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Nitrogen Control in *Corynebacterium glutamicum*: Proteins, Mechanisms, Signals

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Abstract In order to utilize different nitrogen sources and to survive in a situation of nitrogen limitation, microorganisms have developed sophisticated mechanisms to adapt their metabolism to a changing nitrogen supply. In this communication, the recent knowledge of nitrogen regulation in the amino acid producer *Corynebacterium glutamicum* is summarized. The core adaptations of *C. glutamicum* to nitrogen limitation on the level of transcription are controlled by the global regulator AmtR. Further components of the signal pathway are GlnK, a P_{II}-type signal transduction protein, and GlnD. Mechanisms involved in nitrogen control in *C. glutamicum* regulating gene expression and protein activity are repression of transcription, protein-complex formation, protein modification by adenylylation, change of intracellular localization, and proteolysis.

Key words: Ammonium, *Corynebacterium*, global regulation, nitrogen control, nitrogen metabolism, nitrogen regulation

Nearly all macromolecules in a bacterial cell, proteins, nucleic acids (RNA, DNA), and the murein sacculus, contain nitrogen. To provide an optimal supply of this macronutrient, prokaryotes have developed transport and assimilation systems for a variety of nitrogen sources. Furthermore, sophisticated control mechanisms ensure energy-efficient uptake and assimilation and allow an adequate response to situations of nitrogen limitation.

We are interested in nitrogen metabolism and nitrogen regulation in corynebacteria (for reviews, see [10–12, 62]). This group of mycolic acids-containing actinomycetes includes pathogens like *Corynebacterium diphtheriae*, *Corynebacterium jeikeium*, and *Corynebacterium urealyticum* as well as amino acid and nucleotide producers such as *Corynebacterium ammoniagenes*, *Corynebacterium efficiens*, and *Corynebacterium glutamicum*. *C. glutamicum* was isolated by Kinoshita and co-workers in a screening program for L-glutamate-producing bacteria from a soil sample collected at Ueno Zoo in Tokyo [34, 61] and subsequently used for the industrial production of amino acids. Today, large amounts of L-glutamate (more than 1,500,000 tons per year) and L-lysine (more than 560,000 tons per year) are produced by use of *C. glutamicum* strains, in addition to smaller amounts of L-alanine, L-isoleucine, and L-proline and in addition to different nucleotides [21, 36, 37].

Because of the industrial use of *C. glutamicum* for L-glutamate production, the ammonium assimilating, L-glutamate-generating glutamate dehydrogenase (GDH) was characterized already shortly after discovery of this bacterium in the 1960s [33, 44]. Later, research was extended to other enzymes and pathways. During the last five decades, numerous studies piled up enormous knowledge on amino acid biosynthesis pathways, carbon metabolism, and other topics of *C. glutamicum* physiology (for an overview, see [8]). With respect to nitrogen metabolism, detailed information on transport and assimilation of nitrogen sources as well as nitrogen regulation is available on a molecular level today [12]. Genes coding for ammonium, creatinine, glutamate, and urea uptake systems [3, 5, 35, 38, 50, 60] as well as enzymes for ammonium, creatinine, glutamate, and urea utilization [2, 7, 26, 41, 43, 48, 56, 57] have been identified and characterized in *C. glutamicum*. Furthermore, regulatory systems came into the focus of research in the last years, and crucial components for enzyme activity regulation as well as nitrogen-dependent transcription regulation were identified [4, 27, 28, 40, 42, 54]. In this communication, the recent knowledge on nitrogen control mechanisms in *C. glutamicum* is summarized.

NITROGEN CONTROL IN *C. GLUTAMICUM*

Nitrogen regulation systems are found in bacteria and archaea, and distinct components such as the ammonium

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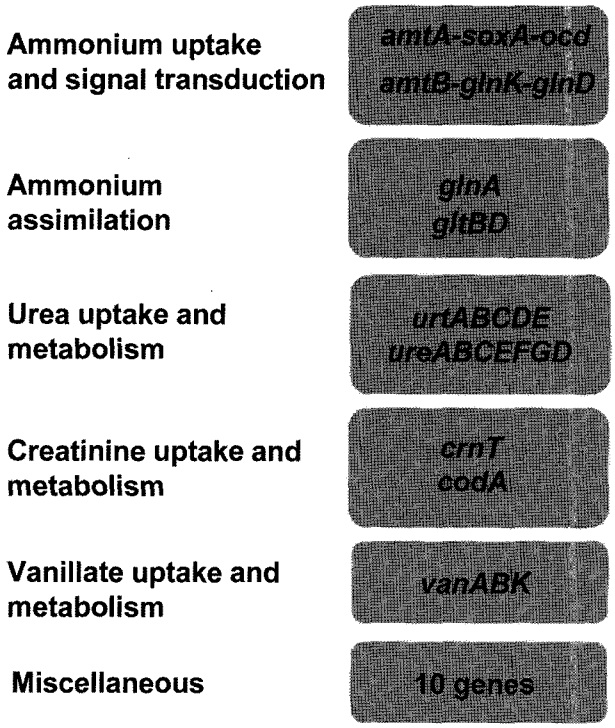

Fig. 1. AmtR-regulated cellular processes.
AmtR controls the core response of *C. glutamicum* to nitrogen limitation. The majority of genes under AmtR-control encode uptake and assimilation systems for different nitrogen sources besides regulatory proteins.

transporter AmtB and signal transduction protein GlnK are conserved in many prokaryotes [29, 30, 58]. However, despite common elements, nitrogen control in bacteria is rather diverse; for example, different regulatory mechanisms are realized in *Escherichia coli*, *Bacillus subtilis*, and in *Streptomyces* species (for recent reviews, see [1, 17, 18, 46]).

In *C. glutamicum*, the expression of genes in response to nitrogen limitation is governed by the TetR-type regulator AmtR, which blocks transcription of various genes during growth in nitrogen-rich medium (Fig. 1). The regulon of this master regulator was characterized recently by a combination of bioinformatics and molecular biology approaches [4]. Based on identified AmtR-binding sequences upstream of the AmtR-regulated genes *amtA* [28], *amtB* [28], *glnA* [41], and *gltB* [2], a computer-assisted genome screening for new AmtR targets was carried out. New AmtR-regulated genes identified by this bioinformatics approach were validated by RNA hybridization experiments, real-time reverse transcriptase PCR, and gel retardation assays [4]. In addition, transcriptome and proteome analyses of wild-type and *amtR* deletion strains were carried out using DNA microarrays. In summary, at least 33 genes are directly controlled by the AmtR repressor, which include genes encoding transporters and enzymes for ammonium assimilation (*amtA*, *amtB*, *glnA*, *gltBD*), creatinine (*codA*, *crnT*) and urea transport and metabolism (*urtABCDE*, *ureABCEFGD*), and a number of biochemically uncharacterized enzymes and transport systems as well as signal transduction proteins (*glnD*, *glnK*). For the *vanABK* operon, an indirect regulatory effect of AmtR was shown. The AmtR-controlled regulator of this gene cluster was not identified [39].

The global analysis of AmtR target genes not only allowed the definition of the AmtR regulon, but also the further characterization of the AmtR binding motif. By the use of bioinformatic tools, an AmtR box was defined (Fig. 2). Interestingly, several positions of this consensus sequence are rather variable, and the position of the AmtR binding motif as well as its numbers upstream of target genes is varying. It was suggested that these features most likely influence the different stringency of repression observed for the different AmtR-controlled genes [4].

INTERACTION OF THE AMTR REGULON AND OTHER REGULATION SYSTEMS

As described above, AmtR governs the core response of *C. glutamicum* to nitrogen starvation. However, when wild-type cells are challenged by nitrogen deprivation, a complex response occurs, which involves changes in the transcription of more than two-hundred genes [51–53]. As indicated by transcriptome analyses, a rearrangement of the cellular transport capacity, of amino acid and protein

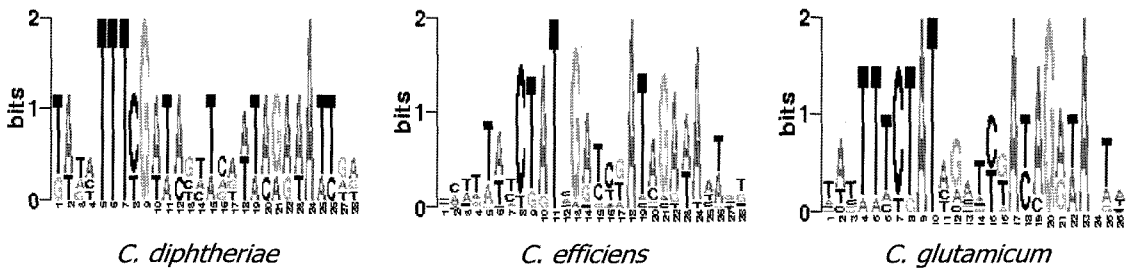

Fig. 2. AmtR binding motifs in different *Corynebacterium* species.
Depending on the number of putative AmtR-regulated genes, the AmtR box in *C. diphtheriae* was composed of four DNA sequences, whereas the boxes for *C. efficiens* and *C. glutamicum* were generated from twenty sequences (adapted from [62]).

synthesis as well as energy metabolism, is taking place under these conditions. Chemostat experiments, in which different growth rates were realized by ammonium limitation, indicated that different global networks for the regulation of growth, the carbon and energy metabolism are involved in this response [53]. The interaction of these global regulatory networks with nitrogen control is not understood today.

THE SIGNAL TRANSDUCTION PATHWAY CONTROLLING AMTR

AmtR is a member of the TetR-family of transcriptional regulators, which are widely distributed in bacteria and found especially often in actinomycetes [9, 45]. Typically, these proteins control transcription depending on the binding of small effector molecules; *e.g.*, antibiotics like tetracycline (for a review, see [45]). Interestingly, AmtR binding to promoter sequences of nitrogen-controlled genes is controlled by protein complex formation. AmtR is released from its target DNAs upon interaction with the trimeric complex of the signal transduction protein GlnK [27, 28, 42]. For this protein-protein interaction, it is essential that the GlnK trimer is present in its modified form [4, 54]. Modification of GlnK occurs at tyrosyl residue 51, located in the T-loop of GlnK, and is carried out by the GlnD protein [42, 54]. Because of its high homology to the *E. coli glnD* gene product, this enzyme was initially described as a uridylyltransferase [27, 42]. However, it was later shown that *C. glutamicum* GlnD functions as an adenylyltransferase [54]. Although not different from the biochemical mechanism, adenylylation as a posttranslational modification of GlnK is interesting from an evolutionary point of view. This kind of GlnK modification has been reported until now for *S. coelicolor* [22] and *C. glutamicum* [54] and might be widely distributed among actinomycetes.

The *C. glutamicum* GlnD protein is a bifunctional enzyme, which catalyzes adenylylation and deadenylylation of GlnK depending on the cellular nitrogen status. A *glnD* mutant allele, which codes for a truncated GlnD protein lacking the C-terminal half of the enzyme, is still active in GlnK modification. However, this truncated protein is unable to carry out the back reaction and to demodify GlnK [54]. Similar mutants were obtained in *Klebsiella pneumoniae* and *S. coelicolor* [20, 22]. Interestingly, deadenylylation of GlnK in response to an ammonium pulse after a starvation period is influenced by the ammonium transporter AmtB. In an *amtB* mutant strain, the GlnK protein was deadenylylated in response to ammonium addition approximately ten times faster than in the wild-type. The underlying regulatory mechanism of this phenomenon is unknown [54]. Somehow, the presence of AmtB has to be sensed by a component of the signal transduction cascade. An obvious and simple mechanism in this respect would be an interaction of AmtB and GlnD. This hypothesis is supported by the localization and operon organization of the corresponding genes, which are conserved among several mycolic acids-containing actinomycetes [11], since it was proposed that conservation of gene order might be a fingerprint of proteins that physically interact [15]. However, experimental evidence for an interaction of AmtB and GlnD is lacking.

Protein sequence analyses showed that the *C. glutamicum* GlnD protein lacks a ligand-binding domain typically found in other GlnD proteins [59]. This observation suggests that GlnD has no sensory properties and hints to the existence of an independent sensor of nitrogen supply in *C. glutamicum*. This idea is further supported by the fact that overexpression of *glnD* led to a deregulation of nitrogen control in *C. glutamicum* [42], a result that makes a function of GlnD as a sensor protein rather unlikely.

EFFECTOR MOLECULES AND PUTATIVE SENSOR PROTEINS

To react appropriately to changes in nitrogen availability, cells have to sense alterations in the concentration of nitrogen sources or marker metabolites, either outside or inside the cell. Since various nitrogen sources such as ammonium, amino acids, creatinine, peptides, and urea can be metabolized by *C. glutamicum* [12], sensing of every single nitrogen source would make several sensors necessary. Typical systems for the sensing of such environmental changes are bacterial two-component signal transduction systems. However, no influence of such systems on nitrogen regulation was detected when corresponding mutant strains were studied [12].

Alternatively, a limited number of key metabolites would be suitable to indicate the nitrogen supply of the cell. Possible candidates would be ammonium, 2-oxoglutarate, L-glutamate, and L-glutamine. L-Glutamate can be excluded to fulfil this function, since *C. glutamicum* accumulates up to 200 mM of this amino acid and the internal L-glutamate pool reacts only slowly to nitrogen starvation, whereas the response on the transcription level is much faster [40, 42]. Obviously, L-glutamate serves as a buffer for the nitrogen and/or carbon supply of the *C. glutamicum* cell, rather than reflecting the nitrogen status. The same seems to be true for L-glutamine, since the pool of this amino acid is also very high compared with other bacteria and reacts only slowly to changes in the nitrogen supply compared with the cellular response on the level of transcription [40, 42]. This is in contrast to the situation in *Salmonella typhimurium* [25] and *Klebsiella pneumoniae* [47]. These Gram-negative bacteria perceive external nitrogen limitation as a decrease of their internal glutamine pool. Also for *Bacillus subtilis*, it is proposed that the cell senses an initial drop in the glutamine pool [23]. However, in

contrast to the situation in enterobacteria, the glutamine pool in *B. subtilis* is already replenished during starvation, most likely by degradation of nitrogen-containing macromolecules.

Putative nitrogen markers identified by metabolite analyses in *C. glutamicum* are 2-oxoglutarate and ammonium [40, 42]. Deduced from studies in other organisms (for a review, see [1]), the most likely sensor protein of 2-oxoglutarate might be the signal transduction protein GlnK. However, direct biochemical evidence for nitrogen sensing by *C. glutamicum* is missing.

OTHER POSTTRANSCRIPTIONAL CONTROL MECHANISMS OF NITROGEN METABOLISM

As described above, regulation of transcription by AmtR is controlled by the GlnD-GlnK signal transduction pathway, which involves adenylylation and deadenylylation of GlnK. In addition to this pathway, further posttranslational control mechanisms were identified, which are involved in nitrogen regulation in *C. glutamicum*.

Membrane Sequestration, GlnK-AmtB Interaction, and Proteolysis of GlnK

Besides its modification status, the GlnK protein changes its intracellular localization in response to changes of the cellular nitrogen supply. While it is present in the cytoplasm during nitrogen starvation, the GlnK protein is sequestered to the cytoplasmic membrane in response to an ammonium pulse following a nitrogen starvation period. About 2 to 5% of the GlnK pool is located at the cytoplasmic membrane after ammonium addition. GlnK binding to the cytoplasmic membrane depends on the ammonium transporter AmtB, which is encoded in the same transcriptional unit as GlnK and GlnD, the *amtB-glnK-glnD* operon. In contrast, the structurally closely related methylammonium/ammonium permease AmtA does not bind GlnK [54]. Binding of GlnK proteins to AmtB ammonium permeases was also shown for other bacteria such as *Azotobacter vinelandii* [14], *B. subtilis* [16], and *E. coli* [14, 29].

Upon ammonium addition, the major amount of *C. glutamicum* GlnK is degraded. Proteolysis is GlnK-specific and cannot be observed for other proteins involved in nitrogen metabolism such as glutamine synthetase [54]. In contrast to the situation in *C. glutamicum*, in *E. coli* [6, 14] or *S. coelicolor* [22], GlnK seems to be stable for longer time periods. In *C. glutamicum*, only about 2 to 5% of the GlnK protein is protected from proteolysis by an unknown mechanism. Both proteolysis and protection are dependent on the presence of the ammonium permease AmtB, an observation that emphasizes the multiple functions of this protein.

The complex formation between AmtB and GlnK was interpreted as an ancient prokaryotic nitrogen control system [30], which was conserved during evolution. This would hint to a crucial role for the cell. In addition, the high redundancy of nitrogen control mechanisms hint to an essential function. However, in the laboratory, mutants of nitrogen control show no growth phenotype. Obviously, control seems to be very robust and might be more important in the soil, the natural habitat of *C. glutamicum*.

A small proteolytic modification of the N-terminal region of GlnK was reported for *S. coelicolor* [22]. In response to an ammonium pulse following a starvation period, three amino acids, methionine, lysine, and leucine, are cleaved off from the N-terminus of *S. coelicolor* GlnK. The function of this proteolytic processing is unclear [22, 46]. The three amino acid residues are conserved in *C. glutamicum* GlnK; however, cleavage was not observed here [54].

Posttranslational Regulation of Ammonium Assimilation

C. glutamicum assimilates ammonium almost exclusively *via* glutamate dehydrogenase (GDH) and the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. A significant contribution of other ammonium assimilating enzymes (for example, alanine dehydrogenase) was not observed, when *in vivo* flux measurements of ammonium assimilation were carried out [57]. When ammonium is present in high concentrations, it is primarily assimilated by GDH. During the reaction, GDH oxidizes one mol NADPH per mol ammonium assimilated. Since GDH has a low affinity to ammonium (K_m=3.08 mM [49]), at ammonium concentrations below approximately 5 mM, the glutamine synthetase/glutamate synthase (GS/GOGAT) system takes over [56], although at the cost of an extra mol of ATP per mol ammonium assimilated. Nevertheless, even under nitrogen surplus, GS must remain at least partially active to satisfy the cellular demand of glutamine. *In vivo* flux analyses revealed that without nitrogen limitation, 28% of the ammonium is assimilated *via* the GS reaction in *C. glutamicum* [57]. This is an unusually high fraction, and it was speculated that this situation reflects the higher glutamine demand of *C. glutamicum* for cell wall synthesis [57].

Because of the high energy demand, it is advantageous to downregulate GS when glutamine is present at sufficient concentrations, to prevent a waste of ATP. As described above, the GS/GOGAT pathway is significantly upregulated in response to nitrogen deprivation on the level of transcription (see above). Additionally, GS is posttranslationally modified to regulate enzyme activity. Nucleotide sequence analyses of the corynebacterial *glnA* gene, coding for glutamine synthetase, revealed a typical adenylylation site, a tyrosyl residue at amino acid position 405 [26]. Subsequently carried out biochemical analyses showed that GS activity is controlled by adenylylation/deadenylylation. A *glnA* allele encoding a phenylalanyl instead of a tyrosyl residue at position 405 shows wild-type GS activity but has lost posttranslational regulation and activity regulation [27]. The

gene encoding the bifunctional enzyme adenylyltransferase, *glnE*, has been identified during the systematic sequencing of the *C. glutamicum* genome and its function has been verified by deletion analysis [41]. In analogy to the signal transfer shown in *E. coli*, a signal relay *via* UTase, GlnK (see below), and ATase to GS was assumed [42]. However, later enzyme activity measurements in a *glnK* deletion strain showed that ATase works independently from GlnK in *C. glutamicum* [54], a situation that is similar in *S. coelicolor* [22].

CONSERVATION OF NITROGEN CONTROL IN CORYNEBACTERIA

The complete genome sequence has been determined and published for *C. diphtheriae* [13], *C. efficiens* [19], *C. glutamicum* [24, 32], and *C. jeikeium* [55] (for a global comparison of these genomes, see [31]). With respect to nitrogen control, the nosocomial pathogen *C. jeikeium* shows a loss of central regulatory proteins. Only the *glnK* gene can still be found, whereas GlnD- and AmtR-encoding genes are missing in this bacterium [9, 62]. In contrast to the situation in *C. jeikeium*, nitrogen control is intact in

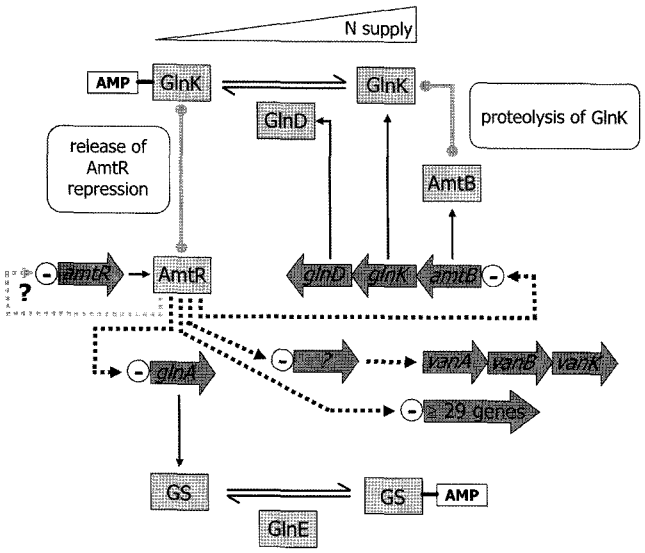

Fig. 3. Schematic representation of nitrogen control mechanisms in *C. glutamicum*.

Crucial components of nitrogen regulation are shown as green squares. Orange arrows, connected with AmtR by black dotted lines, represent AmtR-controlled genes. A putative autoregulation of AmtR based on the presence of an AmtR binding motif could not be shown (grey dotted line and question mark) [4]. The *vanABK* operon is regulated indirectly by AmtR, the direct regulator (arrow with question mark) has not been identified. Interaction (grey lines with circles) of GlnK~AMP and AmtR is crucial for release of AmtR repression [4], while interaction of GlnK with AmtB leads to its degradation [54]. Adenylylation/deadenylylation of GlnK and glutamine synthetase (GS) by GlnD and GlnE depends on nitrogen supply. The signals for modification/demodification are unkown.

C. diphtheriae and *C. efficiens* and especially the latter species shares various genes encoding proteins of metabolic pathways with *C. glutamicum* [62]. The AmtR protein is highly conserved compared with *C. glutamicum* AmtR in *C. efficiens* (86% identical amino acids) and *C. diphtheriae* (70% identical amino acids). When the *C. glutamicum* AmtR binding motif was used to screen the *C. diphtheriae*, *C. efficiens*, and *C. jeikeium* genomes for putative AmtR binding sites, a number of genes that are connected to nitrogen metabolism and that are nitrogen-regulated in *C. glutamicum* were identified in *C. diphtheriae* and *C. efficiens*, whereas no AmtR box was found in *C. jeikeium*. For *C. diphtheriae*, the genes identified include the *glnA2* and *gdh* genes, and for *C. efficiens*, the *glnA* gene and the *gltBD*, *amtB-glnK-glnD*, *amtA-ocd-soxA* and *ureABCEFGD* gene cluster. From the data obtained, species-specific AmtR binding motifs were generated (Fig. 2).

PERSPECTIVES

The most urgent question related to nitrogen control in *C. glutamicum*, which has to be answered to fully understand this global regulatory network (depicted in Fig. 3), is concerning the small effector molecule(s) indicating the cellular nitrogen status and the corresponding sensor protein(s). Global metabolome analyses might help to identify crucial nitrogen metabolites. For unequivocal identification, a set-up of *in vitro* systems will be necessary.

Other features of *C. glutamicum* nitrogen regulation also deserve attention. For example, a considerable number of different regulatory mechanisms have already been found to be involved in the control of nitrogen metabolism in this organism; *i.e.*, adenylylation/deadenylylation, change of intracellular localization, proteolysis, and repression of transcription. However, totally unexplored mechanisms are control of mRNA stability and small regulatory RNA molecules. It is very likely that these are used by *C. glutamicum*, and in fact, hints for a regulation of *amtA* mRNA stability were already observed (L. Nolden, H. Merkens, and A. Burkovski, unpublished).

Future studies will aim to understand the connection of different regulatory networks within the cell. For this purpose, a powerful combination of bioinformatics, transcriptome, and proteome analyses as well as flux and metabolome analyses will be necessary, leading towards a systems biology approach [63] and a holistic view on the *C. glutamicum* cell.

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