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AmtR, a global repressor in the nitrogen regulation system of *Corynebacterium glutamicum*

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Summary

The uptake and assimilation of nitrogen sources is effectively regulated in bacteria. In the Gram-negative enterobacterium *Escherichia coli*, the NtrB/C two-component system is responsible for the activation of transcription of different enzymes and transporters, depending on the nitrogen status of the cell. In this study, we investigated regulation of ammonium uptake in *Corynebacterium glutamicum*, a Gram-positive soil bacterium closely related to *Mycobacterium tuberculosis*. As shown by Northern blot hybridizations, regulation occurs on the level of transcription upon nitrogen starvation. In contrast to enterobacteria, a repressor protein is involved in regulation, as revealed by measurements of methylammonium uptake and β-galactosidase activity in reporter strains. The repressor-encoding gene, designated *amtR*, was isolated and sequenced. Deletion of *amtR* led to deregulation of transcription of *amt* coding for the *C. glutamicum* (methyl)ammonium uptake system. *E. coli* extracts from *amtR*-expressing cells were applied in gel retardation experiments, and binding of AmtR to the *amt* upstream region was observed. By deletion analyses, a target motif for AmtR binding was identified, and binding of purified AmtR protein to this motif, ATCTATAGN$_{1-4}$ATAG, was shown. Furthermore, the binding of AmtR to this sequence was proven *in vivo* using a yeast one-hybrid system. Subsequent studies showed that AmtR not only regulates transcription of the *amt* gene but also of the *amtB–glnK–glnD* operon encoding an *amt* paralogue, the signal transduction protein P$_{II}$ and the uridylyltransferase/uridylyl-removing enzyme, key components of the nitrogen regulatory cascade. In summary, regulation of ammonium uptake and assimilation in the high G+C content Gram-positive bacterium *C. glutamicum* differs significantly from the mechanism found in the low G+C content Gram-positive model organism *Bacillus subtilis* and from the paradigm of nitrogen control in the Gram-negative enterobacteria.

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Introduction

Global regulatory networks allow bacteria to survive conditions of stress and starvation and are indispensable for an appropriate adaptation of the cellular metabolism to a changing environment. Among these regulatory networks, the system responsible for controlling uptake of nitrogen sources and their assimilation is of major significance.

Most microorganisms use ammonium (NH_4^+) as the preferred nitrogen source. Although its uncharged form, ammonia (NH_3), is membrane permeable, energy-dependent transport systems for ammonium were described in many bacteria (for a review, see Kleiner, 1993). In general, these carriers are inhibited by glutamine or its analogues (Kleiner and Castorph, 1982; Jayakumar *et al.*, 1986). The intracellular pool of glutamine is supposed to reflect directly the nitrogen supply of the cell, and this regulatory signal thus ensures that uptake of nitrogen sources corresponds exactly to the requirements of the organism. In addition, carrier synthesis is regulated by the availability of ammonium itself (Kleiner, 1985). Intracellular ammonium accumulation via an uptake system, however, may lead to an energy-wasting futile cycle, during which energy-dependent uptake is counteracted by diffusion of ammonia out of the cell, a process designated cyclic retention. Thus, absence of carrier synthesis under conditions of sufficient nitrogen supply restricts energy-consuming ammonium uptake to situations when cells are starving for nitrogen.

In *Corynebacterium glutamicum*, a Gram-positive soil bacterium widely applied in the industrial production of amino acids, two genes encoding (putative) ammonium uptake carriers were described. The isolation of *amt* (Siewe *et al.*, 1996) was the first report on the sequence of a gene coding for a bacterial ammonium uptake system combined with the characterization of the corresponding protein. As shown by Siewe *et al.* (1996), transport of (methyl)ammonium via the Amt system was only active during nitrogen starvation. Recently, a second *amt* gene with so far unknown function was isolated. This gene was

designated *amtP* for *amt* paralogue (Jakoby *et al*., 1999). Based on the great number of homologues cloned in different bacteria, sequence similarity analyses and its genetic organization, this system is designated *amtB* now.

In this study, we describe the regulatory mechanism of *amt* and *amtB* expression. In contrast to the situation observed in *Escherichia coli* (for reviews, see Merrick and Edwards, 1995; Magasanik, 1996; Reitzer, 1996) or in *Bacillus subtilis* (for a review, see Fisher, 1999), no activation of transcription via a NtrB/C-like two-component system or the TnrA system was found; however, regulation by a repressor protein was found. The corresponding gene, *amtR*, was isolated and sequenced, and binding of the AmtR protein to different target DNAs was characterized *in vitro* and *in vivo*.

Results

Regulation of amt *transcription*

In a previous study (Siewe *et al*., 1996), *amt* expression was analysed indirectly via (methyl)ammonium uptake measurements in the absence and presence of nitrogen sources and chloramphenicol. (Methyl)ammonium uptake was induced upon nitrogen deprivation; this induction was prevented by the addition of chloramphenicol. Based on these data, it was assumed that synthesis of *amt* is regulated on the level of transcription. To exclude an indirect effect of chloramphenicol, transcription of the *amt* gene was analysed directly in this study. For this purpose, Northern blot hybridizations with different *amt* probes were carried out. Total RNA was isolated from *C. glutamicum* cells before and after nitrogen starvation and was hybridized with a digoxigenin-labelled *amt* antisense RNA probe. Although no *amt* transcript was detected in cells grown under nitrogen excess, hybridization signals were observed within 5–10 min after cells were pelleted and transferred to a nitrogen-free medium (Fig. 1A). These results prove that (methyl)ammonium uptake is in fact regulated on the level of *amt* transcription. In the dot blot experiments, *amt* transcription seemed to be completely abolished under conditions of nitrogen surplus. Sometimes, however, a faint hybridization signal was detected. RT-PCR experiments were carried out in order to decide whether this signal corresponds only to an unspecific background labelling or whether *amt* is constitutively transcribed, at least at a low level. By this approach, a basal level of *amt* transcript was detected (Fig. 1B). A contamination of the mRNA preparation with chromosomal DNA was excluded in these experiments (data not shown).

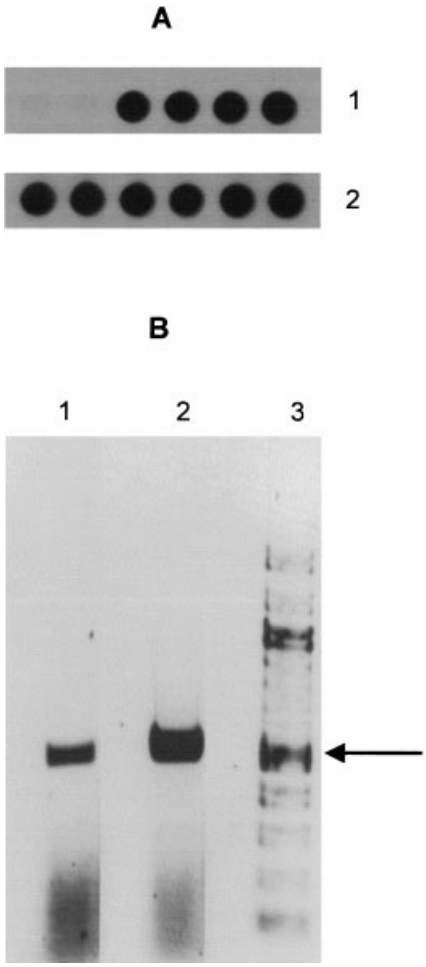

Fig. 1. A. Dot blots of total RNA isolated before and after 5, 10, 20, 30 and 45 min of nitrogen deprivation. 1, Wild-type probed for *amt* transcription; 2, wild-type probed for 16S rRNA as a control.
B. RT-PCR using *amt*-specific primers. As a template, either RNA from cells grown under nitrogen excess (1) or from cells starved of nitrogen for 30 min (2) was used; 3, standard (100 bp ladder; New England Biolabs; prominent bands indicate, from top to bottom, 1.0, 0.5 and 0.1 kb). The RT-PCR product is indicated by an arrow.

Effect of a high amt *copy number on Amt synthesis*

As shown by Siewe *et al*. (1996), (methyl)ammonium uptake activity is negligible in the wild type, when cells are grown in the presence of excess ammonium. Upon nitrogen deprivation, methylammonium uptake rate increases from approximately 0.5 to 6 nmol min^{-1} mg^{-1} dry weight. When we introduced additional copies of the *amt* gene on a high copy number plasmid, a partial deregulation of methylammonium uptake activity leading to an initial rate of approximately 9 nmol min^{-1} mg^{-1} dry weight was observed even without nitrogen deprivation (Fig. 2A). Upon starvation, methylammonium uptake increased to 14 nmol min^{-1} mg^{-1} dry weight, similar to the wild type. These results indicate the presence of a repressor protein which was titrated out by the additional *amt* copies and which otherwise prevents *amt* transcription in the wild type during high ammonium supply.

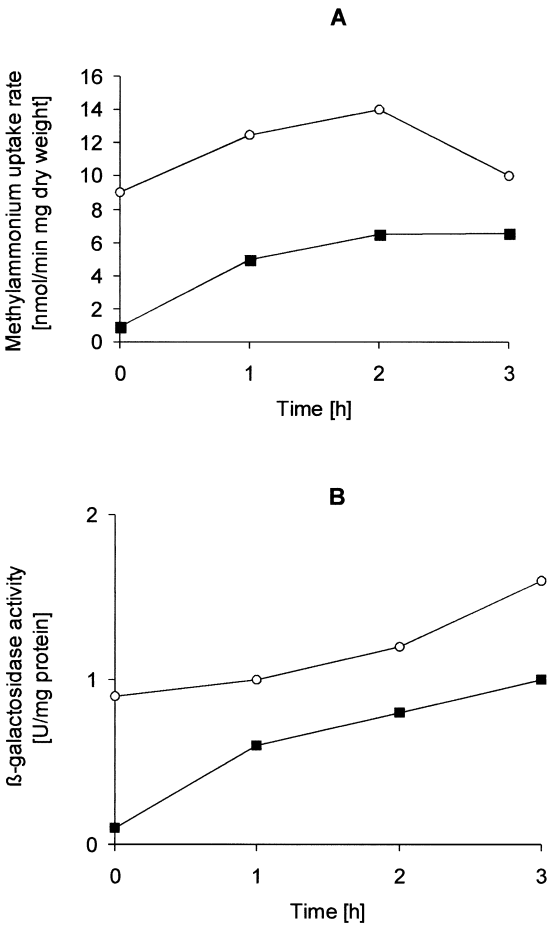

Fig. 2. A. Methylammonium uptake rate depending on *amt* copy number. Cells of the wild type carrying plasmid pJC1 as a control (solid squares) or pJCAmtHII (open circles) were starved of nitrogen for the indicated times, and (methyl)ammonium uptake rates were determined.
B. β-Galactosidase activity depending on *amt–lacZ* copy number. Cells of the wild type carrying a single *pamt–lacZ* fusion in the chromosome, ATCC 13032::pKdlz (solid squares), and on a high copy number plasmid, ATCC 13032 pJCdlz (open circles), were starved of nitrogen. β-Galactosidase activity was determined at the indicated times.

Characterization of amt–lacZ *fusions*

To verify the data obtained for the regulation of *amt* transcription by methylammonium uptake measurements, and to exclude indirect effects on the level of activity regulation of the transporter, a reporter gene assay was established. A set of experiments under conditions applied for the uptake measurements were carried out using strain ATCC 13032 carrying either a *pamt–lacZ* fusion integrated in the chromosome or on a high copy number plasmid. As observed for methylammonium uptake, β-galactosidase activity was induced by nitrogen starvation in a chromosomal *pamt–lacZ* fusion, whereas a high copy number of *pamt–lacZ* resulted in a deregulated *lacZ* expression (Fig. 2B), further arguing for the model of a repressor-regulated transcription.

Screening for the gene encoding the repressor of amt *transcription*

The plasmid-encoded *pamt–lacZ* fusion was expressed not only in *C. glutamicum* but also in *E. coli*, i.e. β-galactosidase activity was detectable in an *E. coli lacZ* mutant strain carrying plasmid pJCdlz. Based on this observation, a screening assay was established to isolate the gene encoding the *amt* repressor. A *C. glutamicum* gene bank of 2–5 kb chromosomal DNA *Sau*3A fragments cloned in vector pUC19 was introduced in *E. coli* DH5α*mcr* carrying plasmid pJCdlz. Approximately 80 000 clones were screened on LB medium agar plates containing carbenicillin, kanamycin and Xgal. Although the majority of colonies were blue, i.e. showed a β-galactosidase-positive phenotype, approximately 100 white clones were detected, 12 of which were further analysed. After confirming the phenotype by retransformation of these plasmids, restriction analysis, ligation of fragments and transformation of the resulting plasmids, subsequent rounds of screening revealed that the DNA encoding the putative repressor of *amt* transcription was located on a 1.8 kb *Bam*HI fragment. The corresponding pUC19 plasmid carrying this fragment was designated pUC11-1.8.

Characterization of the amtR *gene*

The nucleotide sequence of the 1.8 kb *Bam*HI fragment in pUC11-1.8 was determined (GenBank accession no. AJ133719). Computer-assisted analyses revealed three open reading frames, namely *yjcc*, an open reading frame similar to *E. coli, yjcc*, a gene encoding a putative regulator of transcription, and *citE*, coding for a putative citrate lyase fragment (Fig. 3A). Further analyses of the 669 bp composing the gene encoding the putative repressor, designated *amtR*, revealed that the deduced protein has low but significant similarity to members of the TetR/ArcR family of transcriptional regulators, especially in the typical helix–turn–helix motif (Fig. 3B). AmtR consists of, for example, 43% identical amino acids with a TetR/ArcR transcriptional regulator of *Aquifex aeolicus* (AE000776), 42% identity with the LanK protein of *Streptomyces cyanogenus* (AF080235) and 37% identical amino acids with a TetR/ArcR family transcriptional regulator of *Deinococcus radiodurans* (AE002049).

For the *amtR* gene, no regulation on the level of transcription was found. Northern blot hybridizations with total RNA prepared before and after nitrogen restriction and a probe against *amtR* mRNA showed low amounts of a constitutively expressed transcript. The constitutive

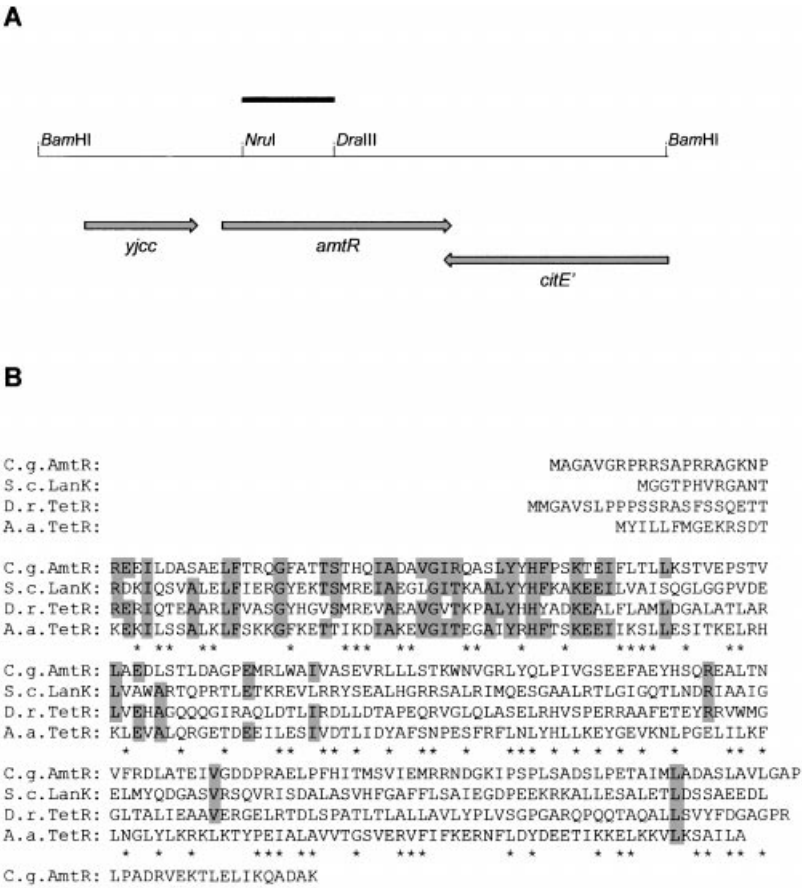

Fig. 3. A. Chromosomal organization of the *amtR* locus. Identified reading frames are indicated by arrows and the deletion introduced in the *amtR* gene is indicated by a black bar.
B. Alignment of AmtR with LanK of *S. cyanogenus* and with TetR/ArcR transcriptional regulators of *D. radiodurans* and of *A. aeolicus* (from top to bottom). Amino acid residues identical in at least three repressor proteins are shaded in grey, residues identical in two proteins are indicated by asterisks.

expression of *amtR* was verified by RT-PCR (data not shown). The size of the mRNA, 1 kb, indicated a monocistronic organization of *amtR* (data not shown).

Deletion of the amtR *gene*

To elucidate the role of AmtR in transcriptional control of *amt*, an unmarked deletion of *amtR* was introduced in *C. glutamicum* wild-type strain ATCC 13032, as described earlier (Schäfer *et al*., 1994). The deletion in the resulting strain, MJ6-18, was verified by PCR (data not shown; for position of the deleted fragment, see Fig. 3A).

First, the effect of deleting *amtR* on transcription of the *amt* gene was tested. Northern blot hybridizations were carried out using mRNA isolated from wild-type strain ATCC 13032 and *amtR* deletion strain MJ6-18 before and after onset of nitrogen starvation (Fig. 4). An *amt* antisense probe was used to detect an *amt* transcript, whereas 16S rRNA was probed as a control using a digoxigenin-labelled DNA probe. In contrast to the wild type, in which *amt* was expressed only upon nitrogen starvation, in strain MJ6-18 a hybridization signal with an *amt* probe was observed even without nitrogen deprivation. The *amt* gene is constitutively transcribed in this strain.

When uptake of [^{14}C]-methylammonium was tested in strain MJ6-18, a methylammonium uptake rate of approximately 6 nmol mg^{-1} dry weight min^{-1} was measured in contrast to the wild type, even without nitrogen starvation. Obviously (methyl)ammonium uptake is controlled mainly on the level of transcription of the *amt* gene via AmtR. Interestingly, an additional increase in methylammonium uptake rate of 4 nmol mg^{-1} dry weight min^{-1} in *amtR* deletion strain MJ6-18 was observed when cells were starved for 3 h of nitrogen. As this cannot be attributed to

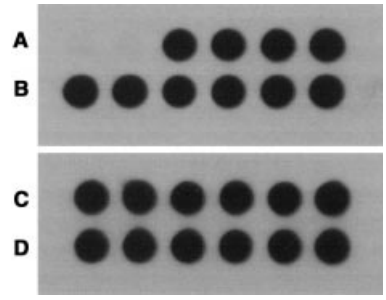

Fig. 4. Dot blots of total RNA isolated before and after 5, 10, 20, 30 and 45 min of nitrogen starvation.
A. Wild type probed for *amt* transcription.
B. *amtR* deletion mutant MJ6-18 probed for *amt* transcription.
C. Wild type probed for 16S rRNA as a control.
D. MJ6-18 probed for 16S rRNA.

a regulation on expression level upon starvation, as in the experiments in which additional *amt* copies were introduced in the wild type on a high *amt* copy number plasmid (Fig. 2A), the increase of transport activity in strain MJ6-18 upon nitrogen deprivation might indicate a second regulatory mechanism, probably on the level of activity of the carrier protein. First experiments hint to a role of glutamine synthetase in Amt activity regulation (M. Jakoby and J. Meier-Wagner, unpublished).

AmtR binds to two DNA motifs upstream of amt

To show binding of AmtR to the *amt* 5′ region, gel retardation tests were carried out using different digoxigenin-labelled DNA fragments and using *E. coli* cell extract of strain DH5α*mcr* pUC18 as a negative control and DH5α*mcr* pUC11-1.8 as a host for *amtR* expression. Although no retardation of the digoxigenin-labelled DNA fragment was observed using the control extract, increasing amounts of AmtR-containing *E. coli* extract resulted in three differently shifted bands (Fig. 5A). Two palindromic DNA sequences were found on the DNA fragment shifted by AmtR, consisting of the sequence ATCTATAGN$_4$ATAG. The sequence designated *amt*$_1$ is located at position −97 to −86 relative to the *amt* start codon, motif *amt*$_2$ is at position −61 to −46 (Table 1). To investigate whether

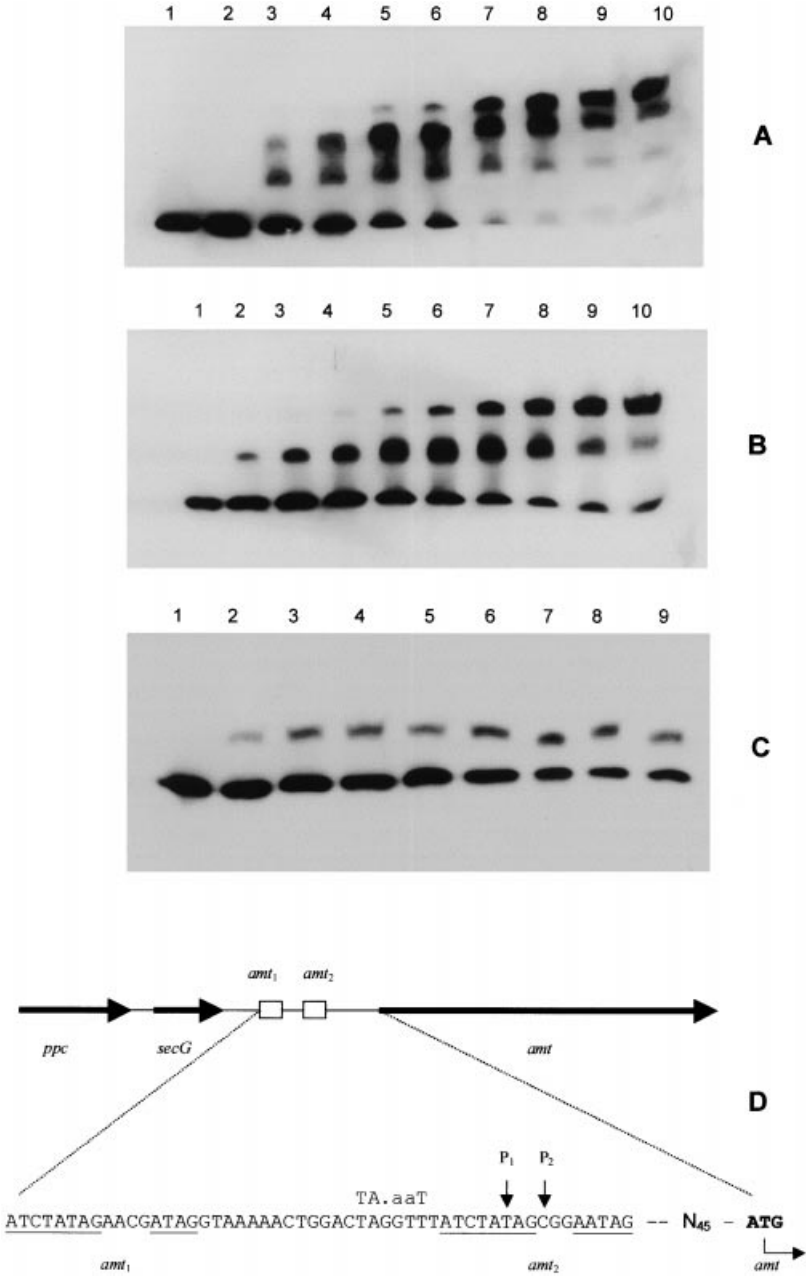

Fig. 5. A. Gel shift of the *amt* upstream region. The *amtR* gene was heterologously expressed in *E. coli*. Lane 1, control using DH5α*mcr* pUC18 extracts (10 μg); lanes 2–10, using rising amounts (2–10 μg, in 1 μg steps) of DH5α*mcr* pUC11-1.8 extracts.
B. Deletion of the AmtR binding motif *amt*$_1$. The gel retardation experiment was carried out as described in A.
C. Deletion of the AmtR binding motif *amt*$_2$. The gel retardation experiment was carried out as described in A.
D. Physical map of the *amt* upstream region. The AmtR binding motifs *amt*$_1$ and *amt*$_2$ and the ATG start codon of the *amt* gene are shown. P_1 and P_2 indicate the transcriptional start sites determined for the wild type. Compared with the promoter consensus sequences defined by Pátek *et al.* (1996) for *C. glutamicum*, a putative −10 consensus sequence was identified (shown on top of the corresponding *amt* upstream sequence), whereas no similarity with the described −35 consensus sequence ttGcca was found.

Table 1. AmtR-binding consensus motif.

Nucleotide sequence	5′ region
ATCTATAGAACG**ATAG**	amt_1
ATCTATAGGCGG**ATAG**	amt_2
ATCTATAGA **ATAG**	*amtB*
ATCG**ATA**CCAA **ATAG**	*amtR*
↑ ↑	

Nucleotides of the putative consensus motif are shown in bold letters. Deviations found in the *amtR* 5′ region leading to loss of binding are marked with arrows.

these sequences are responsible for AmtR binding, deletion analyses were carried out. For this purpose, either the complete amt_1 or the amt_2 motif or both motifs were deleted without changing the remaining *amt* upstream sequence. In fact, both elements were independently able to bind AmtR, resulting in band shifts (Fig. 5B and C), whereas a DNA fragment carrying the exact deletions of both motifs was not shifted by AmtR-containing extracts, excluding an unspecific binding of AmtR (data not shown).

To gain information on the physiological role of the two motifs upstream of the *amt* gene, the start site(s) of the *amt* transcript were determined. In the wild type, two transcripts were detected starting 56 and 53 bp upstream of the ATG start codon of *amt* (data not shown), i.e. in the amt_2 motif (for an overview, see Fig. 5D). These transcriptional start sites were found when cells were grown either under nitrogen excess or under nitrogen deprivation and also in *amtR* deletion strain MJ6-18 (data not shown). In amt_2 deletion strain Δ268/283, *amt* expression was still under the control of AmtR, and transcription started upon nitrogen starvation 39 bp upstream of the *amt* gene (data not shown). This result indicates that both AmtR binding sites are occupied in *C. glutamicum* when cells grow under nitrogen excess. The occurrence of two AmtR motifs upstream of the *amt* gene might ensure a tight expression control. This hypothesis is in accord with the observation that in amt_1 deletion strain Δ304/320 repression of *amt* transcription upon nitrogen excess is, although still present, slightly released (data not shown). In this strain, however, *amt* transcription is significantly reduced.

Purified AmtR binds to its target DNA

The retardation of DNA fragments in gel shift experiments was strictly dependent on the presence of the *C. glutamicum amtR* gene. To exclude side-effects of *E. coli* proteins and to elucidate whether AmtR alone is able to bind to its target DNA, the AmtR repressor protein was purified. For this purpose, AmtR was synthesized in *E. coli* strain BL21 pMamtR as a maltose binding protein–AmtR fusion. After isolation, the fusion protein was cleaved by factor Xa, and AmtR was further purified (Fig. 6A). For this purpose, gel filtration experiments were carried out.

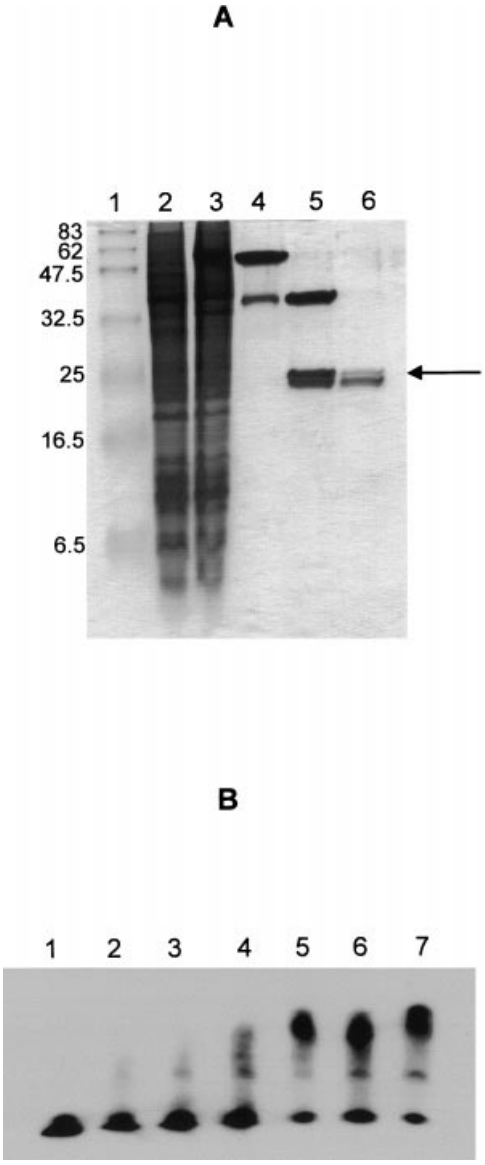

Fig. 6. A. Purification of AmtR. An SDS polyacrylamide gel was loaded as follows: 1, marker proteins (M_r indicated at the left); 2, BL21 pMamt extract before induction of *malE–amtR* transcription; 3, cell extract after IPTG induction; 4, maltose eluate from amylose column; 5, endoproteinase Xa digest of MBP–AmtR fusion protein; 6, AmtR-containing eluate after gel filtration (the AmtR protein is marked by an arrow).
B. Gel retardation experiments using isolated AmtR protein. A DNA fragment comprising the *amt* upstream region was incubated without (1) and with rising amounts (2.5, 5, 10, 15, 20 and 25 μg) of purified AmtR protein (2–7).

Analysis of the elution profile via comparison with standard proteins revealed that AmtR forms a dimer under the purification conditions used (data not shown). Subsequently, the recombinant AmtR protein was applied in gel shift experiments. The isolated and purified AmtR protein led to an identical pattern of shifted *pamt*-carrying DNA fragments to *E. coli* extracts containing AmtR,

whereas control DNA without protein addition was not retarded (Fig. 6B). This result proves that AmtR binds upstream of the *C. glutamicum amt* gene. For a successful shift, however, unexpectedly high amounts of the recombinant AmtR protein were necessary. This observation as well as the double band formed by AmtR on SDS polyacrylamide gels (Fig. 6A) prompted us to determine the N-terminal sequence of the purified protein. The amino acid sequence of the lower band exhibited the N-terminal sequence RAGKNPREEILDASAELFTRQGF, indicating a loss of 13 amino acids. In less than 10%, the N-terminal arginyl residue (R) was also lost. As the double band was only observed after binding of the fusion protein to the amylose column and cleavage with factor Xa, it is obvious that this endoproteinase cleaves in the maltose binding protein–AmtR fusion at an additional non-standard site.

AmtR is functional in a yeast one-hybrid in vivo *assay*

In the gel retardation *in vitro* approach, AmtR, either present in *E. coli* whole cell extracts or as a purified recombinant protein, was able to bind to its target motif. To show this binding activity also *in vivo*, we applied a yeast one-hybrid system. The AmtR target motif was cloned into plasmid pHISi-1, which was linearized afterwards and subsequently introduced in the genome of *Saccharomyces cerevisiae* strain YM4271. As its parental strain, the resulting strain, YLN1, showed a histidine-negative phenotype due to the mutation in the *HIS3* locus. When plasmid pGAD424amtR carrying the *amtR* gene was introduced in strain YLN1, the resulting transformants exhibited, in contrast to a control transformed with plasmid pGAD424, a histidine-prototrophic phenotype (data not shown). This result showed that AmtR, fused to the GAL4 activation domain, interacted with the chromosomally integrated *amtR* binding motif and led to an activation of the *HIS3* gene located on the inserted pHISi-1 vector. Obviously, AmtR is active not only *in vitro* but also *in vivo* in *S. cerevisiae*.

AmtR is a global regulator of nitrogen control

To identify other putative AmtR-controlled genes, *C. glutamicum* DNA sequences available in databases were screened for the AmtR binding sequence ATCTATAGN$_4$ATAG. No obvious recognition motif could be identified upstream of *gdh*, coding for glutamate dehydrogenase (Börmann *et al.*, 1992), *glnA*, encoding glutamine synthetase (Jakoby *et al.*, 1997), or *gltBD*, encoding glutamate synthase (GOGAT; SWISSPROT accession no. BAA75930). In contrast, one putative binding site with the sequence ATCGATACN$_3$ATAG was found upstream of *amtR* itself and another one, ATCTATAGN$_1$ATAG, was identified upstream of the *amtB* gene, encoding an *amt* paralogue with putative ammonium transport function which was formerly designated *amtP* (Jakoby *et al.*, 1999).

The spacing between the 5′ and 3′ part of the consensus seems to be unusually variable in the different putative binding sites (Table 1). This observation was verified by changing the spacing between the two submotifs of the amt_2 sequence. Without loss of AmtR

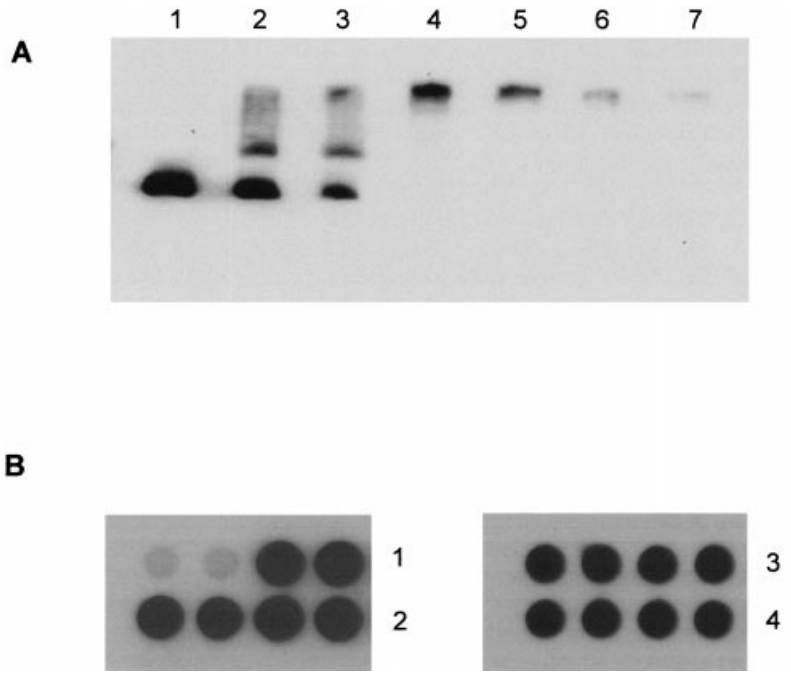

Fig. 7. A. Binding of AmtR to the *amtB* upstream region. Experiments were carried out as described in Fig. 5A with the exception that an 0.2 kb DNA fragment of the *amtB* upstream region was used. Lane 1, control using DH5α*mcr* pUC18 extracts (10 μg); lanes 2–7, using rising amounts (1, 2, 4, 6, 8 and 10 μg) of DH5α*mcr* pUC11-1.8 extracts. B. Dot blot of total RNA isolated from wild type and *amtR* deletion strain MJ6-18 hybridized with an *amtB* (1) and a 16S rRNA probe (2). RNA was isolated before and after 5, 10, 20, 30 and 45 min of nitrogen deprivation.

binding, it was reduced to only one or enlarged to up to 10 nucleotides (data not shown). The physiological role of this observation is unclear.

When binding of AmtR to the *amtR* upstream region was analysed in gel retardation experiments, no shifted DNA fragment, i.e. no binding of AmtR to the ATCGA-$TACN_3ATAG$ sequence, was detected (data not shown). The two nucleotides changed in comparison with the *amt* upstream motif; changes of T to G at position 4 and G to C at position 8 were sufficient to prevent AmtR binding. These naturally occurring mutations in the AmtR consensus emphasize the central role of these nucleotides in the consensus motif. The function of the modified AmtR binding motif upstream of the *amtR* gene remains unclear.

The putative binding motif upstream of *amtB* was also analysed. Gel shift experiments showed that AmtR binds to this region, resulting in a shift pattern less complex than that found for the *amt* upstream region because only one AmtR binding motif is located upstream of *amtB* (Fig. 7A). In this case, however, the AmtR binding motif was located on the DNA strand complementary to the *amtB* gene. To show that *amtB* transcription is in fact regulated by binding of AmtR to this site, total RNA isolated from the wild type and from the *amtR* deletion strain MJ6-18 was hybridized against an *amtB* probe and a 16S rRNA probe for control. In strain MJ6-18, *amtB* is, in contrast to the wild type, constitutively expressed and is not dependent on nitrogen starvation (Fig. 7B). This result shows that AmtR is responsible for the regulation of both ammonium transporter-encoding genes in *C. glutamicum*.

On the *C. glutamicum* chromosome, *amtB* is located upstream of the *glnK* (formerly designated *glnB*) and *glnD* genes coding for the signal transduction protein P_{II} and uridylyltransferase/uridylyl-removing enzyme respectively (Jakoby *et al.*, 1999). The fact that *amtB* homologues are frequently clustered with *glnK* homologues in a wide

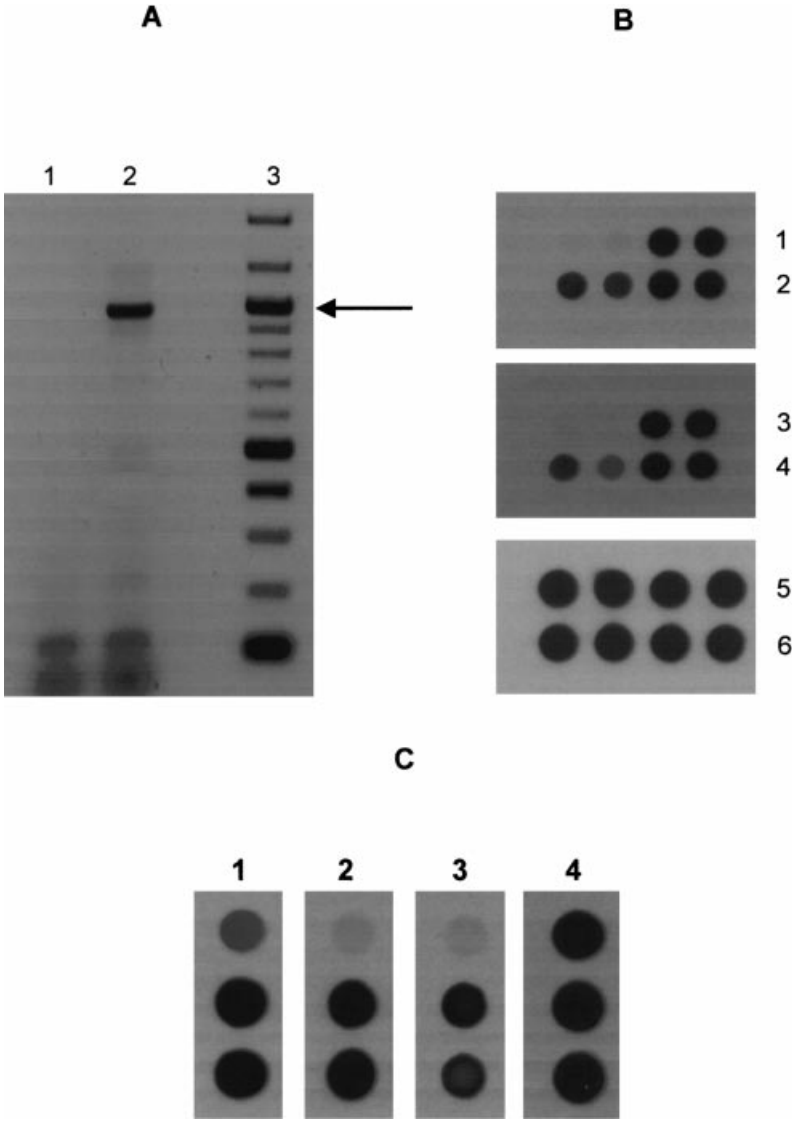

Fig. 8. A. RT-PCR of the *amtB–glnK–glnD* operon. The forward primer annealed in the *amtB* gene, the reversed primer in *glnD*. The *amtB–glnK–glnD*-spanning product is indicated by an arrow. 1, Control without reverse transcriptase; 2, RT-PCR reaction using total RNA isolated from nitrogen-starved cells as template; 3, 100 bp ladder. Prominent bands indicate, from top to bottom, 1, 0.5 and 0.1 kb (New England Biolabs). The 960 bp *′amtB–glnK–glnD′* fragment is indicated by an arrow.
B. Dot blots of total RNA isolated from wild type (1, 3, 5) and from *amtR* deletion strain MJ6-18 (2, 4, 6) hybridized with an *glnK* (1, 2), *glnD* (3, 4) and a 16S rRNA probe (5, 6) for control. Total RNA was isolated before and after 5, 15 and 30 min of nitrogen starvation.
C. Dot blots of total RNA isolated from wild-type strain ATCC 13032 transformed with plasmid pJC1 (chromosomal *amt* copy; lane 1) and pJCAmtHII (high copy number of *amt*, lane two) and of *amtR* deletion strain MJ6-18 (lane 3). Cells were grown in medium with excess nitrogen. Samples were hybridized with probes for *amtB* (1), *glnK* (2), *glnD* (3) and 16 S rRNA (4).

number of archaea and bacteria such as *E. coli* or *Mycobacterium tuberculosis* (Thomas *et al*., 2000) and the lack of terminator structures downstream of *C. glutamicum amtB* gave rise to the idea that *amtB* might form an operon with *glnK* and *glnD*. In this case, AmtR would not only regulate the synthesis of (putative) (methyl)ammonium permeases but also of key components of the nitrogen regulatory cascade in *C. glutamicum*. In RT-PCR experiments, primers were used for annealing in the 3′ part of *amtB* and in the 5′ part of *glnD*, and, in fact, a 960 bp DNA fragment was detected, indicating a common transcript of *amtB*, *glnK* and *glnD* (Fig. 8A). As expected from this result, Northern blot hybridization analyses, which were subsequently carried out, revealed that the expression of *glnK* and *glnD* is also regulated via AmtR and depends on the nitrogen status of the cells (Fig. 8B). Under nitrogen excess, a low basal level of transcription was found, which, however, was detectable only by RT-PCR (data not shown). Upon nitrogen starvation, *glnK* and *glnD* expression is strongly upregulated, similar to *amtB* transcription. Repression of *glnK* and *glnD* transcription was relieved in *amtR* deletion strain MJ6-18 (Fig. 8B). Furthermore, site-directed mutagenesis of the AmtR binding motif upstream of *amtB* in wild-type strain ATCC 13032 resulted in the loss of repression of *amtB*, *glnK* and *glnD* transcription (L. Nolden, unpublished).

Provided that AmtR is a global regulator which represses not only transcription of the *amt* gene but also of the *amtB–glnK–glnD* operon, an out-titration of AmtR by a high copy number of the *amt* upstream region should result in a deregulated expression of these genes. In fact, Northern blot hybridizations revealed a deregulated transcription of *amtB*, *glnK* and *glnD* when the *amt* gene was present in multiple copies (Fig. 8C).

Discussion

Transcription of the *amt* gene encoding the high-affinity (methyl)ammonium uptake system of *C. glutamicum* is regulated by a specific repressor protein. The purified AmtR protein, heterologously synthesized in *E. coli*, was found to bind specifically to its target DNA, therefore no cofactors seem to be necessary for AmtR/DNA interaction. The signal for the release of AmtR from its target DNA, resulting in the start of transcription upon nitrogen starvation, remains unclear. Preliminary studies on the internal amino acid pool of the cells and feeding experiments seem to exclude low molecular weight metabolites such as glutamate, glutamine or 2-oxoglutarate (M. Jakoby, unpublished) and suggest a protein–protein interaction as the regulatory mechanism. A candidate for this type of regulation is P_{II}, the central signal transduction protein for the regulation of nitrogen metabolism in *C. glutamicum* (Jakoby *et al*., 1999; L. Nolden, unpublished). In future studies, we will address this possible interaction.

A recognition motif of AmtR, $ATCTATAGN_{1-4}ATAG$ (Table 1), was identified by deletion analyses and by gel retardation assays. In addition to this *in vitro* approach, binding of AmtR to its target DNA was also shown *in vivo* using a *S. cerevisiae* one-hybrid system. Future studies will show which is the minimal consensus sequence in this motif, e.g. the first two bases, A and T, may be dispensable although these nucleotides are conserved throughout the motifs found. The complex pattern of AmtR binding to the amt_1 and amt_2 motif resulting in three shifted bands hints at a co-operative DNA–protein interaction, which might ensure a tight regulation of *amt* transcription. This idea is supported by the partially released repression when amt_1 is deleted.

The AmtR binding motif was found not only upstream of the *amt* gene but also upstream of *amtB*, an *amt* paralogue with so far unknown function. Interestingly, *amtB* is organized in an operon together with *glnK* (formerly designated *glnB*) and *glnD.* Although the *glnK* gene encodes the central signal transduction protein P_{II}, *glnD* is coding for the uridylyltransferase/uridylyl-removing enzyme (Jakoby *et al*., 1999). As P_{II} and the uridylyltransferase/uridylyl-removing enzyme are involved in the nitrogen regulatory cascade, indirectly AmtR also controls glutamine synthetase activity. No AmtR binding sites were observed upstream of other *C. glutamicum* genes known to be involved in nitrogen assimilation and regulation, i.e. *gdh* and *glnA* encoding glutamate dehydrogenase and glutamine synthetase (Börmann *et al*., 1992; Jakoby *et al*., 1997). Also upstream of the *amtR* gene, no AmtR binding could be detected, excluding an autoregulation of *amtR* transcription by the AmtR repressor, as shown in the case of *B. subtilis* transcriptional regulators GlnR and TnrA (for a review, see Fisher, 1999). Northern blot hybridization and RT-PCR experiments indicated that *amtR* is constitutively expressed in *C. glutamicum*.

To our knowledge, this is the first observation of a repressor protein controlling ammonium uptake in bacteria. In *E. coli*, an activation of transcription of the *glnK–amtB* operon depending on the Ntr system was shown. Activation of *amtB* transcription by the NtrB/C two-component system was shown for *E. coli* (van Heeswijk *et al*., 1996; Atkinson and Ninfa, 1998), *Azospirillum brasiliense* (Van Dommelen *et al*., 1998) and *Azorhizobium caulinodans* (Michel-Reydellet *et al*., 1998). Consistent with an NtrC-independent regulation of *amt* transcription in *C. glutamicum*, no consensus motif of σ^{54} promoter $TGGCAN_6GTGCTT$ (Beynon *et al*., 1983) was found upstream of *amt*. Activation of a gene encoding a (methyl)ammonium uptake system, namely *amt1*, was also described for *Synechocystis* sp. PCC 6803 (Montesinos *et al*., 1998). In this organism, transcription is

activated by a specific nitrogen control transcription factor, NtcA. In *B. subtilis*, the Gram-positive model organism, regulation does not depend on an Ntr-like system but on different regulatory proteins (for a review, see Fisher, 1999). In this bacterium, transcription of *nrgA*, encoding a putative ammonium uptake system, and *nrgB*, encoding a P_{II}-type protein, depends on activation *via* the TnrA protein (Wray *et al*., 1994, 1998).

In summary, we find a unique mixture of different regulatory modules for nitrogen control in *C. glutamicum*. The data obtained for the transcriptional control of *amt*, *amtB* and *glnK* differ from the situation found in other bacteria, including *B. subtilis.* However, repression in general is common in nitrogen control in this Gram-positive model bacterium. Regulation of glutamine synthetase activity via adenylylation and a cascade of uridylylation/deuridylylation of P_{II} via uridylyltransferase/uridylyl-removing enzyme shown in *C. glutamicum* (Jakoby *et al*., 1997, 1999) is similar to the enterobacterial activity regulation. The new type of nitrogen control found in *C. glutamicum* supports Fisher (1999), who stated that beside the paradigm of nitrogen regulation, i.e. the Ntr system in enterobacteria, a number of different mechanisms might be realized in other organisms.

Experimental procedures

Strains and growth conditions

Strains and plasmids used in this study are listed in Tables 2 and 3. Bacteria were routinely grown in Luria–Bertani (LB) medium (Sambrook *et al*., 1989) at 30°C (*C. glutamicum*) or 37°C (*E. coli*). If appropriate, antibiotics were added in standard concentrations (Ausubel *et al*., 1987). LB medium for *C. glutamicum* strains was supplemented with 2% glucose (final concentration). To study the effects of nitrogen starvation under highly similar conditions, a standard inoculation scheme was applied. A fresh *C. glutamicum* culture in LB medium was used to inoculate minimal medium (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 0.5, and cells were grown 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 resuspended 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). 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). Transcriptional start sites were determined using the 5′-RACE system, as recommended by the supplier (Gibco BRL).

RNA preparation and Northern blot hybridization analysis

Total RNA was prepared after disruption of the *C. glutamicum* cells by glass beads using the RNeasy Mini Kit, as recommended by the supplier (Qiagen). The RNA was either size fractionated using agarose gels containing formaldehyde and blotted onto positively charged nylon membranes (Sambrook *et al*., 1989) or spotted directly onto nylon membranes using a Schleicher and Schuell Minifold I Dot Blotter. Hybridization of digoxigenin-labelled RNA probes was detected with a Fuji luminescent image analyser LAS1000 or Kodak X-OMAT X-ray films using alkaline phosphatase-conjugated anti-digoxigenin Fab fragments and CSPD as light-emitting substrate, as recommended by the supplier (Roche Diagnostics). For RT-PCR, the Enhanced Avian RT-PCR Kit was used as described by the manufacturer (Sigma).

Digoxigenin-labelling of DNA and gel retardation experiments

Digoxigenin-labelled DNA fragments were prepared using digoxigenin-labelled primers obtained from MWG for PCR reactions. Bands were detected with a Fuji luminescent

Table 2. Strains used in this study.

Strains	Relevant genotype/description	Reference
C. glutamicum		
ATCC 13032	Wild type	Abe *et al*. (1967)
Δ268/283	Wild type carrying a chromosomal deletion of the amt_2 motif	This study
Δ304/320	Wild type carrying a chromosomal deletion of the amt_1 motif	This study
MJ6-18	Δ*amtR*	This study
E. coli		
BL21	F^- *ompT gal [dcm] [lon]* $hsdS_B$ (r_B^- m_B^-)	Studier *et al*. (1990)
DH5α*mcr*	*supE44 hsdR17 recA1 endA1 gyrA96 thi1 relA mcrA* Δ(*mrr-hsdRMS-mcrBC*)	Grant *et al*. (1990)
S17-1	*thi-1 endA1 hsdR17 supE44* λ^- *pro*	Simon *et al*. (1983)
S. cerevisiae		
YLN1	YM4271carrying plasmid pHISi-1Pamt integrated in the *HIS3* locus	This study
YM4271	*MATa ura3-52 his3-200 ade2-101 lys2-801 leu2-3112 trp1-901 tyr1-501 gal4Δ512 gal80Δ538 ade5::hisG*	Wilson *et al*. (1991); Liu and Herskowitz (1993)

Table 3. Plasmids used in this study.

Plasmids	Description	Reference
pAmtR	pUC18 carrying the *amtR* gene for cloning in pGAD424	This study
pGAD53m	7.8 kb positive control plasmid, contains mouse *p53* gene in frame with the GAL4 activation domain gene	Clontech Laboratories
pGAD424	6.6 kb negative control plasmid, for expressing the GAL4 activation domain gene	Clontech Laboratories
pGAD424amtR	pGAD424 carrying the *amtR* gene	This study
pGEM3+	*E. coli* plasmid for *in vitro* transcription, Ap^r	Promega
pGEMamt	0.5 kb internal *amt* fragment in pGEM3+	This study
pGEMamtP	1.0 kb internal *amtB* fragment in pGEM3+	This study
pGEMglnB	0.3 kb internal *glnK* fragment in pGEM3+	This study
pGEMglnD	0.7 kb internal *glnD* fragment in pGEM3+	This study
pHISi-1	5.4 kb vector for integration at the *HIS3* locus, carries the *HIS3* reporter gene	Clontech Laboratories
pHISi-1Pamt	plasmid pHISi-1 carrying the AmtR target motif	This study
pJC1	$ori_{E.\ c.}$ $ori_{C.\ g.}$, *E. coli–C. glutamicum* shuttle vector, Km^r	Cremer *et al.* (1990)
pJCAmtHII	*amt* in pJC1	This study
pJCdlz	*pamt–lacZ* fusion in pJC1	This study
pJL28	pBR322 carrying *lacZY'*	G. Unden (personal communication)
pK19Δ268/283	pK19*mobsacB* derivative which carries a 600 bp *amt* upstream fragment with an amt_2 deletion	This study
pK19Δ304/320	pK19*mobsacB* derivative which carries a 600 bp *amt* upstream fragment with an amt_1 deletion	This study
pK19ΔamtR	pK19*mobsacB* carrying a 1.5 kb DNA fragment from pUC11-1.7 used for *amtR* deletion	This study
pK19*mobsacB*	*ori* pUC, Km^r, *mob sacB*	Schäfer *et al.* (1994)
pKdlz	pK19*mobsacB* carrying a *pamt–lacZ* fusion	This study
pMal-c2	*malE*, Ap^r	Guan *et al.* (1987)
pMamtR	*amtR* in pMal-c2	This study
pUC11-1.7	pUC11-1.8, carrying an 0.3 kb deletion in the *amtR* gene	This study
pUC11-1.8	1.8 kb *Bam*HI fragment of *C. glutamicum* chromosomal DNA carrying *yjcc amtR citE'* ligated to pUC19	This study
pUC18/19	*plac*, Ap^r	Yanisch-Perron *et al.* (1985)
pUCΔ268/283	pUCP carrying an amt_2 deletion	This study
pUCΔ304/320	pUCP carrying an amt_1 deletion	This study
pUCamt1/2	pUC18 which carries the amt_1 and amt_2 binding motif (bp −120 to −26 of the *amt* upstream region)	This study
pUCamtR	pUC18 with *amtR* gene for cloning in pMal-c2	This study
pUCdlz	pUC18 carrying a *pamt–lacZ* fusion on a *Bam*HI fragment	This study
pUCdppc	pUC18 carrying a 3 kb *Bam*HI fragment of *C. glutamicum* chromosomal DNA including *'ppc secG amt ocd'*	Siewe *et al.*, 1996
pUCPamt	pUC18 carrying the *amt* 5′ region and 5′ part of *amt* (bp −364 to +276)	This study

Ap^r, resistance to ampicillin; Km^r, resistance to kanamycin.

image analyser LAS1000 or Kodak X-OMAT X-ray films using alkaline phosphatase-conjugated antidigoxigenin Fab fragments to probe the digoxigenin-labelled DNA and CSPD as light-emitting alkaline phosphatase substrate, as recommended by the supplier (Roche Diagnostics).

Construction of a C. glutamicum *gene bank*

Chromosomal DNA was isolated according to Eikmanns *et al.* (1994) and was partially digested with *Sau*3A. Resulting DNA fragments with a size of 2–5 kb were isolated and ligated to *Bam*HI-restricted and dephosphorylated pUC19 plasmid DNA.

Construction of pamt–lacZ *fusions*

A DNA fragment spanning 1.3 kb upstream of *amt* and its first five codons was fused to the *lacZ* gene from vector pJL28. For this purpose, plasmid pUCdppc was digested with *Ksp*I, treated with Klenow DNA Polymerase and restricted with *Bgl*II. The remaining plasmid DNA was ligated with a *Bam*HI–*Dra*I fragment of pJL28 carrying a start codon-deprived *lacZ* gene. From the resulting plasmid, pUCdlz, the *pamt–lacZ* fusion was isolated via *Bam*HI restriction and was cloned either into *Bam*HI-digested and dephosphorylated *E. coli–C. glutamicum* shuttle vector pJC1 to investigate the fusion when present in high copy number or into insertion vector pK19*mobsacB* to study its effect when present in a single chromosomal copy.

Construction of deletion mutants

A chromosomal deletion of the *amtR* gene was introduced in the *C. glutamicum* genome according to the protocol described by Schäfer *et al.* (1994), using plasmid pK19*mobsacB* for deletion in *C. glutamicum* and *E. coli* strain S17-1 for conjugation. For deletion, plasmid pUC11-1.8 carrying *amtR* and *citE'* on a 1.8 kb *Bam*HI fragment was digested with *Nru*I and *Dra*III, resulting in a loss of 0.3 kb within the *amtR* gene. After treatment with Klenow DNA polymerase to fill in DNA overhangs, the plasmid DNA was religated, leading to pUC11-1.7. A 1.5 kb *Bam*HI fragment carrying the flanking regions of *amtR* and the truncated *amtR* gene was isolated from this plasmid and was subsequently ligated to *Bam*HI-restricted

and dephosphorylated plasmid pK19*mobsacB*, leading to pK19ΔamtR, which was applied to introduce the deletion in *C. glutamicum* wild-type strain ATCC 13032. The resulting *amtR* deletion strain was designated MJ6-18.

The same method was also used to delete the amt_1 and amt_2 motif upstream of the *amt* gene. The *amt* upstream region and the 5′ part of *amt* (bp −364 to +276) was amplified via PCR and was ligated to pUC18 using the Sure Clone Ligation Kit (Amersham Pharmacia Biotech). The resulting plasmid, pUCPamt, was used as a template to generate defined deletions by PCR using primers that have a corresponding gap between their 5′ ends (Imai *et al*., 1991). After PCR with these primers, treatment with Klenow DNA polymerase to remove 3′ overhangs and phosphorylation, the amplified linear DNA was self-ligated, leading to plasmid pUCΔ304/320 in the case of a defined amt_1 deletion and to pUCΔ268/283 for the deletion of amt_2. These plasmids were digested with *Eco*RI and *Bam*HI and the resulting 0.6 kb DNA fragments were ligated to pK19*mobsacB*. The corresponding ligation products pK19Δ304/320 and pK19Δ268/283 were used for deletion of the corresponding motifs in *C. glutamicum* wild-type ATCC 13032.

Construction of a malE–amtR *fusion and purification of AmtR*

The maltose binding protein fusion technique was applied to purify the *C. glutamicum* AmtR protein. For this purpose, the *amtR* coding sequence was amplified via PCR using primers 5′-AAAAGTACTGCAGGAGCAGTGGGACGCCCC-3′ (introducing a *Sca*I site, underlined) and 5′-TCCTCTAGATTATTTCGCGTCAGCCTGCTT-3′ (introducing an *Xba*I site, underlined). The 0.7 kb PCR product was subcloned into plasmid pUC18, resulting in plasmid pUCamtR, and was sequenced for control. Subsequently, the *amtR* fragment was isolated after *Sca*I–*Xba*I restriction of pUCamtR and was ligated to the *Xmn*I–*Xba*I-resticted and dephosphorylated pMal-c2 vector downstream of the *malE* gene, so that the *malE′* and *′amtR* reading frames were properly aligned. The fusion protein encoded by this plasmid, pMamtR, carries an endoprotease Xa cleavage site directly in front of the AmtR protein; after cleavage by the protease, the recombinant AmtR carries a threonine residue instead of methionine at its N-terminus.

For the production of the maltose binding protein–AmtR fusion, strain BL21 was used as a host. LB medium containing 0.2% glucose and carbenicillin (25 μg ml^{-1}, final concentration) was inoculated with an overnight culture of strain BL21 pMamtR to an OD_{600} of approximately 0.05. When the exponential growth phase was reached (OD_{600} approx. 0.5), transcription of the *malE–amtR* fusion was induced by the addition of 300 μM IPTG. After 4 h, cells were harvested by centrifugation, resuspended in buffer (20 mM tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM DTT) and frozen as recommended (New England Biolabs). After thawing, the cells were broken by French press treatment, cell debris was removed by centrifugation (0.5 h, 9000 *g*) and the lysate was bound to an amylose matrix. Purification steps were carried out as recommended by the supplier (New England Biolabs). Elution fractions were analysed by SDS–PAGE (Laemmli, 1970). To cleave the maltose binding protein–AmtR fusion, factor Xa (1 μg mg fusion protein) was added to fusion protein-containing fractions, samples were incubated overnight at 4°C and were concentrated via dialysis against buffer containing 20% PEG 20 000. Using a Superdex 75 column (Amersham Pharmacia Biotech), AmtR was separated from the maltose binding protein. The AmtR-containing eluate was desalted using a HiPrep 26/10 column (Amersham Pharmacia Biotech).

Construction of the yeast one-hybrid assay

The Matchmaker One-Hybrid-System (Clontech Laboratories) was used as recommended by the supplier. To integrate the AmtR binding motif in the genome of strain YM4271, a 100 bp DNA fragment carrying both AmtR binding sites upstream of *amt* (bp −120 to −20) was amplified by PCR and ligated to pUC18, digested with *Sma*I and dephosphorylated using the Sure Clone Ligation Kit (Amersham Pharmacia Biotech). After testing for the desired orientation, the resulting plasmid pUCamt1/2 was restricted with *Eco*RI–*Xba*I and the 100 bp target motif was introduced in *Eco*RI–*Xba*I-treated integration vector pHISi-1, leading to pHISi-1Pamt, which was integrated in the *HIS3* locus. The *amtR* gene, from start to stop codon, was amplified by PCR and was cloned into plasmid pUC18 (see above), leading to plasmid pAmtR. After restriction with *Eco*RI–*Bam*HI, the *amtR* gene was ligated to *Eco*RI–*Bam*HI-restricted and dephosphorylated plasmid pGAD424.

Enzyme activity measurements, transport assays and miscellaneous methods

β-Galactosidase activity was assayed as described by Miller (1972). Uptake of methylammonium was measured using a rapid filtration approach (Siewe *et al*., 1996). The protein contents of samples was determined according to Bradford (1976).

Nucleotide sequence accession number

The nucleotide sequence data reported were submitted to GenBank (EMBL) and assigned accession no. AJ133719.

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