Nitrogen Metabolism and Nitrogen Control in Corynebacteria: Variations of a Common Theme

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Key Words

Ammonium • *Corynebacterium* • Glutamine synthetase • Nitrogen control • Nitrogen regulation

Abstract

The published genome sequences of *Corynebacterium diphtheriae*, *Corynebacterium efficiens*, *Corynebacterium glutamicum* and *Corynebacterium jeikeium* were screened for genes encoding central components of nitrogen source uptake, nitrogen assimilation and nitrogen control systems. Interestingly, the soil-living species *C. efficiens* and *C. glutamicum* exhibit a broader spectrum of genes for nitrogen transport and metabolism than the pathogenic species *C. diphtheriae* and *C. jeikeium*. The latter are characterized by gene decay and loss of functions like urea metabolism and nitrogen-dependent transcription control. The global regulator of nitrogen regulation AmtR and its DNA-binding motif are conserved in *C. diphtheriae*, *C. efficiens* and *C. glutamicum*, while in *C. jeikeium*, an AmtR-encoding gene as well as putative AmtR-binding motifs are missing.

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Introduction

Almost all of the macromolecules in a bacterial cell, e.g. proteins, nucleic acids and cell wall components, contain nitrogen. Thus, prokaryotes have developed elaborate mechanisms to provide an optimal nitrogen supply for metabolism and to overcome and survive situations of nitrogen starvation. This communication focuses on nitrogen metabolism in corynebacteria. The first member of the genus, *Corynebacterium diphtheriae*, was described at the end of the 19th century [Lehmann and Neumann, 1896] and today approximately 100 *Corynebacterium* species and subspecies have been identified (http://www.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=Corynebacterium&show_all_details=1).

The uptake and assimilation of nitrogen sources in corynebacteria has been investigated intensively for the amino acid producer *Corynebacterium glutamicum* and a considerable number of uptake systems for nitrogen sources as well as assimilatory pathways and regulatory proteins were identified and characterized (for reviews, see Burkovski [2003a, b, 2005]). Based on these functional data, we compared nitrogen assimilation and nitrogen control systems of four *Corynebacterium* species with published genome sequence, i.e. *C. diphtheriae* [Cerdeno-Tarraga et al., 2003], *C. efficiens* [Fudou et al., 2002], *C. glutamicum* [Ikeda and Nakagawa, 2003; Kalinowski et

Table 1. Corynebacterial genes and proteins involved in nitrogen uptake, assimilation and control

Gene	Function (in <i>C. glutamicum</i>)	Species ^a			
		Cdi	Cef	Cgl	Cje
<i>amtA</i>	ammonium uptake system [Siewe et al., 1996]	–	+	+	–
<i>amtB</i>	ammonium uptake system [Meier-Wagner et al., 2001]	–	+	+	+
<i>amtR</i>	transcription regulator [Jakoby et al., 2000] ^b	+	+	+	–
<i>codA</i>	creatinine deaminase [Bendt et al., 2004]	–	–	+	–
<i>crnT</i>	creatinine permease [Bendt et al., 2004]	–	–	+	–
<i>gdh</i>	glutamate dehydrogenase [Börmann et al., 1992]	+	+	+	+
<i>glnA</i>	glutamine synthetase, GS I-β type [Jakoby et al., 1997]	–	+	+	+
<i>glnA2</i>	glutamine synthetase, GS I-α type [Nolden et al., 2001b]	+	+	+	+
<i>glnD</i>	GlnK-regulating adenylyltransferase [Jakoby et al., 1999; Nolden et al., 2001b; Strösser et al., 2004] ^b	+	+	+	–
<i>glnE</i>	GS-regulating adenylyltransferase [Nolden et al., 2001b]	+	+	+	+
<i>glnK</i>	signal transduction protein [Jakoby et al., 1999; Nolden et al., 2001b]	+	+	+	+
<i>gltB</i>	glutamate synthase subunit [Beckers et al., 2001; Schulz et al., 2001]	–	+	+	+
<i>gltD</i>	glutamate synthase subunit [Beckers et al., 2001; Schulz et al., 2001]	–	+	+	+
<i>ureA</i>	urease subunit [Nolden et al., 2000; Puskás et al., 2000]	–	+	+	–
<i>ureB</i>	urease subunit [Nolden et al., 2000; Puskás et al., 2000]	–	+	+	–
<i>ureC</i>	urease subunit [Nolden et al., 2000; Puskás et al., 2000]	–	+	+	–
<i>ureD</i>	urease subunit [Nolden et al., 2000; Puskás et al., 2000]	–	+	+	–
<i>ureE</i>	urease subunit [Nolden et al., 2000; Puskás et al., 2000]	–	+	+	–
<i>ureF</i>	urease subunit [Nolden et al., 2000; Puskás et al., 2000]	–	+	+	–
<i>ureG</i>	urease subunit [Nolden et al., 2000; Puskás et al., 2000]	–	+	+	–
<i>ureR</i>	putative regulator of urease genes [Beckers et al., 2004]	–	–	+	–
<i>urtA</i>	subunit of urease uptake system [Beckers et al., 2004]	–	+	+	–
<i>urtB</i>	subunit of urease uptake system [Beckers et al., 2004]	–	+	+	–
<i>urtC</i>	subunit of urease uptake system [Beckers et al., 2004]	–	+	+	–
<i>urtD</i>	subunit of urease uptake system [Beckers et al., 2004]	–	+	+	–
<i>urtE</i>	subunit of urease uptake system [Beckers et al., 2004]	–	+	+	–

^a Cdi = *Corynebacterium diphtheriae*, Cef = *Corynebacterium efficiens*, Cgl = *Corynebacterium glutamicum*, Cje = *Corynebacterium jeikeium*.

^b Functional analysis in *C. diphtheriae* [Nolden et al., 2002].

^c Two copies present in the genome.

al., 2003] and *C. jeikeium* [Tauch et al., 2005] (for a global comparison of these genomes, see Kalinowski [2005]).

Results and Discussion

Genes Encoding Proteins Involved in Nitrogen Metabolism and Nitrogen Control in *Corynebacteria*

The available corynebacterial genome sequences at the NCBI genomes database (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>) were screened for genes involved in the uptake of nitrogen sources, their metabolism and nitrogen control. On the basis of components already characterized in *C. glutamicum*, the comparison of the differ-

ent species revealed that *C. glutamicum* and *C. efficiens* have a broader spectrum of genes encoding proteins involved in nitrogen metabolism than *C. diphtheriae* and *C. jeikeium*, species which are characterized by a loss of various genes (table 1). A *gdh* gene encoding glutamate dehydrogenase was annotated in all species. The same is true for *glnA2* encoding a GS I-α type glutamine synthetase. Interestingly, a typical high-affinity ammonium assimilation GS/GOGAT pathway consisting of GS I-β type glutamine synthetase and glutamate synthase (GOGAT) seems to be absent in *C. diphtheriae*. This is correlated with a reduced number of ammonium transport proteins in this organism. While two genes encoding ammonium transporters are present in *C. glutamicum* and *C. efficiens* (*amtA* and *amtB*), *C. jeikeium* has one of

Table 2. Corynebacterial genes and proteins with predicted functions in nitrogen uptake, nitrogen assimilation and nitrate/nitrate usage

Gene	Predicted function	Species ^a			
		Cdi	Cef	Cgl	Cje
<i>glsK</i>	glutaminase	–	+	+	–
<i>narI</i>	respiratory nitrate reductase γ chain	+	+	+	–
<i>narJ</i>	nitrate reductase δ chain	+	+	+	–
<i>narH</i>	respiratory nitrate reductase oxidoreductase	+	+	+	–
<i>narG</i>	nitrate reductase α subunit	+	+	+	–
<i>narK</i>	nitrate/nitrite transporter, nitrite extrusion protein	+	+	+	–
^c	ferredoxin-nitrite reductase	–	+	–	–
^c	nitrate transport ATP binding protein	–	+	–	–
^c	nitrate transport permease protein	–	+	–	–
^c	nitrate transport protein	–	+	–	–
^c	nitrate transport protein	–	+	–	–
^c	urea carboxylase	–	+	–	–

^a Cdi = *Corynebacterium diphtheriae*, Cef = *Corynebacterium efficiens*, Cgl = *Corynebacterium glutamicum*, Cje = *Corynebacterium jeikeium*.

^b Two copies present in the genome.

^c No gene designation given.

these genes (*amtB*) while no *amt* gene was annotated in *C. diphtheriae*. Urease-encoding genes were only annotated in *C. efficiens* and *C. glutamicum*. In these species also genes encoding a urea uptake system were found. Based on the genome data, the previously reported observation of urease activity in a non-virulent *C. diphtheriae* strain is doubtful [Nolden et al., 2002]. The *ureR* gene encoding a putative regulator of urease operon transcription was present in *C. glutamicum* only. However, an effect of a knock-out mutation was not observed [Beckers et al., 2004]. The gene is missing in *C. efficiens*. The *crnT* gene, coding for a creatinine uptake system, and *codA*, encoding a creatinine deaminase, were only annotated in *C. glutamicum*; this nitrogen source seems to be exclusively metabolized by this species. Besides the mentioned uptake and assimilation proteins, the presence of a number of regulatory proteins can be predicted. A glutamine synthetase-regulating adenylyltransferase encoded by *glnE* and a P_{II}-type signal transduction protein encoded by the *glnK* gene was observed in all organisms. Interestingly, a gene encoding the master regulator of nitrogen-dependent transcription control, AmtR, and a *glnD* gene encoding the GlnK-modifying enzyme was found in all species besides *C. jeikeium*, where these seem to be absent.

Based on the annotations of putative proteins (table 2), the presence of a glutaminase enzyme (encoded by *glsK*) can be predicted in *C. efficiens* and *C. glutamicum*. Besides the urease, two urea carboxylases are annotated for

C. efficiens in addition to a second glutamate dehydrogenase-encoding gene. Furthermore, the annotation of the *narJIHG* gene cluster indicates that *C. diphtheriae*, *C. efficiens* as well as *C. glutamicum* might have the potential to use nitrate reduction for energy generation. However, the annotation of various genes for nitrate uptake systems might indicate that *C. efficiens* is best equipped for anaerobic nitrate and nitrite respiration, while transport might be limiting in *C. glutamicum*.

Conservation of AmtR-Binding Motifs

AmtR was identified as the master regulator of nitrogen control in *C. glutamicum* and its binding sites were characterized upstream of genes transcribed in response to nitrogen starvation [Beckers et al., 2005]. The AmtR protein is highly conserved in *C. efficiens* (86% identical amino acids) and *C. diphtheriae* (70% identical amino acids), while it is absent in *C. jeikeium* [Brune et al., 2005]. We used the *C. glutamicum* AmtR-binding motif to screen the *C. diphtheriae*, *C. efficiens* and *C. jeikeium* genome for putative AmtR-binding sites. In fact, a number of genes which are connected to nitrogen metabolism and which are nitrogen-regulated in *C. glutamicum* were identified in the AmtR-exhibiting strains *C. diphtheriae* and *C. efficiens* by this approach. For *C. diphtheriae*, these include the *glnA2* and *gdh* gene, for *C. efficiens*, the *glnA* gene and the *gltBD*, *amtB-glnK-glnD*, *amtA-ocd-sox*A and *ureABCEFGD* gene cluster. From the data obtained, species-specific AmtR-binding motifs were generated, which

Fig. 1. AmtR-binding motifs for *C. diphtheriae* (A), *C. efficiens* (B) and *C. glutamicum* (C) represented by sequence logo plots. Depending on the number of putative AmtR-regulated genes, the AmtR box in *C. diphtheriae* was composed of 4 DNA sequences, while the boxes for *C. efficiens* and *C. glutamicum* were generated from 20 sequences.

show some variations compared to the *C. glutamicum* AmtR box (fig. 1). In contrast, searches in *C. jeikeium* gave no significant results (data not shown), which is in accord with the absence of an *amtR* gene in this bacterium.

Analysis of Nitrogen-Dependent Transcription

From the genome data, predictions about nitrogen control in the different species were possible. It was already shown that nitrogen control in *C. diphtheriae* is regulated by an AmtR-dependent mechanism as found in *C. glutamicum* [Nolden et al., 2002]. While AmtR is conserved in *C. diphtheriae*, *C. efficiens* and *C. glutamicum*, the lack of an *amtR* gene homolog as well as the lack of a *glnD* gene, which was shown to be essential for nitrogen signal transduction in *C. glutamicum* [Nolden et al., 2001a; Strösser et al., 2004] gave rise to the idea that *C.*

Fig. 2. Transcription of genes encoding proteins involved in ammonium uptake and assimilation in *C. jeikeium*. RNA was isolated from cells grown in BYT medium and from cells incubated in nitrogen-free minimal medium. Samples were hybridized with the indicated digoxigenin-labeled antisense probes. Since the *amtB* hybridization revealed very low signal intensity, exposition of the X-ray film was carried out overnight. Dot blots of all other probes were exposed for 5–15 min.

jeikeium does not have a nitrogen control system. To test this hypothesis, RNA hybridization experiments were carried out. *C. jeikeium* cells were grown in nitrogen-rich BYT medium, samples for RNA preparation were taken and remaining cells were transferred to prewarmed *C. glutamicum* minimal medium without nitrogen source as described in a previous publication for *C. diphtheriae* [Nolden et al., 2002]. After 15, 30 and 60 min of incubation, samples were taken. RNA preparations were hybridized with probes directed against *amtB*, *gdh*, *glnA*, *glnE*, *gltB* and *gltD* (fig. 2). These experiments indicated that *amtB* is expressed, although at very low level, up-regulation of transcription in response to nitrogen starvation was not observed. A significant up-regulation of expression in response to nitrogen deprivation was also not observed for *gdh*, *glnA* and *glnE*. Only *gltB* and *gltD* expression was strongly induced upon starvation. On the first glance, this result seems to hint to an AmtR-independent nitrogen control mechanism of *gltBD* transcription. However, further studies are necessary to examine whether

this is a specific effect of nitrogen deprivation or the result of general starvation or growth limitation response.

Analysis of Ammonium Uptake

In order to verify the genome annotations and especially total the absence of ammonium-transporter-encoding *amt* genes in *C. diphtheriae*, transport measurements were carried out. Uptake of the [^{14}C]-labeled ammonium analog methylammonium was determined for cells supplied with nitrogen and for nitrogen-deprived cells. To address the question of different substrate affinities, low (100 μM), medium (500 μM) and high (1,000 μM) methylammonium concentrations were used.

For *C. diphtheriae*, no methylammonium transport was detectable, which is in accordance with the absence of an ammonium transporter-encoding *amt* gene in this organism. Astonishingly, also *C. jeikeium* revealed no methylammonium uptake, although RNA hybridization experiments hint to an at least low-level expression of the ammonium transporter-encoding *amtB* gene (fig. 2). Either the *C. jeikeium* AmtB transporter is highly selective for ammonium and the substrate analog [^{14}C]-methylammonium is not recognized, or the protein is not synthesized in sufficient amounts or it is inactive. In *C. efficiens* and *C. glutamicum*, an identical pattern of methylammonium uptake was observed. Transport was not detectable, when cells were grown in nitrogen-rich BHI medium. This is in accord with the repression of *amtA* and *amtB* transcription by AmtR. In response to nitrogen starvation, the transporters are synthesized (for *C. glutamicum* data, see Meier-Wagner et al. [2001] and Beckers et al. [2005]) and uptake occurs. In general, the transporters of *C. efficiens* have a lower activity compared to those of *C. glutamicum*, and also their affinity seems to be lower. In the presence of 100 μM methylammonium a transport rate of $0.5 \pm 0.6 \text{ nmol min}^{-1} (\text{mg dry weight})^{-1}$ was determined for *C. efficiens* and $6.5 \pm 0.8 \text{ nmol min}^{-1} (\text{mg dry weight})^{-1}$ for *C. glutamicum*. In the two species, uptake rates increased with higher substrate concentration (500 and 1,000 μM) to 5.0 ± 2.2 and $6.9 \pm 2.3 \text{ nmol min}^{-1} (\text{mg dry weight})^{-1}$ in *C. efficiens* and to 9.3 ± 1.1 and $11.6 \pm 0.4 \text{ nmol min}^{-1} (\text{mg dry weight})^{-1}$ in *C. glutamicum*.

Regulation of Glutamine Synthetase Activity

As in the case of methylammonium transport, glutamine synthetase activity was determined for cells supplied with nitrogen and for nitrogen-deprived cells. For *C. diphtheriae*, no GS activity was detectable. This species has only a *glnA2* gene, which encodes a GSI α -type glu-

tamine synthetase, a situation comparable to a *C. glutamicum* *glnA* deletion strain. Also in *C. glutamicum*, GS activity was not observed in a *glnA2* background [Nolden et al., 2001b]. *C. efficiens* and *C. glutamicum* reveal an almost identical GS activity pattern. In nitrogen-rich medium, activity is low ($0.28 \pm 0.02 \text{ } \mu\text{mol (mg protein)}^{-1} \text{ min}^{-1}$ for *C. efficiens* and $0.07 \pm 0.04 \text{ } \mu\text{mol (mg protein)}^{-1} \text{ min}^{-1}$ for *C. glutamicum*), while upon nitrogen starvation activity increases significantly to $0.86 \pm 0.28 \text{ } \mu\text{mol (mg protein)}^{-1} \text{ min}^{-1}$ for *C. efficiens* and $0.67 \pm 0.10 \text{ } \mu\text{mol (mg protein)}^{-1} \text{ min}^{-1}$ for *C. glutamicum*. Regulation most likely occurs on the level of transcription, controlled by AmtR, and on the level of activity, regulated by modification/demodification of GS via the *glnE* gene product [Nolden et al., 2001b]. GS activity in *C. jeikeium* is low ($0.09 \pm 0.02 \text{ } \mu\text{mol (mg protein)}^{-1} \text{ min}^{-1}$) and under the experimental conditions used not regulated in response to the nitrogen supply.

Modification of GlnK

In *C. glutamicum*, signal transduction protein GlnK is crucial for nitrogen control [Nolden et al., 2001a]. It is synthesized in response to nitrogen limitation, immediately adenylylated and in this form competent to interact with AmtR and induce transcription of nitrogen-controlled genes. When nitrogen supply improves, GlnK~AMP is demodified to cease interaction with AmtR and to stop transcription of the AmtR regulon [Beckers et al., 2005]. Using *C. glutamicum* GlnK-specific antibodies, we tested GlnK synthesis and modification/demodification in *C. diphtheriae*, *C. efficiens* and *C. jeikeium*. Western blot experiments gave no immunoreaction with *C. diphtheriae* and *C. jeikeium* proteins (data not shown), either due to lack of cross-reactivity or of GlnK synthesis. For *C. efficiens* increasing synthesis of the protein upon starvation and demodification in response to ammonium addition was shown (fig. 3). Again, these results support the idea that nitrogen regulation is identical in *C. glutamicum* and *C. efficiens*.

Concluding Remarks

In this study, the available genomes sequences of corynebacteria were compared in respect to nitrogen metabolism and regulation. In general, nitrogen metabolism and control components are conserved among these species. However, specific differences were found. While the soil-living species *C. efficiens* and *C. glutamicum* have a broad set of genes coding for nitrogen source uptake systems, assimilatory enzymes and regulatory proteins, a clear tendency towards gene decay was observed for the

Fig. 3. Immunodetection of GlnK in *C. efficiens*. Cell extracts were prepared from cells grown in BHI medium (1), incubated for 1 h in nitrogen-free mineral salt medium (2) and after re-suspension of nitrogen-starved cells in BHI medium (3). Proteins were separated by SDS-PAGE, blotted and incubated with *C. glutamicum* GlnK-specific antiserum (M, marker proteins, from top to bottom: 24, 17 and 11 kDa).

pathogens *C. diphtheriae* and *C. jeikeium*. This includes ammonium and urea uptake systems, ammonium assimilation pathways, urea-cleaving enzymes and regulatory proteins like AmtR and GlnD. A similar tendency towards loss of genes in pathogenic bacteria was observed for mycobacteria. Especially *Mycobacterium leprae* shows a drastic gene reduction and decay compared for example to *Mycobacterium smegmatis* [Cole et al., 2001; Vissa and Brennan, 2001].

In *C. diphtheriae*, *C. efficiens* and *C. glutamicum* nitrogen-dependent gene expression seems to be controlled by the master regulator AmtR. Interestingly, not only the corresponding gene but also the DNA-binding motif of this global nitrogen regulator seems to be conserved during evolution. A similar result was obtained for the DtxR protein, which acts as a transcriptional regulator of iron metabolism in *C. glutamicum*. With the characterization of the DtxR box and analysis of DtxR-regulated genes and operons in *C. glutamicum* [Brune et al., 2006; Wennerhold and Bott, 2006], it was possible to predict corresponding binding motifs and regulons in *C. diphtheriae*, *C. efficiens* and *C. jeikeium* [Brune et al., 2006].

In summary, the screening of genomes for specific genes and transcriptional regulator-binding sites can give valuable information about physiology and uncharacterized metabolic pathways in different bacterial species.

Experimental Procedures

Bacterial Strains and Growth

C. diphtheriae DSM44123, *C. efficiens* YS-314 [Fudou et al., 2002] and *C. glutamicum* ATCC13032 [Abe et al., 1967] were cultivated in Brain Heart Infusion (BHI) while *C. jeikeium* K411 was grown in BYT medium [Tauch et al., 2004]. To induce nitrogen starvation, cells were harvested by centrifugation and re-suspended in CgXII minimal medium [Keilhauer et al., 1993], which was used without addition of ammonium and urea.

Methylammonium Uptake

Methylammonium uptake was measured as described [Siewe et al., 1996]. In brief, cells grown to the mid-log phase were harvested by centrifugation, washed with MES/Tris buffer (adjusted with NaOH to pH 8.0), and suspended in the same buffer to a final OD₆₀₀ of 3–4. Uptake measurements were started by the addition of 100, 500 and 1,000 μ M [¹⁴C]-labeled methylammonium. Samples were taken in 15-sec intervals over a period of 2 min. Cells were separated from the surrounding medium by rapid filtration, washed twice, and subjected to scintillation counting. Experiments were carried out at least in duplicate and transport rates (mean $\pm$ error) were calculated.

Glutamine Synthetase Activity

GS activities (mean $\pm$ SD) were determined as described [Jakoby et al., 1997] from three independent experiments.

SDS-PAGE and Western Blotting

Corynebacterium cells were disrupted using glass beads and a Q-BIOgene FastPrep FP120 instrument (Q-BIOgene, Heidelberg, Germany). Proteinase inhibitor (Complete, Roche, Basel, Switzerland) was added as recommended by the supplier. Protein concentrations were determined using a modified Lowry method [Dulley and Grieve, 1975]. SDS-PAGE was carried out as described [Schägger and von Jagow, 1987]. Gel-separated proteins (30 μ g/lane) were transferred onto a polyvinylidene difluoride membrane by electroblotting and incubated with *C. glutamicum* GlnK-specific antiserum. Antibody binding was visualized by using anti-antibodies coupled to alkaline phosphatase and the BCIP/NBT alkaline phosphatase substrate.

RNA Hybridization Experiments

Probes for the analysis of *C. jeikeium* gene transcription were generated by PCR and subsequent labeling with DIG RNA-labeling mix and T7 polymerase (Roche, Mannheim, Germany). Oligonucleotide primers for the amplification of 0.3-kb fragments (*amtB*: 5'-CCTCCGGAATTCGGCTTGG-3'/5'-GGGCCCTA-ATACGACTCACTATAGGGGCCGATGTCGATGGCGTTGG-3', *gdh*: 5'-CAGCGCAACGCGGGGAG-3'/5'-GGGCCCTA-ATACGACTCACTATAGGGCTGGCAGTGTGAAGGGCTTG-3', *glnA*: 5'-CGTCCGTGCGCGGCTTCA-3'/5'-GGGCCCTA-ATACGACTCACTATAGGGGCCACCGATCGGCAGGCCG-3', *glnE*: 5'-CGCGTTCCTCCGTGCCAAC-3'/5'-GGGCCCTAAT-ACGACTCACTATAGGGGCTGCTTCCACTCCGCCG-3', *gltB*: 5'-CGTGTGGTGTGCGCTTTGTG-3'/5'-GGGCCCTAATAC-GACTCACTATAGGGGGTTCAGGCCCTCTTCTCC-3', *gltD*: 5'-GGCTGATCCACGCGGATTTC-3'/5'-GGGCCCTAATAC-GACTCACTATAGGGCGGGTCATCGCCGATACCC-3'), chromosomal DNA as template and the following PCR program were

used: 3 min at 94°C, 30 cycles of 15 s at 94°C followed by 15 s at 60°C and 1 min at 72°C, 10 min at 72°C, cooling to 4°C.

For RNA preparation, bacteria were harvested by centrifugation, suspended in buffer (*NucleoSpin^hRNA II-Kit*, Macherey-Nagel, Düren, Germany) and immediately frozen in liquid nitrogen. Aliquots were thawed on ice and immediately disrupted using glass beads and a Q-BIOgene FastPrep FP120 instrument (Q-BIOgene, Heidelberg, Germany). After removal of cell debris, the RNA was isolated using the *NucleoSpin^hRNA II-Kit* following the recommendations of the supplier. The RNA was spotted onto nylon membranes using a Schleicher & Schuell (Dassel, Germany) Minifold I Dot Blotter. Hybridization of digoxigenin-labeled

RNA probes was detected using Kodak X-OMAT X-ray films, alkaline phosphatase-conjugated anti-digoxigenin Fab fragments and CSPD as light-emitting substrate (Roche, Germany).

Bioinformatics

AmtR-binding sites were searched for in the *C. diphtheriae* and *C. efficiens* genomes by using the *PredictRegulon* web service (<http://210.212.212.6/prindex.html> [Yellaboina et al., 2004]) with the known binding sites from *C. glutamicum* [Beckers et al., 2005] as input sequences. The sequence logo plots in figure 1 were made with the *SEQLOGO* web service (<http://ep.ebi.ac.uk/EP/SEQLOGO/>).

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