revealed that AmtR is able to bind GlnK only when the protein is present in its adenylylated form, i.e. when the cells face nitrogen limitation. Unmodified GlnK, which indicates nitrogen-rich conditions, is not bound (Fig. 2A). Because the active form of GlnK is a trimer, not only homotrimeric completely modified or unmodified protein complexes, but also heterotrimers with one or two adenylylated and corresponding numbers of unmodified GlnK proteins were observed in *C. glutamicum* (Jakoby *et al*., 1999; Nolden *et al*., 2001a). Native polyacrylamide gel electrophoresis was used to study, which GlnK forms were present in the fractions used for interaction studies (Fig. 2B). While completely adenylylated GlnK

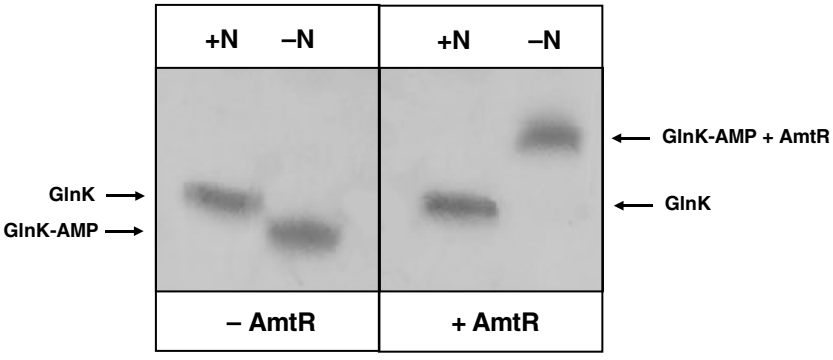



**Fig. 1.** Analysis of AmtR–GlnK interaction by native gel electrophoresis. ATCC 13032 pEKEXglnK cells were grown in the presence of 0.5 mM IPTG to induce *glnK* expression. Depending on the nitrogen supply (+N, –N) GlnK was present in its unmodified or adenylylated form. Twenty-five microgram of cytoplasmic protein fractions of these cells were subjected to native polyacrylamide gel electrophoresis, either in the absence (left panel) or presence (right panel) of 1 μg of purified AmtR~$His_6$. Cell extract containing GlnK-AMP resulted in a shift of AmtR motility.

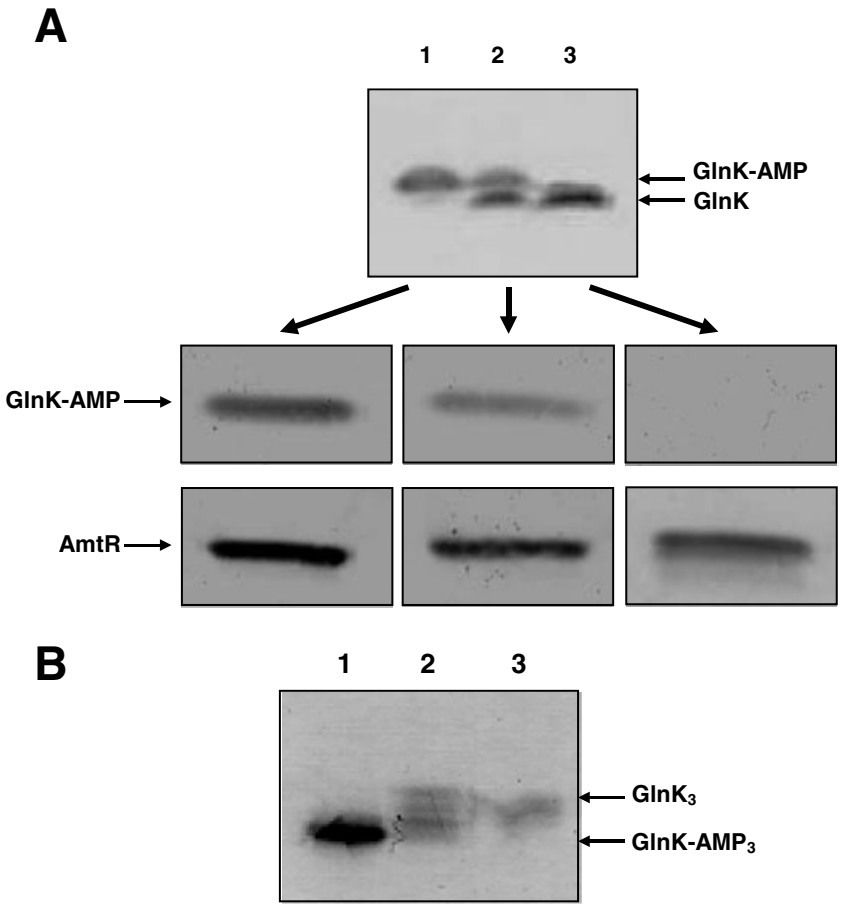



**Fig. 2.** AmtR–GlnK complex formation shown by pull-down experiments.
A. By variation of nitrogen supply, *C. glutamicum* cell extract were prepared, which contained exclusively GlnK-AMP (Western blot of SDS-PAGE, upper panel, lane 1, cells incubated for 1.5 h in nitrogen-free minimal medium), a mixture of GlnK-AMP and GlnK (lane 2, cells harvested 5 min after an ammonium pulse), or only GlnK (lane 3, cells harvested 10 min after an ammonium pulse). Twenty-five microgram of these protein extracts were loaded on columns with immobilized AmtR protein. Washing and elution fractions were tested for the presence of AmtR and GlnK by Western blotting. Lower panels show sections of blots representing elution fractions of these columns probed with AmtR- and GlnK-specific antisera. While AmtR was eluted from all columns, GlnK was detectable only when present in its modified form. The adenylylation of GlnK, indicated by the apparent molecular mass of the protein, was validated by mass spectrometry (data not shown).
B. Native gel electrophoresis of protein fractions. Twenty-five microgram of the protein fraction applied in the pull-down experiments described in A were separated by native PAGE. The gel was blotted and probed with GlnK-specific antisera. Trimeric GlnK complexes in these protein fractions were either fully adenylylated ($GlnK\text{-}AMP_3$, lane 1), a mixture of $GlnK\text{-}AMP_3$, $GlnK\text{-}AMP_2$/GlnK, GlnK-AMP/$GlnK_2$ and $GlnK_3$ (lane 2), or trimers of fully unmodified GlnK ($GlnK_3$, lane 3).

trimers were present in the cell extract of nitrogen-starved cells and completely unmodified GlnK trimers were observed in cells incubated for 30 min in the presence of ammonium, cells harvested shortly after ammonium pulse contained a mixture of $(GlnK\text{-}AMP)_3$ $(GlnK\text{-}AMP)_2$/GlnK, GlnK-AMP/$(GlnK)_2$ and $GlnK_3$. The data obtained indicated that only the fully adenylylated GlnK trimer binds to AmtR (Fig. 2B).

### *AmtR–GlnK interaction is sufficient to stop AmtR repression*

In order to demonstrate a physiological function of AmtR interaction with GlnK-AMP, gel retardation experiments were carried out. A promoter fragment of *amtB*, carrying a putative AmtR binding site (Jakoby *et al*., 2000) was mixed with isolated AmtR protein. Due to the binding of AmtR, a retardation of this DNA fragment was observed (Fig. 3). The rapid electrophoretic mobility of the free DNA was recovered by the addition of cell extract from nitrogen-starved cells, which contained GlnK-AMP, but not with cell extract containing unmodified GlnK protein (Fig. 3, upper panel). This strongly indicates that GlnK-AMP is able to release the repression of AmtR. The result obtained was independent from the time of preincubation of AmtR with GlnK (with time points between 0 and 30 min tested). Addition of GlnK-AMP results in an immediate loss of AmtR binding to its target DNA. Because cell extract was applied in these experiments, the presence of additional effectors could not be excluded. Therefore, the experiments were repeated with elution fractions from the AmtR-affinity column. Also in this case, AmtR was released from its target DNA in the presence of GlnK-AMP only (Fig. 3, lower panel), which indicates that GlnK-AMP alone is sufficient to unblock AmtR-dependent repression. For control, gel retardation experiments were repeated with the *gltB* upstream region. As the ammonium transporter-encoding *amtB* gene, expression of the glutamate synthase encoding *gltBD* operon is AmtR-controlled (Beckers *et al*., 2001) and identical results were obtained when the *gltB* promoter was used instead of the *amtB* upstream region in the two sets of experiments using cell extracts or immuno-purified GlnK (data not shown).

### *Bioinformatic screening of new AmtR-controlled genes*

A number of AmtR-regulated genes has been identified and binding motifs have been characterized. These

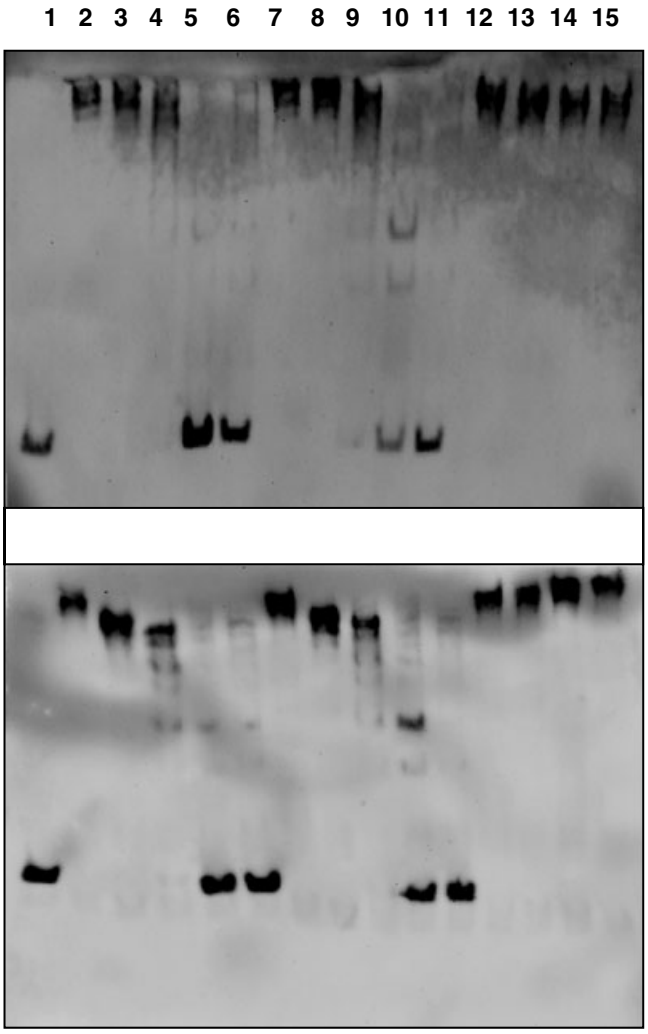



**Fig. 3.** Control of AmtR repression by GlnK-AMP. Upper panel: Gel retardation experiments were carried out with *amtB* promoter DNA (0.4 ng $\mu l^{-1}$), which was mixed with 50 ng $\mu l^{-1}$ purified AmtR protein. To test the influence of GlnK, cell extract, which contained exclusively GlnK-AMP (lanes 3–5), a mixture of GlnK-AMP and GlnK (lanes 8–10), or only GlnK (lanes 13–15) was applied. GlnK concentrations in the different protein fractions were quantified by Western blot analyses and identical amounts of the different GlnK forms were applied. Lanes 1, 6, 11 labelled DNA without AmtR addition, lanes 2, 7, 12, *amtB* fragment plus AmtR. Lower panel: In this set of experiments, instead of cell extract, GlnK from corresponding elution fractions from AmtR affinity columns (see Fig. 2) were added (protein concentrations used were 0.1, 0.2 and 0.5 $\mu g\ \mu l^{-1}$) with lanes 12–15 being negative controls without GlnK-AMP.

include extensive motifs like ATCTATAG-$N_4$-ATAG upstream of the *amtA* gene as well as ATCTATAG-$N_1$-ATAG upstream of the *amtB-glnK-glnD* operon, mid-size sequences like ATCTATAG upstream of the *gltBD* operon, and small, truncated forms like ATCTAT upstream of the *glnA* gene. These known AmtR binding sequences were used for a genome-wide screening for new AmtR-regulated candidate genes using Blast searches and Hidden-Markov models. The results of this approach are summarized in Table 1. In summary, binding sites upstream of 14 different genes were predicted by computer analyses, including new putative AmtR binding sites upstream of the genes *gdh*, *gluA*, *hkm*, NCgl1099, NCgl1100 and NCgl1915. However, because the AmtR binding motif might be located on the sense or antisense strand, in case of divergently organized genes like *gltB* and *hkm* or NCgl1099 and 1100 it is unclear which gene is in fact regulated by AmtR. Consequently, experimental verification of AmtR regulation is crucial.

*Transcriptome analyses using DNA microarrays*

As a first experimental validation of putative AmtR-regulated genes identified by the genome-wide bioinformatics screening, DNA microarray analyses were carried out. The transcriptome of the *C. glutamicum* wild-type strain ATCC 13032 and the corresponding *amtR* deletion strain MJ6-18 was investigated in cells grown in nitrogen-rich standard minimal medium. Alterations of gene expression is represented by the ratio of each of the genes, which was obtained by dividing the intensity of the hybridization signal obtained for RNA of the *amtR* deletion strain by that of the wild type. Ratios beyond a threshold of 2 ($< 0.5$ and $> 2.0$) were taken as significant for gene regulation by AmtR. 28 genes with derepessed transcription in the mutant strain were observed (see Table 2). Out of these 10 were identified by bioinformatics analysis. The reason, why the latter approach gave less targets, results from the fact, that AmtR does not only repress expression

**Table 1.** Putative AmtR-regulated genes identified by bioinformatics. Sequence and position of AmtR binding sites are indicated.

| Gene (NCBI accession no.) | AmtR binding sequence | Position | Reference |
|---|---|---|---|
| *amtA* (NCgl1521) | TTTTTAC**CTAT**CGTTCT**A**T**AG**ATTTCTG | –103 to –76 | Jakoby et al. (2000) |
| | GTATTTT**CTAT**TCCGCT**A**T**AG**AT**A**AACC | –66 to –39 | |
| *amtB* (NCgl 1983) | ATATTTT**CTAT**AGTTTA**A**C**AG**GT**A**ATTT | –186 to –159 | Jakoby *et al.* (2000) |
| | GCTCTAA**CTAT**AGACCT**A**C**AG**AA**A**CTAA | –118 to –91 | |
| *codA* (NCgl0075) | ATCTATT**CTAT**TGCTTGAC**AG**GT**A**TTAG | –86 to –59 | Bendt *et al.* (2004) |
| *crnT* (NCgl0075) | ATACTTT**CTAT**AGATTG**A**T**AG**AA**A**TGTG | –81 to –54 | Bendt *et al.* (2004) |
| | CCCTTTT**CTAT**CAATCT**A**T**AG**AA**A**CTTG | –35 to –8 | |
| *gdh* (NCgl 1999) | TACAAAT**T**GATAACCT**AA****AG**AA**A**TTTT | –370 to –343 | This study |
| *glnA* (NCgl2133) | AAAGTTTTG**AT**AGATCG**A**C**AG**GT**A**ATGC | –151 to –124 | Nolden *et al.* (2000) |
| *gluA* (NCgl 1875) | GCAATAT**CTAT**CATGTG**A**T**AG**GT**A**AATT | –195 to –168 | This study |
| *gltB* (NCgl0181) | CGTTTTC**CTAT**AGGTTG**A**TC**G**AA**A**GTAA | –117 to –90 | Beckers *et al.* (2001) |
| | TTATTAT**C**G**A**ACGATTG**ATAG**AA**A**CAGG | –48 to –21 | |
| *hkm* (NCgl0180) | TTATTAT**C**G**A**ACGATTG**A**T**AG**AA**A**CAGG | –181 to –154 | This study |
| | CGTTTTC**CTAT**AGGTTG**A**TC**G**AA**A**GTAA | –112 to –85 | |
| NCgl1099 | TTATTTT**CTAT**CAATCT**A**T**AG**AT**A**CTAG | –42 to –15 | This study |
| NCgl1100 | TTATTTT**CTAT**CAATCT**A**T**AG**AT**A**CTAG | –43 to –16 | This study |
| NCgl 1915 | CAAATTT**CTAT**CAAACT**A**T**AG**AA**A**GATA | –220 to –193 | This study |
| *ureA* (NCgl0083) | AGTAGCG**CTA**CATCTGC**A**T**A**TCT**A**CCCC | –135 to –108 | Beckers *et al.* (2004) |
| *urtA* (NCgl0893) | CAACTAC**CTAT**AGGCTG**A**C**AG**AA**A**CTCT | –70 to –43 | Beckers *et al.* (2004) |
| | CTAAAAA**CTAT**AGAGCT**A**T**AG**AA**A**CTTT | –44 to –17 | |

Highly conserved positions in these sequences are shown in bold. References indicate first report of AmtR-control of the corresponding genes.

of monocistronic genes, but of whole operons as well. For example, a transcriptional coupling of *amtB-glnK-glnD* (Jakoby *et al*., 2000), *gltBD* (Beckers *et al*., 2001), *ureABCEFGD* and *urtABCDE* (Beckers *et al*., 2004) was shown. The operon structure of other genes was addressed in an additional set of experiments (see below).

Another reason for differing results might be a change of expression below the detection limit of microarrays. For example the *hkm* gene was described to be moderately nitrogen starvation-dependent (Schulz *et al*., 2001) was identified by the bioinformatics approach, but not in microarray experiments.

### *Organization of AmtR-regulated genes*

For the putative AmtR-regulated genes identified by the bioinformatics approach or by the DNA microarray experiments transcription units were deduced from the organization of the genome. To prove the resulting putative operon structures, reverse transcriptase polymerase chain reaction (RT-PCR) experiments were carried out using total RNA isolated from the wild type as well as from strain MJ6-18 and primer pairs annealing to the first and last gene of the putative transcription units. Control PCR experiments carried out without RT verified that the RNA preparations used were DNA-free, while the RT-PCR experiments revealed cotranscription of *amtA*, *ocd* and *soxA* coding for a methylammonium/ammonium permease, a putative ornithine cyclodeaminase and a putative sarcosine oxidase, a common transcript of the *gluABCD* cluster encoding the primary glutamate uptake system of *C. glutamicum*, and a transcription unit comprising the NCgl1915–1918 genes encoding a putative ABC oligopeptide uptake system (Fig. 4).

The analyses of transcriptional organization revealed that especially the last genes of some operons (*amtB-glnK-glnD*, *gluABCD*, *oppABCD*) were not detected in microarray experiments. Possible reasons for this effect might be an inefficient hybridization of the probe immobilized on the chip, RNA degradation or operon polarity effects.

### *RNA hybridization analyses*

To validate the results obtained by DNA microarray analyses and genome screening for AmtR binding motifs, RNA hybridization experiments were carried out using antisense probes for the different genes and total RNA isolated from the wild type and *amtR* deletion strain MJ6-18 grown in nitrogen-rich minimal medium and starved for nitrogen. Most candidate genes identified by the bioinformatics approach and DNA microarray analysis were AmtR-regulated (Fig. 5). Only for *gdh* and *hkm* transcription, which was found by the bioinformatics approach, and for NCgl2787, which was detected in microarray experiments, an influence of *amtR* deletion was not detectable (data not shown).

While the majority of genes investigated in the wild type showed a low basal transcription during growth in nitrogen-rich medium, which was increased upon nitrogen starvation, two genes showed a more complex expression pattern. When transcription of *gluA* and NCgl1915 coding for components of ABC transporters for the uptake of glutamate and a putative oligopeptide uptake system was

**Table 2.** DNA microarray experiment.

| Gene (NCBI accession no.) | Factor of transcription (mean ± SD)[a] | Transcriptional organization (reference) | Function of gene product |
|---|---|---|---|
| Transport proteins | | | |
| *amtA* (NCgl1521) | 4.11 ± 2.02 | *amtA-ocd-soxA* (this study) | Methylammonium/ammonium permease |
| *amtB* (NCgl1983) | 2.25 ± 0.19 | *amtB-glnK-glnD* (Jakoby *et al*., 2000) | Ammonium transporter |
| crnT (NCgl0075) | 2.7 ± 2.28 | *crnT* (Bendt *et al*., 2004) | Creatinine transporter |
| *gluA* (NCgl1875) | 3.05 ± 0.80 | *gluABCD* (this study) | Glutamate uptake system, ATP binding protein |
| *gluB* (NCgl1876) | 2.71 ± 0.58 | *gluABCD* (this study) | Glutamate uptake system, L-glutamate binding protein |
| *gluC* (NCgl1877) | 2.20 ± 0.41 | *gluABCD* (this study) | Glutamate uptake system, integral membrane component |
| NCgl 1915 | 4.37 ± 1.90 | NCgl 1915 | Putative oligopeptide uptake system, |
| | 4.01 ± 1.49 | –NCgl 1918 (this study) | oligopeptide-binding protein |
| NCgl 1916 | 3.62 ± 1.90 | NCgl 1915–NCgl 1918 (this study) | Putative oligopeptide uptake system, permease component |
| NCgl 1917 | 2.70 ± 0.64 | NCgl 1915–NCgl 1918 (this study) | Putative oligopeptide uptake system, permease component |
| *urtA* (NCgl0893) | 3.75 ± 2.21 | *urtABCDE* (Beckers *et al*., 2004) | Urea binding protein |
| *urtB* (NCgl0894) | 4.09 ± 0.96 | *urtABCDE* (Beckers *et al*., 2004) | ABC transporter for urea uptake, permease component |
| *urtC* (NCgl0895) | 2.84 ± 0.60 | *urtABCDE* (Beckers *et al*., 2004) | ABC transporter for urea uptake, permease component |
| *vanK* (NCgl2302) | 4.22 ± 1.46 | *vanABK* (Merkens *et al*., 2005) | Putative of major facilitator superfamily |
| | 3.46 ± 2.12 | | |
| Ammonium assimilation | | | |
| *gltD* (NCgl0182) | 2.82 ± 0.18 | *gltBD* (Beckers *et al*., 2001) | Glutamate synthase (GOGAT), small subunit |
| *glnA* (NCgl2133) | 2.94 ± 1.50 | *glnA* (Nolden *et al*., 2001a) | Glutamine synthetase I (β-subtype) |
| Use of alternative nitrogen sources | | | |
| *codA* (NCgl0075) | 2.70 ± 2.28 | Not determined | Creatinine deaminase |
| *ureA* (NCgl0083) | 2.79 ± 0.59 | *ureABCEFGD* (Beckers *et al*., 2004) | Urease, γ subunit |
| *ureB* (NCgl0084) | 3.54 ± 1.20 | *ureABCEFGD* (Beckers *et al*., 2004) | Urease, β subunit |
| *ureC* (NCgl0085) | 3.08 ± 1.05 | *ureABCEFGD* (Beckers *et al*., 2004) | Urease, α subunit |
| *ureE* (NCgl0086) | 4.35 ± 1.93 | *ureABCEFGD* (Beckers *et al*., 2004) | Urease accessory protein |
| *ureF* (NCgl0087) | 2.22 ± 0.77 | *ureABCEFGD* (Beckers *et al*., 2004) | Urease accessory protein |
| Signal transduction | | | |
| *glnK* (NCgl1982) | 2.36 ± 0.74 | *amtB-glnK-glnD* (Jakoby *et al*., 2000) | GlnK ($P_{II}$-type signal transduction protein) |
| Miscellaneous or unknown functions | | | |
| NCgl2787 | 2.70 ± 0.64 | Not determined | Putative flavoprotein involved in $K^+$-transport |
| *ocd* (NCgl1520) | 2.88 ± 0.61 | *amtA-ocd-soxA* (this study) | Putative ornithine decarboxylase |
| *soxA* (NCgl1519) | 2.92 ± 0.98 | *amtA-ocd-soxA* (this study) | Putative sarcosine oxidase |
| *vanA* (NCgl2300) | 12.05 ± 8.98 | *vanABK* (Merkens *et al*., 2005) | Vanillate demethylase |
| | 12.05 ± 8.96 | | |
| *vanB* (NCgl2301) | 6.83 ± 1.67 | *vanABK* (Merkens *et al*., 2005) | Vanillate demethylase |
| | 6.83 ± 1.67 | | |
| NCgl1099 | 2.17 ± 0.96 | Not determined | Putative hydrolase |

The transcriptome of the wild type and of *amtR* deletion mutant MJ6-18 grown in nitrogen-rich standard minimal medium was compared in three independent experiments (biological replicates). Each DNA microarray covered the genome threefold (technical replicates).
**a.** For some genes two different PCR products were spotted on the microarray.

investigated, these genes revealed a high basal expression in the presence of ammonium and urea. In response to depletion of nitrogen sources by centrifugation and suspension in prewarmed medium without nitrogen sources, expression of the two genes decreased in the first 10 min, but later increased to a slightly higher level than before starvation. In *amtR* deletion strain MJ6-18, transcription of these genes was significantly, but only slightly enhanced compared with the wild type. These results hint to more than one regulatory mechanism controlling expression of these genes, e.g. carbon catabolite repression or regulation by RNA degradation.

### *Quantification of AmtR repression by real-time PCR*

As an independent assay for the quantification of the *C. glutamicum* response to nitrogen deprivation, real-time PCR experiments were carried out for 12 genes (Table 3) which represent a total of 32 nitrogen-controlled genes due to their transcriptional organization in operons. For an exact quantification of real-time PCR results, dilution series were tested for each pair of oligonucleotide primers to determine its PCR efficiency and the total amount of RNA applied in the real-time PCR experiments was exactly determined using RiboGreen. This method allows normalization of the results obtained without additional control reactions using housekeeping genes or 16S rRNA (Hashimoto *et al*., 2004).

AmtR-dependent regulation was verified for 10 of the 12 genes tested by real-time RT-PCR. Factors for derepression of transcription in the *amtR* deletion strain compared with the wild type, which were obtained by this

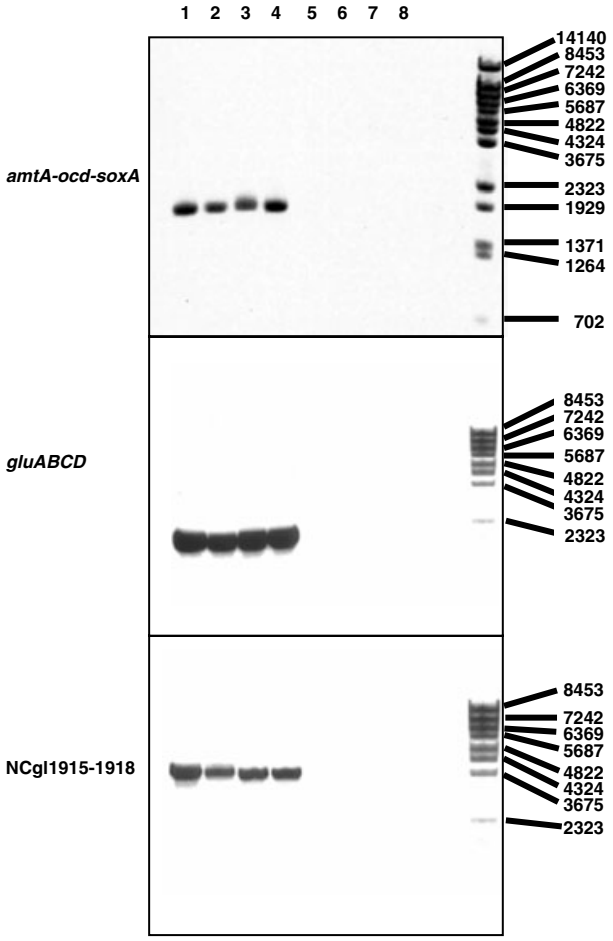



**Fig. 4.** Transcriptional organization of AmtR-regulated gene clusters. Reverse transcriptase (RT)-PCR using primers annealing to (A) *amtA* and *soxA* (B) *gluA* and *gluD* and to (C) NCgl 1915 and NCgl 1918. Total RNA was used as a template isolated from wild-type strain ATCC 13032 grown in nitrogen-rich minimal medium (1, 5) and after 30 min of nitrogen starvation (2, 6), and from *amtR* deletion strain MJ6-18 grown under nitrogen surplus (3, 7) and starved for nitrogen (4, 8). The RT-PCR products showed the expected size for the different operons (1.8, 2.1 and 3.5 kb respectively). Control reactions without the addition of RT (5, 6, 7 and 8) gave no PCR product validating that the RNA preparations used were DNA-free. Sizes of DNA marker bands are indicated.

approach, were in general higher than in the DNA microarray experiments. Obviously, the microarray technique leads to a quenching of the response signal. Only for *gluA* and NCgl1915 regulation could not be verified, most likely due to the 'non-standard' regulation of these genes (see Fig. 5).

### *Gel shift experiments*

In addition to the RNA hybridization experiments, for the genes *codA*, *crnT*, *glnA*, NCgl1099 and NCgl1100 as well as for the operons *amtB-glnK-glnD*, *gluABCD*, *gltBD* and NCgl 1915-NCgl 1918, *ureABCEFGD* and *urtABCDE* AmtR-dependent regulation was verified by gel retardation experiments. Digoxygenin-labelled primers or PCR fragments comprising the DNA motif(s) identified by the bioinformatics approach were used together with cell

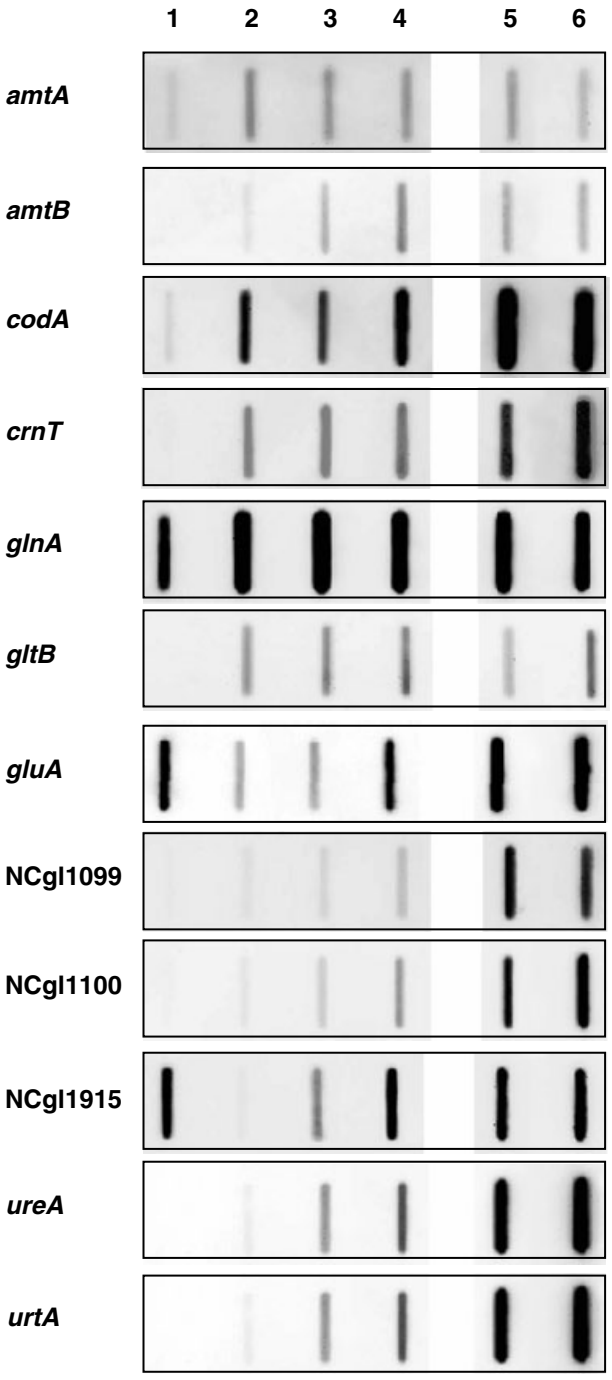



**Fig. 5.** RNA hybridization experiments. Total RNA (1 μg per slot) was prepared from wild type grown in nitrogen-rich minimal medium (lane 1) and after 5, 15 and 30 min of nitrogen limitation (lanes 2–4) as well as from *amtR* deletion strain MJ6-18 grown in nitrogen-rich minimal medium (5) and incubated in nitrogen-free medium for 30 min (6).

**Table 3.** Real-time RT-PCR.

| Gene name | NCgl number | Factor of upregulation |
|---|---|---|
| *crnT* | NCgl0074 | 168 ± 2 |
| *codA* | NCgl0075 | 35 ± 2.0 |
| *ureA* | NCgl0083 | 24 ± 3 |
| *gltB* | NCgl0181 | 591 ± 2 |
| *urtA* | NCgl0893 | 1581 ± 2 |
| – | NCgl1099 | 32 ± 1 |
| – | NCgl1100 | 41 ± 2 |
| *amtB* | NCgl1983 | 137 ± 2 |
| *amtA* | NCgl1521 | 415 ± 2 |
| *glnA* | NCgl2133 | 8 ± 2 |
| *gluA* | NCgl1875 | 1.0 ± 1.6 |
| – | NCgl1915 | 1.5 ± 1.4 |

RNA isolated from the wild-type ATCC 13032 and *amtR*-deletion mutant MJ6-18 grown in nitrogen-rich minimal medium was used as template for quantification of expression of the indicated genes.

extracts of *E. coli* cells carrying an *amtR* expression plasmid or the vector without *amtR* gene for control. For all DNA regions mentioned above, a gel shift was observed. This could be prevented by the addition of unlabelled primers comprising the estimated binding sequences. In all cases, a surplus of these oligonucleotides suppressed binding of AmtR to the digoxigenin-labelled PCR fragment (Fig. 6). Interestingly, a direct correlation of the number of predicted AmtR binding sites and the number of observed bands with decreased electrophoretic mobility was only observed in the case of *glnA* and *urtA*. For several promoters, namely the *amtB*, *crnT*, *gltB*, NCgl1099 and NCgl1100 upstream region less shifted bands than expected were found, while for *codA*, *glnA*, NCgl1915 and *ureA* the number of shifted bands exceeded the predicted binding sites. These results might reflect different binding affinities and binding of AmtR multimers.

Only one gene cluster, which was found to be highly expressed in response to nitrogen starvation and which showed deregulated transcription in an *amtR* deletion strain, the *vanABK* operon, exhibits no AmtR binding site and shows consequently no electrophoretic motility shift in response to AmtR addition in the gel retardation assay (Merkens *et al*., 2005). Obviously, these genes are regulated indirectly by AmtR. The exact control mechanism is unclear.

### *Two-dimensional gel electrophoresis*

In principle, the alteration observed on the level of gene expression should be reflected by the protein pattern of the cell. To show such changes in the concentration of distinct proteins, proteome analyses were carried out. Cytoplasmic protein fractions from wild type and *amtR* mutant cells grown in nitrogen-rich standard minimal medium were prepared and separated by two-dimensional gel electrophoresis. After staining with colloidal Coomassie brilliant blue several spots with increased size and intensity were observed in the protein pattern of *amtR* deletion strain MJ6-18 (Fig. 7). These spots were excised and subjected to tryptic in-gel digest and MALDI-TOF-MS fingerprint analysis. By this approach five out of seven urease subunits, namely UreA, UreB, UreC, UreE and UreF, were identified, besides creatinine deaminase, glyceraldehyde-3-phosphate dehydrogenase, subunit A of vanillate demethylase, acetyl-CoA hydrolase, the NCgl1099 gene product, and a putative catalase.

While urease subunits, creatinine deaminase and NCgl1099 were shown to be under direct AmtR control by bioinformatics and transcript analyses and vanillate demethylase is indirectly regulated by AmtR (see transcriptome analyses presented above and Merkens *et al*., 2005), three proteins identified were encoded by genes

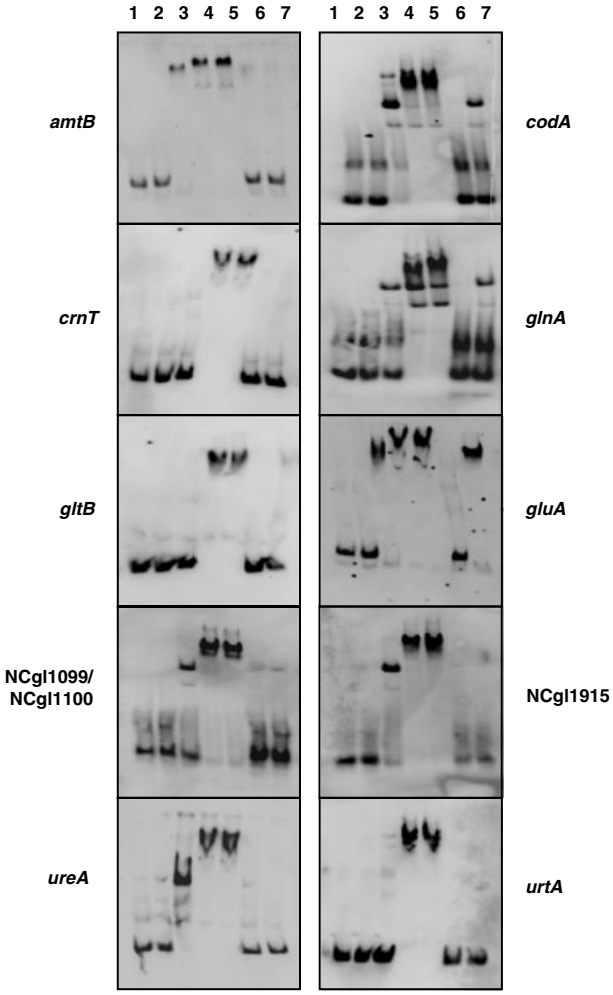
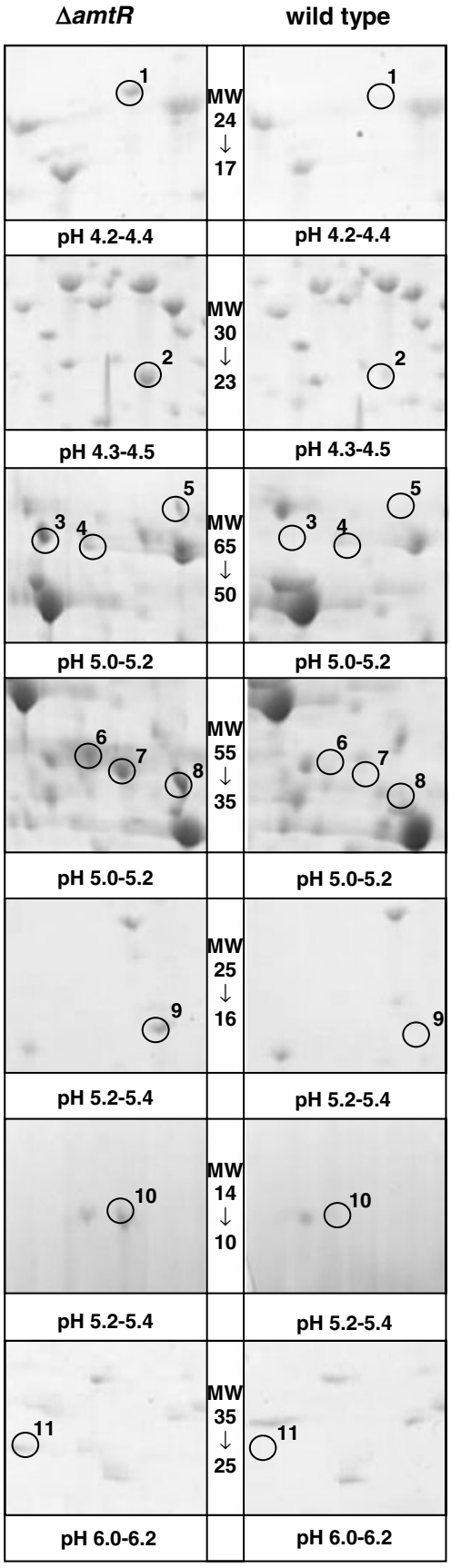



**Fig. 6.** Gel retardation experiments. Increasing amounts of AmtR-containing *E. coli* cell extracts (lanes 1–5: 0, 0.5, 5, 50 and 100 ng protein, respectively) were added to a digoxigenin-labelled DNA fragments (0.4 ng) from the different promoter regions spanning the identified putative AmtR binding sites. As a control, 10 ng of unlabelled oligonucleotides spanning the putative AmtR binding site were added to 5 ng (6) or 50 ng AmtR (7) to suppress binding to the labelled DNA.




which were not observed in the bioinformatics or transcriptome analyses and are therefore considered to be not part of the AmtR regulon. The appearance of these proteins, namely glyceraldehyde-3-phosphate dehydrogenase, acetyl-CoA hydrolase and a putative catalase, might be the result of a post-translational processing event due to metabolic perturbations caused by the absence of AmtR. For example, phosphorylation which would result in the appearance of a new spot shifted to the acidic pH range of the gel was already shown for *C. glutamicum* glyceraldehyde-3-phosphate dehydrogenase (Bendt *et al.*, 2003).

## Discussion

### *Methodological considerations*

Different methods to analyse AmtR-dependent transcription were used in this study. When transcriptome analyses using DNA microarrays were performed, in general low factors of transcriptional changes, not higher than 20-fold, were determined. This low signal intensity of DNA microarrays was also observed by others (for review, see Conway and Schoolnik, 2003), making an independent quantification of transcriptional changes, for example by real-time RT-PCR assays, necessary. In fact, real-time PCR approaches carried out in parallel in this study showed factors for the increase of expression higher than 1500-fold and reflect the results of RNA hybridization experiments much better than the microarray data, e.g. for the strictly regulated ammonium transporter-encoding genes *amtA* and *amtB*, for the *gltBD* operon coding for glutamate synthase, and for *urtA*, the first gene of the urea transporter-encoding *urtABCDE* operon. Nevertheless, transcriptome analyses are a valuable tool for the global characterization of bacterial regulatory networks, especially in combination with other global analysis techniques.

### *AmtR, a new interaction partner of GlnK in* C. glutamicum

*Corynebacterium glutamicum* GlnK is a typical member of the $P_{II}$-type signal transduction protein family (for review, see Arcondéguy *et al.*, 2001). Binding of these proteins to the adenylyltransferase regulating glutamine synthetase activity (Jiang *et al.*, 1998), two-component sensor kinase

**Fig. 7.** Proteome analysis by two-dimensional gel electrophoresis. Protein profiles of Δ*amtR* strain MJ6-18 (left column) and wild type (right column) grown in minimal medium with high nitrogen supply were compared. Sections of cytoplasmic protein maps which revealed differences in three independent biological and technical replicates are shown. The following proteins were identified by peptide mass fingerprint analyses: (1) UreB, (2) UreG, (3) UreC, (4) acetyl CoA hydrolase, (5) a putative catalase, (6) vanillate demethylase subunit A, (7) creatinine deaminase, (8) glyceraldehyde-3-phosphate dehydrogenase, (9) UreE, (10) UreA and (11) the NCgl1099 gene product.

NtrB (Jiang and Ninfa, 1999) and ammonium transport protein AmtB (Coutts *et al*., 2002; Javelle *et al*., 2004) was described for *E. coli*. In *Rhodobacter capsulatus* $P_{II}$ proteins interact with nitrogenase switch-off system DraT/DraG, NtrB, NifA1 and NifA2 (Hallenbeck, 1992; Pawlowski *et al*., 2003) and in the diazotrophic bacterium *Azotobacter vinelandii* interaction of $P_{II}$ with the NifA-NifL system was reported (Little *et al*., 2000). Recently, novel $P_{II}$ interaction partners were characterized in cyanobacteria (for recent review, see Herrero, 2004). For *Synechococcus elongatus*, it was shown that the $P_{II}$ signal transduction protein controls arginine synthesis by complex formation with N-acetyl-L-glutamate kinase (Heinrich *et al*., 2004; Maheswaran *et al*., 2004). Besides in bacteria, protein interactions with GlnK were also reported in archaea. For example, stable complexes of GlnK with glutamine synthetase were reported in the methanogenic archaeon *Methanosarcina mazei* recently and a new regulatory influence of GlnK on glutamine synthetase activity was suggested (Ehlers *et al*., 2005).

Interaction of GlnK with different proteins in the various organisms depends on the modification status of the protein. Unmodified GlnK activates for example phosphatase activity of NtrB in enterobacteria, while activation of NifA in *Rhodospirillum rubrum* requires the modified, i.e. uridylylated form of this $P_{II}$-protein (Zhang *et al*., 2004). In *C. glutamicum* interaction of native GlnK with the AmtB ammonium transport protein was suggested based on the AmtB-dependent membrane sequestration of GlnK after an ammonium pulse following a period of nitrogen starvation (Strösser *et al*., 2004). In this study, we identified a novel GlnK receptor, the AmtR protein. AmtR, the master regulator of nitrogen control in *C. glutamicum*, is a TetR-type repressor protein with an N-terminally located helix-turn-helix motif for DNA binding. Typically, these class of transcriptional regulators bind small effector molecules, e.g. tetracycline, in order to fulfil their function. In this case, however, protein complex formation between AmtR and GlnK was shown. Only the adenylylated form of GlnK, which is present in a situation of nitrogen limitation is able to bind AmtR. If small effector molecules influence or modulate the interaction of the two proteins has to be investigated in future studies.

### *Characterization of the AmtR regulon*

The *C. glutamicum* AmtR regulon was characterized by a combination of bioinformatics approaches and transcriptome analyses, which was also applied successfully to investigate the *Bacillus subtilis* Fur and TnrA regulon (Baichoo *et al*., 2002; Yoshida *et al*., 2003). Based on these investigations, AmtR is the master regulator of nitrogen metabolism on the level of transcription in *C. glutamicum*. Expression of 33 genes is directly controlled by AmtR as indicated by bioinformatics and transcriptome analyses in this study. For further three genes, the *vanABK* operon, an indirect regulatory effect was shown (Merkens *et al*., 2005).

Different functional categories of genes regulated directly by AmtR, which were identified by bioinformatics approaches, DNA microarray analyses and operon determinations, have been observed. This includes genes coding for proteins involved in transport (*amtA*, *amtB*, *crnT*, *gluABCD* AND NCgl1915–1918, *urtABCDE*), which allow the uptake of alternative and low concentrated nitrogen sources, ammonium assimilation (*glnA*, *gltBD*), utilization of alternative nitrogen sources (*codA*, *ureABCEFGD*), signal transduction (*glnD*, *glnK*) and proteins of unknown or putative function (NCgl1099, NCgl1100, *ocd*, *soxA*). Obviously, *C. glutamicum* cells challenged with a situation of ammonium limitation try to scavenge nitrogen sources from the surrounding medium. A similar strategy was described for *E. coli* (Zimmer *et al*. 2000). In *C. glutamicum*, however, the rearrangement of different metabolic pathways is an equally important response.

An AmtR binding site consensus motif was deduced from the target sequences obtained by the bioinformatics approach, which were subsequently verified by transcriptome analyses and gel retardation assays. The resulting AmtR box with the nucleotide sequence tttCTATN$_6$AtAGat/aA (with bases represented by capital letters being highly conserved) is a palindromic sequence (Fig. 8) and can be located in the promoter region either on the sense or antisense strand. We assume that the strength of AmtR repression is modulated by the number of AmtR binding sites and the conservation of the motif.

## Experimental procedures

### *Strains and growth conditions*

Strains and plasmids used in this study are listed in Table 4. 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 BHI medium was used to inoculate minimal medium (per litre 42 g MOPS, 20 g $(NH4)_2S0_4$, 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 approximately 25–30, was used to inoculate fresh minimal medium to an $OD_{600}$ of approximately 1, and cells were grown for 4–6 h until the exponential growth phase was reached ($OD_{600}$ approximately 4–5). To induce nitrogen starvation, cells were harvested by centrifugation and the pellet was

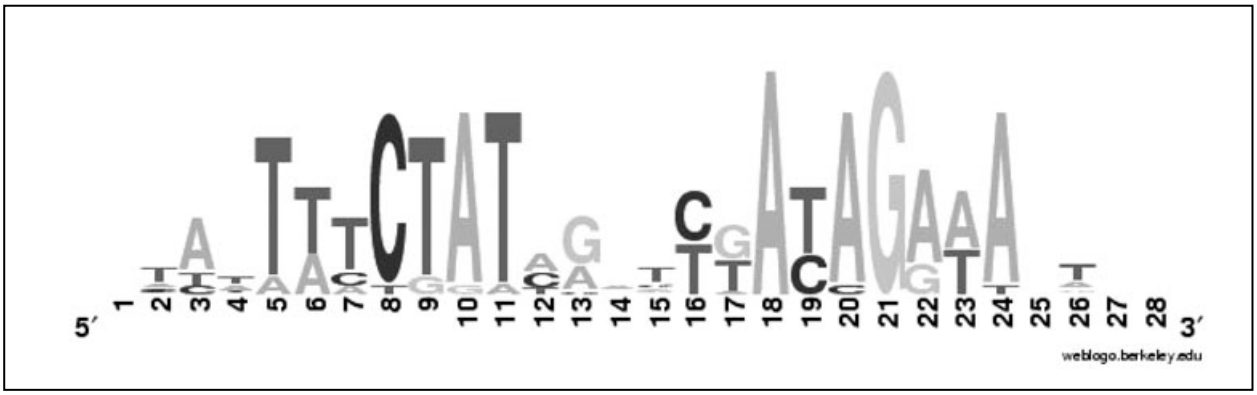



**Fig. 8.** The AmtR box of *C. glutamicum*. An AmtR binding consensus motif was established based on the binding sites predicted by bioinformatics analyses, which were verified by RNA hybridization analyses, real-time RT PCR and gel retardation tests. Putative binding sites upstream of unclear candidate genes (e.g. *gdh* and NCgl2787) and indirectly regulated genes (*vanABK*) were not introduced. The height of letters represents the frequency of the corresponding nucleotides in the AmtR box. The figure was generated using the WebLogo server (Crooks *et al*., 2004).

suspended in and transferred to prewarmed minimal medium without nitrogen source. The nitrogen-deprived cells were incubated at 30°C under aeration.

### *General molecular biology techniques*

For plasmid isolation, transformation, and cloning standard techniques were used (Ausubel *et al*., 1987; Sambrook *et al*., 1989). *E. coli* strains JM109 and JM110 were used as cloning hosts. Competent *C. glutamicum* cells were prepared according to van der Rest *et al*. (1999). Chromosomal *C. glutamicum* DNA was isolated as described (Eikmanns *et al*., 1994). DNA sequence analyses were carried out by the bioanalytics service unit at the Center for Molecular Medicine Cologne.

**Table 4.** Strains and plasmids used in this study.

| Strains/plasmids | Relevant genotype/description | Reference |
|---|---|---|
| *C. glutamicum* | | |
| ATCC 13032 | wild type | Abe *et al*. (1967) |
| MJ6-18 | Δ*amtR* | Jakoby *et al*. (2000) |
| *E. coli* | | |
| JM109 | F′ *traD36 lacI*$^q$ Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/*e14*$^-$ (McrA$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nx$^r$) *endA1 hsdR17* ($r_k^-m_k^-$) *relA1 supE44 recA1* | Yanisch-Perron *et al*. (1985) |
| JM110 | F′ *traD36 lacI*$^q$ Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/rpsL (Sm$^r$) *thr leu thi lacY galK galT ara fhu dam dcm supE44* Δ(*lac-proAB)* | Yanisch-Perron *et al*. (1985) |
| Plasmids | | |
| pEKEX2 | *E. coli–C. glutamicum* shuttle vector, Kn$^r$, *ptac*, *lacI*$^q$ | Eikmanns *et al*. (1991) |
| pEKEXglnK | *glnK* ligated to pEKex2 for overexpression | This study |
| pGEM3z | *E. coli* plasmid for *in vitro* transcription, Ap$^r$ | Promega, Mannheim, Germany |
| pGEMamtA | 0.5 kb internal *amtA* fragment in pGEM3z | Jakoby *et al*. (2000)<br>Nolden *et al*. (2001b) |
| pGEMamtB | 1.0. kb internal *amtB* fragment in pGEM3z | Jakoby *et al*. (2000)<br>Nolden *et al*. (2001b) |
| pGEMcodA | 0.5 kb internal *amtA* fragment in pGEM3z | Bendt *et al*. (2004) |
| pGEMcrnT | 0.5 kb internal *crnT* fragment in pGEM3z | Bendt *et al*. (2004) |
| pGEMgltB | 0.5 kb internal *gltB* fragment in pGEM3z | Beckers *et al*. (2001) |
| pGEMgluA | 0.5 kb internal *gluA* fragment in pGEM3z | This study |
| pGEMNCgl1099 | 0.5 kb internal NCgl1099 fragment in pGEM3z | This study |
| pGEMNCgl1100 | 0.5 kb internal NCgl1100 fragment in pGEM3z | This study |
| pGEMNCgl1362 | 0.5 kb internal NCgl1362 fragment in pGEM3z | This study |
| pGEMNCgl 1915 | 0.5 kb internal NCgl1915 fragment in pGEM3z | This study |
| pGEMglnA | 0.5 kb internal *glnA* fragment in pGEM3z | This study |
| pGEM-urtA | 0.5 kb internal *urtA* fragment in pGEM-3z | Beckers *et al*. (2004) |
| pGEM-ureA | 0.3 kb internal *ureA* fragment in pGEM3z | Beckers *et al*. (2004) |
| pQE60 | His-tag expression vector, Ap$^r$ | Qiagen, Hilden, Germany |
| pQE60-amtR | *amtR* gene in pQE60, C-terminal His-tag | This study |

r (superscript), resistance to; Ap, ampicillin; Kn, kanamycin; Nx, nalidixic acid; Sm, streptomycin.

*Construction of an expression vector for the production of AmtR-His$_6$*

The *amtR* gene was amplified by PCR using chromosomal DNA as template and the following oligonucleotides: 5′-GCGCGC**GAATTC**TATGGCAGGAGCAGTGGGA-3′/5′-GCGCGC**GGATCC**TTTCGCGTCAGCCTGCTTG-3′. The 0.7 kb PCR product was ligated to plasmid pQE60 using the EcoRI and BamHI sites introduced by the oligonucleotide primers (shown in bold) and the resulting plasmid, pQE60-amtR was sequenced for control.

*Construction of a GlnK-delivery plasmid*

The *glnK* gene was amplified by PCR using chromosomal DNA as template and the following oligonucleotides: 5′-GCGCGC**GGATCC**ATGAAACTCATCACCGCAATT-3′/5′-GCGCGC**GAATTC**TTAAAGGGCTGCTTCGCCG-3′. The PCR product was ligated to plasmid pEKEX2 using the BamHI and EcoRI sites introduced by the oligonucleotide primers (shown in bold). The resulting plasmid, pEKEXglnK was sequenced for control.

*Construction of antisense probes*

For the generation of antisense probes internal DNA fragments of the corresponding genes were amplified by PCR and cloned into plasmid pGEM plasmids (Promega, Mannheim, Germany) using restriction sites added to the primer sequence (Table 5). For cloning of the *ureA* gene fragment an EcoRI and an HindIII site was introduced (primer sequences: 5′-GCGCGC**GAATTC**CGTCGCCGTAAAGATCG-3′/5′-GCGCGC**AAGCTT**ATCAAACGTTGCTTCAACC-3′), and for the *urtA* probe an EcoRI and a HindIII restriction site was added (5′-GCGCGC**GAATTC**GGCTTGGACTTTGAAGACG-3′/5-GCGCGC**AAGCTT**CACCGTGATCAACAAATGG-3′) to insert the PCR products into plasmid pGEM-3z. The hybridization probes were produced by *in vitro* transcription using the SP6-polymerase.

*RNA preparation, hybridization analysis and RT-PCR*

Total RNA was prepared after disruption of *C. glutamicum* cells by glass beads using the RNeasy Mini Kit (Qiagen, Hilden, Germany). For hybridization experiments the RNA (approximately 5 μg per sample, diluted in 10× SSC) was spotted onto nylon membranes. After drying and UV fixation (120 mJ cm$^{-2}$), hybridization was carried out as described (Roche, Mannheim, Germany; 'DIG application guide'). For RT-PCR the OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) was used.

*Real-time RT-PCR*

For real-time RT-PCR, a TaqMan device (Applied Biosystems, CA, USA), the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany), 0.5 μM primers (Table 5) and 100 ng of template RNA were used. Reverse transcription was carried out at 50°C for 30 min, the RT was inactivated and the polymerase activated by 15 min incubation at 94°C, PCR was carried out by 40 cycles of the following program: DNA denaturation for 15 s at 94°C, primer annealing for 30 s at 60°C, and DNA polymerization for 30 s at 72°C. All experiments were carried out at least in triplicate with templates from independent cultures. For an exact quantification of real-time PCR results, dilution series were tested for each pair of primers to determine its PCR efficiency and the total amount of RNA applied in the real-time PCR experiments was determined using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR).

*Gel retardation experiments*

To investigate AmtR binding gel-shift assays were carried out. For the upstream region of the different genes PCR-generated DNA fragments spanning the corresponding putative AmtR binding sites were labelled with digoxigenin using the DIG Oligonucleotide 3′-End Labelling Kit, 2nd Generation (Roche, Mannheim, Germany). The labelled DNA was incubated for 20 min on ice with different amounts of AmtR-containing *E. coli* cell extract (DH5α*mcr* pUC11-1.8) or control extract lacking AmtR (DH5α*mcr* pUC18) or with purified AmtR protein. The formation of unspecific DNA–protein complexes was minimized by adding sheared salmon sperm DNA (Eppendorf, Hamburg, Germany) and BSA (AppliChem, Darmstadt, Germany). DNA–protein complexes were separated from free DNA on a non-denaturing 6% polyacrylamide-TBE gel by electrophoresis at 20 mA. The DNA was blotted onto a positively charged nylon membrane (Roche, Mannheim, Germany) and the digoxigenin-labelled DNA was detected with a Fuji luminescent image analyzer LAS1000 (Raytest, Straubenhardt, Germany).

*Transcriptome analyses using DNA microarrays*

Custom-made microarrays were manufactured by GeneScan Europe AG (Freiburg, Breisgau). Altogether 3556 PCR-products were spotted in triplicate on aminosilan-coated glass slides (CMT_GAPS, Corning GmbH, Wiesbaden). For transcriptome analyses 50 μg RNA were concentrated to a volume of 20 μl, mixed with 10 μl random-primers (100 nM, hexamer:octamer ratio 1:1) and denatured at 70°C. After cooling down 3 μl Cy3 or Cy5 dUTP (Amersham Biosciences, Freiburg, Germany) were added. Then a mixture containing 1 μl nucleotide mix (25 mM dATP, dGTP, dCTP and 5 mMdTTP), 10 μl first strand buffer (5×, Gibco, Eggenstein), 5 μl 100 mM DTT and 2 μl superscript II RT (200 U μl$^{-1}$, Gibco, Eggenstein) was apposed and held for 10 min at RT and subsequently for 110 min at 42°C. The RT activity was inactivated incubation for 10 min at 70°C after the addition of 2.5 μl EDTA (20 mM) and 2.5 μl NaOH (500 mM). NaOH was neutralized by the addition of 2.5 μl HCl (500 mM). Purification of the cDNA was rendered possible by the High Pure PCR Product Purification Kit (Roche, Mannheim).

The Cy3 and Cy5 marked cDNAs were mixed in a ratio of 1:1. After the addition of 3 μl herring sperm DNA solution (10 mg ml$^{-1}$), the composition was again denatured for 5 min at 95°C and cooled down rapidly. The solution was then brought onto the microarray. Hybridization was carried out

**Table 5.** Oligonucleotide primers used in this study. Restriction sites added to the primer sequences are written in bold letters.

| Name | Sequence |
|---|---|
| RNA hybridization experiments | |
| codA-1 | GCGCGC**GAATTC**ACGTAGATGTCACCGATCC |
| codA-2 | GCGCGC**AAGCTT**GCATACTGCAAAACGAACC |
| gluA-1 | GCGCGC**GAATTC**TCGAAATCGATGGAAAGG |
| gluA-2 | GCGCGC**CTGCAG**TGCACGATCAGACTTAGGG |
| NCgl1099-1 | GCGCGC**GAGCTC**ATCAGTGGGACACCCTAGC |
| NCgl1099-2 | GCGCGC**CTGCAG**CGGGTTAGCTCGATTGC |
| NCgl1100-1 | GCGCGC**GAATTC**GTGGATATGTGGTCCATGC |
| NCgl1100-2 | GCGCGC**AAGCTT**AGCTTCCCAAAGAAACAGC |
| NCgl 1915-1 | GCGCGC**GAATTC**GCAACACCAACGAAGTAGG |
| NCgl 1915-2 | GCGCGC**CTGCAG**GGTGTAGTCCGCGTTAGG |
| ureA-1 | GCGCGC**GAATTC**CGTCGCCGTAAAGATCG |
| ureA-2 | GCGCGC**AAGCTT**ATCAAACGTTGCTTCAACC |
| urtA-1 | GCGCGC**AAGCTT**CACCGTGATCAACAAATGG |
| urtA-2 | GCGCGC**GAATTC**GGCTTGGACTTTGAAGACG |
| Organization of gene clusters | |
| OB-amt-ocd-soxA-1 | CTGGAAGAAGGGACACCG |
| OB-amt-ocd-soxA-2 | GCCATGCGAAACAGTCGG |
| OB-gluABCD-1 | GATCGTGTGTTGTTCATGGC |
| OB-gluABCD-2 | GTTTGGGAATTGATGAACGG |
| OB-NCgl 1915–1918-1 | GCACGCTGTCCTCCTACTTC |
| OB-NCgl 1915–1918-2 | GTCGATTTACCTGAACCGGA |
| Real-time RT-PCR | |
| amt_realtime 1 | GCGTCCACACAACCTTCCAC |
| amt_realtime 2 | GCGGTACCACCATTGAATCC |
| amtB-realtime 1 | AGTGGTGGTGGCATGGATG |
| amtB-realtime 2 | GAATGCGATGAGCGCAGTAA |
| codA realtime 1 | TCGCCACAGAATGAGACACC |
| codA realtime 2 | CCCGATTTGTCGAAGTCCTC |
| crnT-realtime 1 | GGCTGTTGCTGCGAATACAAC |
| crnT-realtime 2 | GACGGCACCTCCATCAACTC |
| glnA-realtime 1 | TCCATTCCACAGGCACCAA |
| glnA-realtime 2 | GACGTCAGACCGGTGAGGAT |
| gltB-realtime 1 | GCAGCATGCAACTCGTTCTG |
| gltB-realtime 2 | CCCAAGCGAGTGGATTGATC |
| gluA realtime 1 | ACCTGCGATGAACCCAAAGAT |
| gluA realtime 2 | CCATGACGTCCAACACTTCGT |
| NCgl1099 realtime 1 | GTAGTGGCAATGCGCAGTTG |
| NCgl1099 realtime 2 | TTCTGAGCTGGACCGACGAT |
| NCgl1100 realtime 1 | TCCCAGTCCATAGCCGTTTC |
| NCgl1100 realtime 2 | TCGGAATCTGGCACCTCAA |
| NCgl 1915 realtime 1 | CGACGGCGTGAAGTTCATCT |
| NCgl 1915 realtime 2 | GATAGCGTCCAGCACATCCAA |
| ureA realtime 1 | TCGCCCTCATCACGTATGAA |
| ureA realtime 2 | TCAAAATGGTGCTTCCCCAG |
| urtA realtime 1 | TTCTTCCGCCAGTACAACAGC |
| urtA realtime 2 | CGATGCCTCCGACTTCTTCTT |
| Gel retardation experiments | |
| amtB-gr-1 | CCAGCTCTAACTATAGACCTACAGAAACTAATTTC |
| amtB-gr-2 | CAATACATCTATAGAATAGGTTTTGGAGGG |
| amtB-gr-3 | GGATATTTTCTATAGTTTAACAGGTAATTTAATG |
| amtB-GS-1 | CCTTTTGGGATTGACACC |
| amtB-GS-2 | TTTGCCTGGGTACCACC |
| codA-gr | CCAGATGCTATCTATTCTATTGCTTGACAGGTATTAGTG |
| crnT-gr-1 | AAACTCCCTTTTCTATCAATCTATAGAAACTTGCAAAATC |
| crnT-gr-2 | AAGTCATACTTTCTATAGATTGATAGAAATGTGGGGAG |
| crnT-GS-1 | GAGGGCTTTATCATCGCC |
| crnT-GS-2 | CTGGAACATCTAACAGCCC |
| glnA-gr | AAGTTTTGATAGATCGACAGGTAATGCATTATAC |
| gltBD-gr-1 | CCTCCCGTTTTCCTATAGGTTGATCGAAAGTAACC |
| gltBD-gr-2 | CTTATTATCGAACGATTGATAGAAACAGGATTA |
| gltBD-GS-1 | GGTGGATAGGCGAACATC |
| gltBD-GS-2 | CCTTGTGGTTTCATGCGG |
| gluA-gr | AACACGCAATATCTATCATGTGATAGGTAAATTTCGG |
| NCgl1099/1100-gr | CTAAATTATTTTCTATCAATCTATAGATACTAGGTCTATT |
| NCgl1915-gr | CTCACAAATTTCTATCAAACTATAGAAAGATATTTG |
| ureA-gr-1 | CAGTGAAAAGAGAATTCTATAGGACGCCAGACTTAG |
| ureA-gr-2 | GCTTCATTTTTAAGGGGGTAGATATGCAGATGTAGCGC |
| ureA-gr-3 | GCTAAACTAATTTCTGTTACCTGACAGAAAGGGGCAA |
| ureA-GS-1 | TCATCGGTGCAAGCTTACG |
| ureA-GS-2 | AGTGATATGCAATTTTGCCC |
| urtA-gr-1 | CAAAAACAACTACCTATAGGCTGACAGAAACTCTAA |
| urtA-gr-2 | ACTCTAAAAACTATAGAGCTATAGAAACTTTAACTT |
| urtA-GS-1 | GGCTGAAATGTAAGACACG |
| urtA-GS-2 | GGACGGGACATGAATACC |

over night in a hybridization chamber (Vysis HYBrite, Abbott Diagnostika, Wiesbaden) at 50°C. Subsequently, the microarray was washed first two times with 2× SSC, 0.2% SDS, then twice in 1× SSC, 0.1% SDS, followed by one washing step in 0.5% SSC and once in distilled water. Hybridization signals were detected by a GSI-Lumonics laser scanner (ScanArray 400, Perkin Elmer, Wellesley, MA) and analysed using the QuantArray software package version 3.0.0.0 (PackardBioScience, Meriden, CT). For global and local normalization of data and graphical output the GeneSpring 3.2.12 software package (Silicon Genetics, Redwood City, CA) was used. To avoid problems due to different labelling efficiencies by Cy3 and Cy5, dye switch experiments were carried out.

### *Two-dimensional gel electrophoresis and MALDI-TOF-MS*

*Corynebacterium glutamicum* cells were disrupted using glass beads and a Q-BIOgene FastPrep FP120 instrument (Q-BIOgene, Heidelberg, Germany) in the presence of proteinase inhibitor *Complete* as recommended by the supplier (Roche, Mannheim, Germany). Membranes were removed from the cell extract by ultracentrifugation (Hermann *et al*., 1998; 2000; 2001) and the cytoplasmic proteins were further analysed. Protein concentrations were determined using the Roti-Nanoquant assay (Roth, Karlsruhe, Germany).

For isoelectric focusing (IEF) 24 cm precast IPG strips pI 4–7 and an IPGphor IEF unit (Amersham Biosciences, Freiburg, Germany) were used as described (Hermann *et al*., 2000). 120 μg of protein were focused for 68 000 Vh in a sample buffer containing 6 M urea, 2 M thiourea, 4% CHAPS, 0.5% Pharmalyte (3–10) and 0.4% DTT. For the second dimension electrophoresis was carried out using precast 12–14% polyacrylamide linear gradient gels (ExcelGel Gradient XL 12–14, Amersham Biosciences, Freiburg, Germany) in the Multiphor II apparatus as described (Hermann *et al*., 2000; 2001). After electrophoresis 2-D gels were stained with colloidal Coomassie brilliant blue. The Coomassie-stained gels were aligned using the Delta2D software, version 3.1 (Decodon, Greifswald, Germany). MALDI-TOF-MS for peptide mass fingerprint analyses was carried out by the bioanalytics service unit at the Center for Molecular Medicine Cologne.

### *Affinity purification of AmtR and GlnK coprecipitation*

For affinity purification of AmtR~$His_6$ and coprecipitation experiments, *E. coli* cell extract was prepared from strain JM109 pQE60-amtR grown to an $OD_{600}$ of approximately 0.6 and then induced with 1 mM IPTG for 5 h. The cells were lysed using glass beads and a Q-BIOgene FastPrep FP120 instrument (Q-BIOgene, Heidelberg, Germany), cell debris was removed by centrifugation and approximately 45 μg of cell extract were loaded on a 12 ml 'Ni Sepharose HighPerformance' (Amersham Biosciences, Freiburg, Germany). The column was washed with 10 volumes of 300 mM NaCl, 50 mM $NaH_2PO_4$ and 20 mM imidazol (pH 8.0). Subsequently, 25 mg *C. glutamicum* cell extract prepared according to Strösser *et al*. (2004) was applied on the column. The column was washed (see above) and bound proteins were eluted using the following buffer: 300 mM NaCl, 50 mM $NaH_2PO_4$ and 500 mM imidazol (pH 8.0).

### *Bioinformatic analyses*

The recently published genome sequence of *C. glutamicum* type strain ATCC 13032 (Kalinowski *et al*., 2003) was screened for putative AmtR binding sequences essentially as described by Rey and coworkers for the genome-wide screening of McbR binding sites (Rey *et al*., 2005). The AmtR binding sites found upstream of *amtA*, *amtB*, *glnA* and *gltB* were aligned and used to create an Hidden Markov model using the HMMER 2.1.1 software package (http://hmmer.wustl.edu). The calculated HMM was applied to screen the *C. glutamicum* genome sequence for the presence of putative AmtR binding sites. In addition, the FUZZNUC program package (http://www.hgmp.mrc.ac.uk/Software/EMBOSS/) was used to identify new AmtR binding sites.

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