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Ammonium utilization in *Bacillus subtilis*: transport and regulatory functions of NrgA and NrgB

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Bacillus subtilis uses glutamine as the best source of nitrogen. In the absence of glutamine, alternative nitrogen sources such as ammonium can be used. Ammonium utilization involves the uptake of the gas or the ammonium ion, the synthesis of glutamine by the glutamine synthetase and the recycling of the glutamate by the glutamate synthase. In this work, ammonium transport in *B. subtilis* was studied. At high ammonium concentrations, a large fraction of the ammonium is present as ammonia, which may enter the cell via diffusion. In contrast, the ammonium transporter NrgA is required for ammonium utilization at low concentrations or at low pH values when the equilibrium between uncharged ammonia and the ammonium ion is shifted towards ammonium. Moreover, a functional NrgA is essential for the transport of the ammonium analogue methylammonium. NrgA is encoded in the *nrgAB* operon. The product of the second gene, NrgB, is a member of the PII family of regulatory proteins. In contrast to PII proteins from other organisms, there is no indication for a covalent modification of NrgB in response to the nitrogen supply of the cell. It is demonstrated here that NrgB is localized at the membrane, most likely in association with the ammonium transporter NrgA. The presence of a functional NrgB is required for full-level expression of the *nrgAB* operon in response to nitrogen limitation, suggesting that NrgB might relay the information on ammonium availability to downstream regulatory factors and thus fine-tune their activity.

INTRODUCTION

Nitrogen is a macronutrient for all known forms of life. However, in natural ecosystems the bioavailability of nitrogen is often a growth-limiting factor. Therefore, organisms have evolved highly effective systems for nitrogen acquisition and efficient utilization of scarce resources is ensured by a system of selective use of nitrogen sources. This allows nitrogen sources that can be accumulated at low cost to be preferred over those that require a lot of energy to be employed. This control allows optimal growth of the organisms and ensures a good standing of any given organism in competition in natural environments.

For most bacteria, glutamine is an optimal source of nitrogen (Hu *et al.*, 1999; Fisher & Débarbouillé, 2002). It can easily be converted to glutamate, the major donor of nitrogen for amino acid and nucleotide biosyntheses. Thus, several mechanisms are operating to prevent the expression and activity of pathways devoted to the utilization of secondary nitrogen sources in the presence of glutamine (for a recent review, see Burkovski, 2003). In enteric bacteria, the well-studied Ntr system controls both the biochemical activity of glutamine synthetase by adenylation/deadenylation and the expression of the corresponding gene by a two-component regulatory system, NtrB/NtrC (Merrick & Edwards, 1995). In Gram-positive bacteria, different mechanisms of nitrogen regulation are found. In the high-G+C Gram-positive bacteria *Corynebacterium glutamicum* and *Streptomyces coelicolor*, glutamine synthetase activity is also controlled by adenylation/deadenylation (Jakoby *et al.*, 1999; Fink *et al.*, 1999). Transcription of nitrogen-responsive genes is controlled by the AmtR and GlnR regulators, respectively, in these bacteria (Jakoby *et al.*, 2000; Wray *et al.*, 1991; Fink *et al.*, 2002). In the low-G+C Gram-positive bacterium *Bacillus subtilis*, the activity of glutamine synthetase is not modulated by modification. In this organism, synthesis of glutamine synthetase is regulated by the repressor GlnR (Schreier *et al.*, 1989). In addition, the TnrA regulator controls expression of several genes involved in the utilization of secondary nitrogen sources as well as *glnA* encoding glutamine synthetase (Wray *et al.*, 1996; Fisher & Débarbouillé, 2002). Interestingly, the activity of TnrA is modulated by reversible sequestration by glutamine synthetase itself (Wray *et al.*, 2001; Fisher *et al.*, 2002). In addition to glutamine synthetase, glutamate synthase encoded by the *gltAB* operon is essential for ammonium assimilation in *B. subtilis* (Fisher & Débarbouillé, 2002). Expression of this operon is repressed by TnrA in the absence of ammonium and activated by the regulatory protein GltC in response to an as yet unknown signal (Bohannon & Sonenshein, 1989; Belitsky *et al.*, 2000). Induction of the *gltAB* operon requires the presence of

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sugars that can be catabolized via glycolysis and this induction depends on the pleiotropic regulator of carbon metabolism, CcpA (Faires *et al.*, 1999; Blencke *et al.*, 2003; Wacker *et al.*, 2003).

An important component in signalling of nitrogen supply in both Gram-negative and Gram-positive bacteria is exemplified by the *Escherichia coli* PII protein, encoded by *glnB*. This small protein can be uridylated on a tyrosine residue under conditions of nitrogen limitation. The uridylated and non-uridylated forms of PII interact with other regulatory proteins that control either the activity of glutamine synthetase or the phosphorylation state of NtrC, thus relaying the information concerning the nitrogen status of the cell to these interaction partners (Merrick & Edwards, 1995; Arcondéguy *et al.*, 2001; Ninfa & Atkinson, 2000). In cyanobacteria, PII is modified by phosphorylation on a serine residue (Forchhammer & Tandeau de Marsac, 1994). A paralogous PII-like protein is present in *E. coli* and most other prokaryotes. This protein, GlnK, is usually encoded in an operon or gene cluster with an ammonium transporter, AmtB (Thomas *et al.*, 2000b). In *E. coli*, GlnK is also subject to modification by uridylation at low ammonium concentrations. At high ammonium concentrations, free GlnK binds to AmtB and prevents ammonium uptake by this transporter (Coutts *et al.*, 2002).

In *B. subtilis*, the *nrgAB* operon, encoding homologues of AmtB and GlnK, was identified in a search for genes that are induced by nitrogen limitation (Atkinson & Fisher, 1991). NrgA is a membrane protein, whereas NrgB is a member of the PII family (Wray *et al.*, 1994). In contrast to other PII proteins, NrgB does not possess one of the conserved uridylation or phosphorylation sites. Therefore, it is not known whether NrgB is covalently modified in response to the nitrogen source in *B. subtilis*. Moreover, there is no experimental evidence concerning the functions of the NrgA and NrgB proteins.

In this study, we analysed the functions of the two proteins encoded by the *nrgAB* operon. NrgA is required for the transport and utilization of ammonium at low concentrations. NrgB is localized in the membrane fraction of *B. subtilis* in an NrgA-dependent manner. Our data indicate that NrgB may be involved in the modulation of the activity of the transcription factor TnrA.

METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used in this study are listed in Table 1. *E. coli* DH5α and BL21(DE3) (Sambrook *et al.*, 1989) were used for cloning experiments and protein expression, respectively. *B. subtilis* was grown in Spizizen minimal medium (SMM) (Saxild & Nygaard, 1987) containing succinate (0·6 %, w/v) and glucose (0·4 %, w/v) as the carbon sources. Sources of nitrogen were added as indicated. Auxotrophic requirements were added to a final concentration of 50 mg l^{-1}. *E. coli* was grown in Luria–Bertani (LB) medium and transformants were selected on plates containing ampicillin (100 μg ml^{-1}). LB and sporulation (SP) plates were prepared by the addition of 17 g Bacto agar (Difco) per litre of medium.

DNA manipulation. Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures (Sambrook *et al.*, 1989). Restriction enzymes, T4 DNA ligase and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified from agarose gels using the Nucleospin extract kit (Macherey & Nagel). *Pfu* DNA polymerase was used for PCR as recommended by the manufacturer. DNA sequences were determined using the dideoxy chain-termination method (Sambrook *et al.*, 1989). Chromosomal DNA of *B. subtilis* was isolated as described by Kunst & Rapoport (1995).

Transformation and characterization of the phenotype. *B. subtilis* was transformed with plasmid DNA according to the two-step protocol (Kunst & Rapoport, 1995). Transformants were selected on SP plates containing kanamycin (5 μg ml^{-1}) or chloramphenicol (5 μg ml^{-1}). Quantitative assays of *lacZ* expression in *B. subtilis* were performed with cell extracts, using ONPG as the substrate (Kunst & Rapoport, 1995).

Construction of reporter strains. A translational *nrgA–lacZ* fusion was constructed using the vector pAC7 (Weinrauch *et al.*, 1991) which allows the introduction of the fusion into the *amyE* locus of *B. subtilis*. Briefly, the promoter region of the *nrgAB* operon was amplified by PCR using the oligonucleotides CD1 (5′-AAAGA ATTCT CCTTC CTTTC CATCC CTCG-3′) and CD2 (5′-CATAA AAACT GGATC CCCCA TTTGC AT-3′). The PCR product was digested with *Eco*RI and *Bam*HI and cloned into pAC7 linearized with the same enzymes. The resulting plasmid, pGP168, was linearized by *Pst*I and used to transform *B. subtilis* 168 to give strain GP250.

Table 1. *B. subtilis* strains used in this study

Strain	Genotype	Source*
168	*trpC2*	Laboratory collection
GP250	*trpC2 amyE*::(*nrgA–lacZ aphA3*)	pGP168→168
GP253	*trpC2 amyE*::(*nrgA–lacZ aphA3*) Δ*nrgB*::*cat*	pGP183→GP250
GP254	*trpC2 amyE*::(*nrgA–lacZ aphA3*) Δ*nrgA*::*cat*	pGP184→GP250
GP255	*trpC2 amyE*::(*nrgA–lacZ aphA3*) Δ*nrgAB*::*cat*	pGP185→GP250
GP256	*trpC2 amyE*::(*gltA–lacZ aphA3*) Δ*nrgB*::*cat*	pGP183→GP342
GP257	*trpC2 amyE*::(*gltA–lacZ aphA3*) Δ*nrgA*::*cat*	pGP184→GP342
GP342	*trpC2 amyE*::(*gltA–lacZ aphA3*)	Wacker *et al.* (2003)

*Arrows indicate construction by transformation.

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Deletions of the chromosomal copies of *nrgA* and *nrgB* were obtained by cloning of a *cat* resistance gene encoding a chloramphenicol acetyltransferase in place of the central regions of the respective genes. The *nrgA* deletion was constructed as follows. A fragment containing the chromosomal region upstream of *nrgA* and the first 15 codons of *nrgA* was amplified using the primers CD26 (5′-AAAGG ATCCC TCAGT GTATT ATTTG ATGTA GTAC-3′) and CD27 (5′-AAAGA ATTCG AGTAA AGCGC AAAAG AACAT AAA-3′), digested with *Eco*RI and *Bam*HI and cloned into pBluescript-II SK (Stratagene) cut with the same enzymes to yield plasmid pGP179v. Then, the 3′ part of *nrgA* was amplified using the primers CD28 (5′-AAAGA ATTCG GGCTT GACTT AACGA TGCAC GGGGA A-3′) and CD29 (5′-AAAAA GCTTT TTTAC CGTCA CCTGG TGATC CCG-3′), cut with *Eco*RI and *Hin*dIII and cloned into pGP179v linearized with the same enzymes. The resulting plasmid was pGP179. Finally, the *cat* gene was amplified using the primers CATFORWARD (5′-AAAGA ATTCA AAATT GGATA AAGTG GGATA TTTTT A-3′) and CATREV (5′-AAAGA ATTCC TATTA TAAAA GCCAG TCATT AGGCC T-3′) and plasmid pHV33 (Primrose & Ehrlich, 1981) as the template. The PCR product was cloned into the single *Eco*RI site of pGP179 to give plasmid pGP184. To construct the *nrgB* deletion, we first amplified the region upstream of *nrgB* using the primers CD30 (5′-AAAGG ATCCC TTTGG CCTGC ACGGG ATCGG CGGCA C-3′) and CD38 (5′-AAACA ATTGA CCGCT CATAG CGTCA CTCCT CAC-3′). This fragment was digested with *Mfe*I (introduced with CD38) and ligated with the *Eco*RI-digested *cat* cassette. The ligation mix was used for a second round of PCR using the primers CD30 and CATREV. The region downstream of *nrgB* was obtained with the primer pair CD32 (5′-AAACA ATTGG GACCT GAAGC ACTTT AATAT CGGTA C-3′) and CD34 (5′-CCATC GATCA TAGGC GACAA TGGCA TATTC GCTGA-3′). This fragment was cut with *Mfe*I (introduced with CD32) and was ligated to the *Eco*RI-digested CD30/CATREV fragment. Finally, we performed a last round of PCR using CD30 and CD34. The resulting fragment contained the *cat* cassette flanked by the 5′ and 3′ parts of *nrgB*. This fragment was cut with *Bam*HI and *Cla*I and cloned into pBluescript-II SK. The resulting plasmid was pGP183. The *nrgAB* operon deletion was constructed in a similar way. The upstream part of *nrgA* was amplified using the primers CD26 and CD37 (5′-AAACA ATTGG AGTAA AGCGC AAAAG AACAT AAA-3′). This fragment was digested with *Mfe*I and ligated with the *Eco*RI-digested *cat* fragment. The ligation product was amplified using CD26 and CATREV. The *nrgB* downstream fragment was amplified and ligated to the CD26/CATREV fragment as described above. The obtained ligation product was amplified using CD26 and CD34 and cloned between the *Bam*HI and *Cla*I sites of pBluescript-II SK to yield plasmid pGP185. To introduce the deletions into the chromosome, the plasmids were digested with *Sca*I and used to transform the appropriate *B. subtilis* strains (see Table 1).

Overexpression and purification of NrgB. To overexpress NrgB fused to a hexahistidine sequence at the C terminus, plasmid pGP182 was constructed as follows. A DNA fragment corresponding to the *nrgB* open reading frame was amplified by PCR using chromosomal DNA of *B. subtilis* 168 and the primer pair CD35 (5′-AAACA TATGA GCGGT CAAAT GTTCA AGGTA-3′)/CD36 (5′-AAAAT GCATA GATCC ACGCG GAACA AGTGC TTCAG GTCCT TC-3′). The PCR product was digested with *Nde*I and *Nsi*I, and the resulting fragment was cloned into the large fragment of the expression vector pGP807 (Schmalisch *et al.*, 2002) cut with the same enzymes. This expression system is based on the strong T7 promoter and a *lac* operator.

For overexpression of the recombinant NrgB, *E. coli* BL21(DE3) was transformed with pGP182 and cultivated in LB at 28 °C. Expression was induced by the addition of IPTG (final concentration 1 mM) to exponentially growing cultures (OD_{600} of 0·8). Cells were harvested 2 h after induction, resuspended in a phosphate buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH 6·5) and disrupted by using a French press (20 000 p.s.i., 138 000 kPa). The crude extract was passed over a Ni^{2+} HiTrap chelating column (Pharmacia) followed by elution with an imidazole gradient. The Bio-Rad dye-binding assay was used to determine protein concentration. BSA was used as the standard.

Preparation of membrane fractions. Cultures of *B. subtilis* were harvested by centrifugation (4400 ***g***, 10 min, 4 °C). The following steps were done as described by Coutts *et al.* (2002). Briefly, the cells were lysed by sonication, the cellular debris removed, and the fractions of the cell extract were separated by ultracentrifugation. The membrane pellet was washed three times and finally resuspended in phosphate buffer (50 mM Na_2HPO_4, 50 mM NaH_2PO_4, pH 6·8).

Western blot analysis. Purified NrgB–hexahistidine was used to generate rabbit polyclonal antibodies. For Western blot analysis, *B. subtilis* cell extracts and cellular fractions were separated on 12·5 % SDS-PAGE gels. After electrophoresis, the proteins were transferred to a PVDF membrane (Bio-Rad) by electroblotting. Antibodies were visualized by using anti-rabbit IgG–alkaline phosphatase secondary antibodies (Chemikon International) and the CDP* detection system (Roche Diagnostics). For control experiments, the presence of glyceraldehyde-3-phosphate dehydrogenase was assayed using polyclonal antibodies directed against *B. subtilis* GapA (Meinken *et al.*, 2003).

Assays of methylammonium transport. *B. subtilis* strains were grown in SMM in the presence of glutamine (0·2 %, w/v) as the single source of nitrogen. If the culture had reached an OD_{600} value of 0·9, the cells were centrifuged and resuspended in SMM without any source of nitrogen. Nitrogen starvation was exerted for 210 min and transport assays were performed as described previously (Ludwig *et al.*, 2002). Labelled [^{14}C]methylamine hydrochloride (56 mCi $mmol^{-1}$, 2·07 GBq $mmol^{-1}$) was mixed with non-labelled methylamine hydrochloride and added to the cells (final concentration 7·24 μM). Samples were treated as described previously (Ludwig *et al.*, 2002).

RESULTS

Growth properties of *B. subtilis nrgA* mutants

NrgA exhibits high similarity to ammonium transporters from all domains of life (Wray *et al.*, 1994). We asked therefore whether NrgA might be required for growth of *B. subtilis* in the presence of ammonium as the single source of nitrogen. To address this question, we constructed a set of strains in which the *nrgA* or *nrgB* genes or the complete *nrgAB* operon was deleted. Moreover, these strains contained a translational *nrgA–lacZ* fusion that allowed us to simultaneously monitor the expression of the *nrgAB* operon.

The strains were grown in minimal medium in the presence of ammonium. At a concentration of 2 mM ammonium, the wild-type and the Δ*nrgA* and Δ*nrgB* mutant strains grew with virtually identical rates and reached the same final optical density. Similarly, expression of the *nrgA–lacZ* fusion was induced at the onset of the stationary phase to a comparable extent (Fig. 1a). Obviously, neither NrgA nor NrgB played a significant role under the conditions used in this experiment. This may result from the fact that the ammonium supplied is present in the forms of uncharged ammonia (NH_3) and charged ammonium (NH_4^+) in the growth medium. The actual concentration of the two species depends on two factors: the pH of the culture and the total

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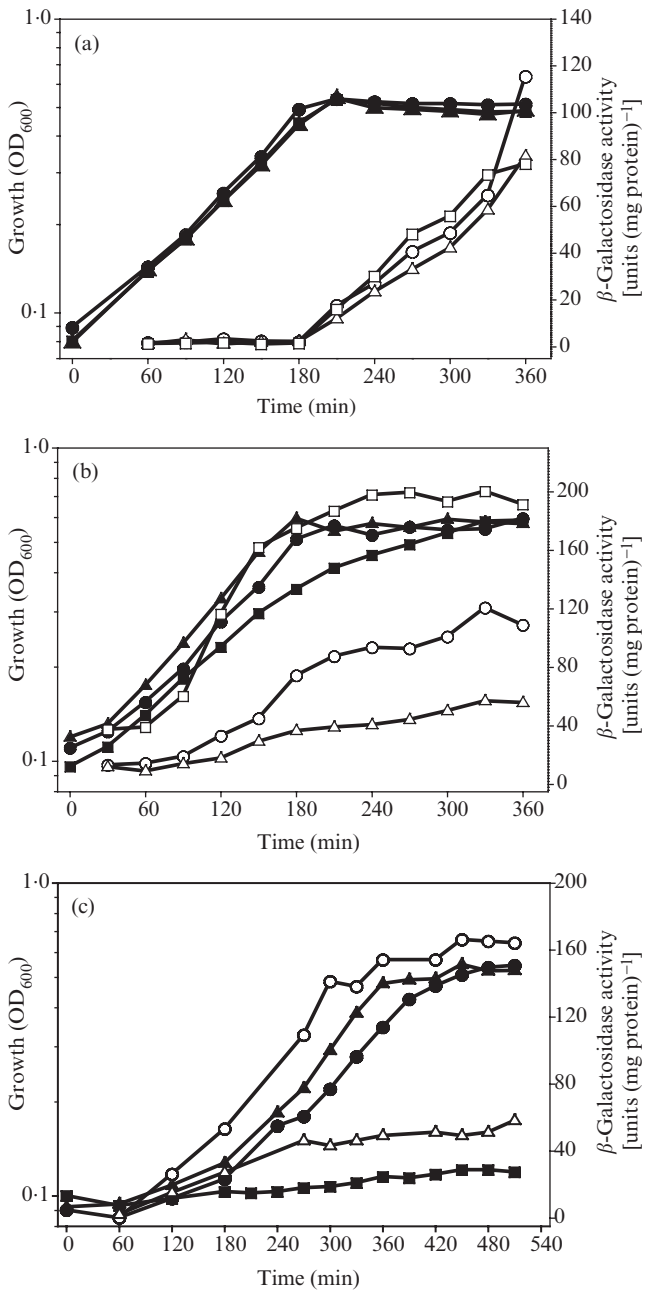

Fig. 1. Influence of the ammonium/ammonia equilibrium on growth and *nrgAB* expression. Growth of the wild-type (GP250) and the Δ*nrgA* (GP254) and Δ*nrgB* (GP253) mutant strains was monitored by measuring the optical density at 600 nm. In addition, expression of the *nrgA–lacZ* fusion was followed by removing samples for β-galactosidase assays at the times indicated. Cultures were grown at 37 °C under vigorous agitation in SMM containing 2 mM ammonium as the sole nitrogen source, at different pH values. Filled symbols indicate optical density of GP250 (●), GP253 (▲) and GP254 (■). Open symbols represent β-galactosidase activity of GP250 (○), GP253 (△) and GP254 (□). (a) Bacteria were grown in the presence of 2 mM ammonium at pH 6·9. (b) Bacteria were grown in the presence of 2 mM ammonium at pH 5·5. (c) Bacteria were grown in the presence of 2 mM ammonium at pH 5·0.

ammonium concentration added to the medium. The minimal medium is regularly buffered with phosphate to get a neutral pH value (pH 6·9). At this pH (and 2 mM total ammonium), the concentration of uncharged ammonia is about 11·2 μM. However, ammonia may freely diffuse through the membrane (Kleiner, 1985), thus circumventing the need for a functional ammonium transporter. We performed therefore further growth experiments at the same ammonium concentration (2 mM) and at acidic pH values at which substantially less uncharged ammonia is present. At a pH of 5·5 ($[NH_3]=350$ nM), both the wild-type strain GP250 and the Δ*nrgA* mutant GP254 were able to grow; however, the Δ*nrgA* mutant grew more slowly (generation time of 156 min vs 93 min for the wild-type strain, see Table 2). Thus, the availability of ammonium may be limiting for the Δ*nrgA* mutant. This assumption is further supported by the finding that the expression of the *nrgA–lacZ* fusion is much stronger in the mutant than in the wild-type strain (see Fig. 1b). At a pH value of 5·0 ($[NH_3]=$ 110 nM), the wild-type grew, although with an increased generation time (see Table 2). In contrast, the Δ*nrgA* mutant was not able to grow under these conditions (Fig. 1c). The same was true for the Δ*nrgAB* double mutant, which was phenotypically very similar to the Δ*nrgA* mutant (see Table 2).

The growth defect of the Δ*nrgA* mutant at pH 5·0 might have resulted from the low concentration of ammonia; however, alternatively, the mutant might be sensitive to the acidic conditions. To distinguish between these possibilities, we replaced the ammonium by glutamine in a medium adjusted to pH 5·0. Under these conditions, all strains grew at an identical rate (data not shown). Therefore, all strains are able to tolerate a pH of 5·0. We may therefore conclude that the observed phenotype of the Δ*nrgA* mutant results

Table 2. Growth rates of *B. subtilis nrgA*, *nrgB* and *nrgAB* mutant strains in response to the ammonium/ammonia equilibrium

Cultures of *B. subtilis* strains GP250 (wild-type), GP253 (Δ*nrgB*), GP254 (Δ*nrgA*) and GP255 (Δ*nrgAB*) were grown at 37 °C under vigorous agitation in SMM supplemented with 2 mM ammonium at the pH values indicated. Growth was monitored by measuring the optical density at 600 nm.

Relevant genotype		Generation time (min)*	
Strain	***trpC2 amyE::(nrg–lacZ aphA3)***	**pH 5·5**	**pH 5·0**
GP250	Wild-type	93 ± 10	143 ± 1
GP253	Δ*nrgB::cat*	86 ± 8	160 ± 17
GP254	Δ*nrgA::cat*	156 ± 6	NG†
GP255	Δ*nrgAB::cat*	148 ± 7	NG

*Generation times were determined from the growth of at least three independent cultures under each condition. Results are shown ± SD.
†NG, No growth.

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from the scarcity of diffusible ammonia. The NrgA protein might be required for the transport of ammonium into the cell.

Studies of ammonium transport in *B. subtilis*

Two lines of evidence suggested that NrgA might function as an ammonium transporter: first, it is a member of the Mep/Amt family of transporters (Thomas *et al.*, 2000a) and, second, our growth experiments indicated a need for a functional NrgA if ammonium is the single nitrogen source. To test the role of NrgA in ammonium transport, we assayed the uptake of a radioactively labelled ammonium analogue, [^{14}C]methylammonium.

The wild-type strain GP250 showed very weak methylammonium uptake after growth in a medium containing the preferred nitrogen source, glutamine. In contrast, the transport was strongly induced after the cultures were exposed to nitrogen starvation (see Fig. 2). This result is in good agreement with the previously reported repression of the *nrgAB* operon by glutamine (Atkinson & Fisher, 1991). The Δ*nrgA* mutant strain GP254 was drastically impaired in the transport of methylammonium even after nitrogen starvation. The ammonium transport of the Δ*nrgB* mutant strain GP253 was nearly identical to that of the wild-type strain. Similarly, the Δ*nrgAB* double mutant GP255 showed uptake rates of methylammonium indistinguishable from the Δ*nrgA* mutant (Fig. 2). Taken together, these results indicate that NrgA is required for the uptake of methylammonium and, therefore, probably also of ammonium. In contrast, NrgB, encoded by the second gene of the *nrgAB* operon, does not seem to be involved in ammonium transport under our experimental conditions.

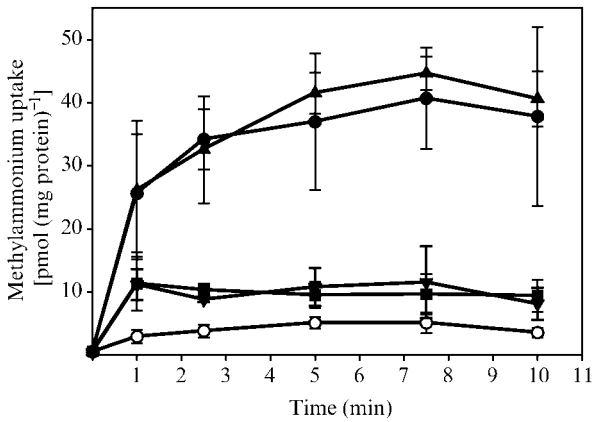

Fig. 2. Function of the gene products of the *nrgAB* operon in [^{14}C]methylammonium uptake. [^{14}C]Methylammonium transport activity of the wild-type (GP250, ●), Δ*nrgA* (GP254, ■), Δ*nrgB* (GP253, ▲) and Δ*nrgAB* (GP255, ▼) strains subjected to a 3·5 h period of nitrogen starvation compared to that of the wild-type strain grown in the presence of 0·2 % (w/v) glutamine (○). The final concentration of [^{14}C]methylammonium was 7·2 μM.

Localization of NrgB in the cytoplasmic membrane of *B. subtilis*

In *E. coli*, the GlnK protein is known to be associated with the cytoplasmic membrane. This association depends on the ammonium transporter AmtB. If uridylated, GlnK dissociates from the membrane (Coutts *et al.*, 2002). We wished therefore to test whether NrgB, the *B. subtilis* GlnK homologue, also bound to the cytoplasmic membrane in an NrgA-dependent manner.

First, we had to ascertain that *nrgB* was expressed in the Δ*nrgA* mutant strain GP254. For this purpose, cell extracts were prepared from the wild-type (GP250) and Δ*nrgA* (GP254), Δ*nrgB* (GP253) and Δ*nrgAB* (GP255) mutant strains after growth in SMM supplemented with glutamate or glutamine. The extracts were subjected to Western blot analysis using polyclonal antibodies directed against *B. subtilis* NrgB (Fig. 3). As expected, NrgB was detectable in the wild-type strain only after growth with the poor nitrogen source glutamate. No NrgB was detected in the Δ*nrgB* and Δ*nrgAB* mutant strains, indicating that the antibodies were specific. In the Δ*nrgA* mutant, NrgB was expressed under nitrogen-limited conditions. Thus, this mutant could be used to test the presumptive role of NrgA in membrane localization of NrgB.

To localize NrgB, the cell extracts were fractionated as

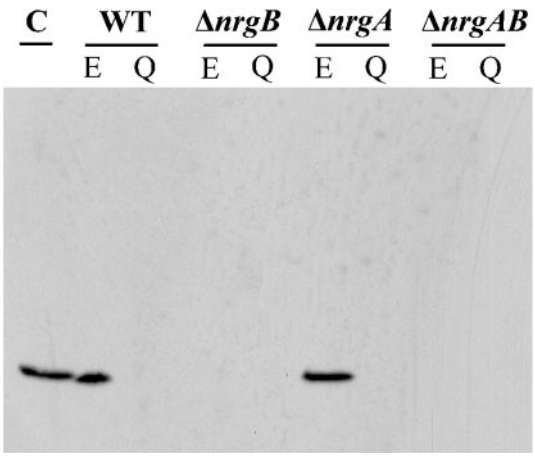

Fig. 3. Synthesis of NrgB in response to the nitrogen source. Crude extracts were prepared from cells grown in SMM containing 0·2 % (w/v) glutamate (E) or 0·2 % (w/v) glutamine (Q) as the sole nitrogen source, representing nitrogen-limited conditions or nitrogen excess, respectively. Samples were subjected to SDS-PAGE followed by Western blot analysis using an anti-NrgB antibody. The strains used are indicated above each lane: wild-type (WT; GP250), Δ*nrgB* (GP253), Δ*nrgA* (GP254), Δ*nrgAB* (GP255). The control lane (C) contains 150 ng of purified *B. subtilis* NrgB.

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described in Methods. The quality of the membrane fractions was tested by Western blot analysis using polyclonal antibodies against a key glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GapA) (Fig. 4). GapA was detected in the cytoplasmic but not in the membrane fractions, confirming that membrane preparations were essentially free of cytoplasmic proteins. In the wild-type strain, most NrgB was found to be associated with the membrane, both under conditions of nitrogen limitation and ammonium shock (Fig. 5a). Binding of NrgB to the membrane was quite tight as suggested by the fact that NrgB was present in the membrane fractions even after a washing step at a high salt concentration (sodium chloride, 600 mM) (data not shown). In contrast, in the Δ*nrgA* mutant strain GP254, no NrgB was present in the membrane fraction. The protein was detected exclusively in the cytoplasmic fraction (Fig. 5b). However, a Coomassie stain indicated that the membrane preparations of the wild-type and mutant strains contained similar amounts of protein (data not shown). Thus, NrgA is required for binding of NrgB to the membrane and we may suppose that the two proteins interact physically.

A function for NrgB in signal transduction

In contrast to its *E. coli* counterpart, NrgB seems not to be modified in response to the nitrogen supply. Moreover, the *B. subtilis* protein binds to the membrane both under nitrogen limitation and nitrogen excess. We asked therefore which function NrgB might fulfil. In the course of our growth experiments, we noticed that the expression of the *nrgAB* operon was threefold reduced in the Δ*nrgB* mutant strain at low ammonia concentrations (2 mM ammonium, pH 5·0) (see Fig. 1). The dependence of *nrgAB* operon expression on a functional NrgB was even more pronounced at lower concentrations of free uncharged ammonia (compare Fig. 1b, c). We may thus assume that NrgB is required for full induction of the *nrgAB* operon under conditions of ammonia limitation.

Next, we asked whether NrgB would be involved specifically in the control of the *nrgAB* operon or more generally in the regulation of nitrogen-controlled promoters. This was tested by studying the expression of the *gltAB* operon encoding glutamate synthase in the wild-type and Δ*nrgA* or Δ*nrgB* mutants. Expression of the *gltAB* operon is repressed by the transcription regulator TnrA, whereas transcription

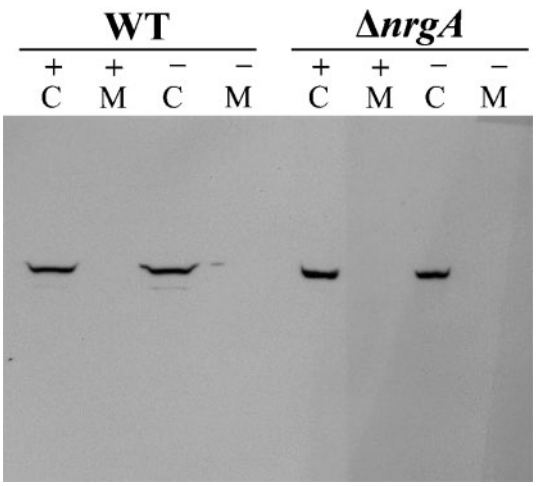

Fig. 4. Prepared membrane fractions are free of cytosolic proteins. Cytoplasmic (C) and membrane (M) fractions of the wild-type (WT; GP250) and Δ*nrgA* (GP254) strains were prepared from cells grown under nitrogen-limited conditions (−) and 30 min after (+) ammonia shock as described in Methods, and were subjected to SDS-PAGE followed by Western blot analysis using an anti-GapA antibody.

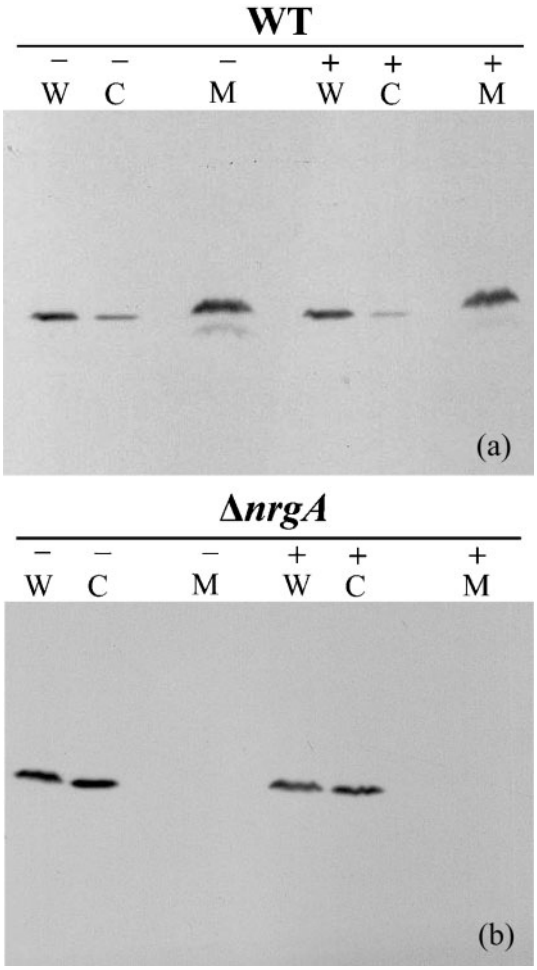

Fig. 5. Membrane association of NrgB is strictly NrgA-dependent. Cells were grown under nitrogen limitation in SMM with 0·2 % (w/v) glutamate as the sole nitrogen source. Whole-cell extracts (W), cytoplasmic (C) and membrane (M) fractions were prepared before (−) and 30 min after (+) ammonia shock and were subjected to SDS-PAGE followed by Western blotting using an anti-NrgB antibody. (a) Fractions prepared from the wild-type (WT) strain GP250. (b) Fractions obtained from the Δ*nrgA* mutant strain GP254.

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of the *nrgAB* operon is activated by TnrA under conditions of nitrogen limitation. Assays of β-galactosidase expression in strains containing a fusion of the *gltAB* promoter to the *lacZ* gene were performed after growth under nitrogen-limited conditions (2 mM ammonium, pH 5·5). The expression of the *gltAB* operon was not affected by the Δ*nrgB* mutation (552 units of β-galactosidase in strain GP256 vs 464 units in the wild-type strain GP342). In contrast, the expression was reduced in the Δ*nrgA* mutant GP257 (212 units). This may result from the lack of internal ammonium due to the transport deficiency of the mutant. Thus, we may conclude that NrgB does not affect the regulation of all TnrA-regulated genes (see Discussion).

DISCUSSION

Ammonium is one of the major sources of nitrogen for bacteria. Ammonium assimilation involves uptake of the ion and its incorporation in organic material to yield glutamine and glutamate. Both steps of ammonium assimilation can be regulated at multiple levels, i.e. expression of the corresponding genes and activities of the encoded enzymes are under strict control. This ensures the preferential utilization of glutamine as the nitrogen source that can be used with the lowest energy cost.

In *B. subtilis*, glutamine synthesis requires the activity of glutamine synthetase since the glutamate dehydrogenase in this organism was proposed to have a catabolic rather than an anabolic function (Fisher & Débarbouillé, 2002; Belitsky & Sonenshein, 1998). In addition, glutamate synthase and ammonium transport are required to provide the substrates for the glutamine synthetase reaction. While both the activity and expression of glutamine synthetase and glutamate synthase have been the subject of extensive previous work (reviewed by Fisher & Débarbouillé, 2002; Belitsky, 2002; see also Faires *et al.*, 1999; Wray *et al.*, 2001; Wacker *et al.*, 2003), ammonium uptake has not yet been studied in *B. subtilis*.

Two lines of evidence suggest that NrgA acts as the major ammonium transporter in *B. subtilis*. First, Δ*nrgA* mutants are unable to utilize ammonium as the single source of nitrogen at low concentrations. Second, the Δ*nrgA* mutant strain is drastically impaired in the transport of methylammonium. This idea has, however, an implication regarding the transport of ammonia. Normally, ammonia and ammonium are present in equilibrium in all solutions. Only at low pH is the equilibrium shifted towards ammonium. Our findings suggest that ammonia may enter the cell by diffusion through the cytoplasmic membrane independent from the ammonium transporter NrgA. As found in this study, an active ammonium transporter (AmtB) is required for ammonium uptake at low concentrations in the nitrogen-fixing proteobacterium *Azospirillum brasilense* (van Dommelen *et al.*, 1998). Interestingly, the high-G+C Gram-positive bacterium *C. glutamicum* possesses two ammonium transporters: the *amt* gene encodes an ammonium/methylammonium transporter, whereas the AmtB protein in this organism is highly specific for ammonium. The existence in *C. glutamicum* of a third, as yet uncharacterized, system for ammonium uptake has been suggested (Siewe *et al.*, 1996; Jakoby *et al.*, 2000; Meier-Wagner *et al.*, 2001). In contrast, the AmtB transporter of *E. coli* was proposed to act specifically as an ammonia rather than an ammonium transporter (Soupene *et al.*, 1998).

In *E. coli*, the activity of the AmtB ammonium transporter is modulated by a small regulatory protein, GlnK. At high ammonium concentrations, the latter protein is present in its free (non-uridylated) form and binds AmtB to prevent further ammonium uptake (Coutts *et al.*, 2002). The *B. subtilis* orthologue of GlnK, NrgB, lacks the conserved modification sites which are targets of either uridylation or phosphorylation (Atkinson & Ninfa, 1999; Forchhammer & Tandeau de Marsac, 1994; Wray *et al.*, 1994). However, the co-ordinated expression of the genes encoding the ammonium transporter NrgA and the presumptive regulator NrgB in the nitrogen-regulated *nrgAB* operon suggested that the two proteins might act in a common function. Our findings clearly indicate that NrgB is not required for ammonium transport. As observed in *E. coli*, NrgB is present in the membrane fraction of *B. subtilis*. This interaction depends on the presence of a functional NrgA, suggesting a direct physical interaction between the two proteins. In contrast to the findings in *E. coli*, the *B. subtilis* NrgB binds to the membrane in an NrgA-dependent manner even under conditions of ammonium limitation (see Fig. 5a; Coutts *et al.*, 2002). Under such conditions, GlnK of *E. coli* is uridylated and unable to bind AmtB. Thus, the proposed permanent interaction between NrgA and NrgB is in good agreement with the hypothesis that NrgB is not subject to covalent modification in *B. subtilis*.

The permanent binding of NrgB to NrgA raises the question concerning the function of this interaction. Our experiments did not indicate any growth disadvantages of the Δ*nrgB* mutant strains under the tested conditions. In a previous study, a role of NrgB was proposed for the utilization of nitrate (Wray *et al.*, 1994). Here, we observed that the extent of activity of the *nrgAB* operon promoter under nitrogen-limited conditions was reduced in the Δ*nrgB* mutant. As both nitrate utilization and *nrgAB* expression depend on transcriptional activation by TnrA we may speculate that NrgB might be involved in fine-tuning the activity of the transcription factor TnrA in response to the availability of ammonium. Moreover, NrgB might negatively affect the activity of NrgA as indicated by the reduced expression of *nrgA* in the Δ*nrgB* mutant while the growth and transport properties of the wild-type and Δ*nrgB* mutant strains are virtually identical. Our results with the *gltAB* operon did not reveal a major role of NrgB in the regulation of its transcription. This discrepancy as compared to the *nrgAB* operon may reflect the regulation factors by TnrA: while *nrgAB* expression is activated by TnrA about 1000-fold, the *gltAB* operon is repressed about 10-fold by TnrA (Wray *et al.*, 1996; Belitsky *et al.*, 2000). Thus, a role for

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NrgB may be confined to the strongly regulated genes or the regulatory effect may be too small to be detected. Clearly, more work is required to elucidate the putative regulatory role of NrgB.

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