The NtcA-Regulated *amtB* Gene Is Necessary for Full Methylammonium Uptake Activity in the Cyanobacterium *Synechococcus elongatus*

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Received 19 March 2007/Accepted 10 August 2007

The Amt proteins constitute a ubiquitous family of transmembrane ammonia channels that permit the net uptake of ammonium by cells. In many organisms, there is more than one *amt* gene, and these genes are subjected to nitrogen control. The mature Amt protein is a homo- or heterooligomer of three Amt subunits. We previously characterized an *amt1* gene in the unicellular cyanobacterium *Synechococcus elongatus* strain PCC 7942. In this work, we describe the presence in this organism of a second *amt* gene, *amtB*, which encodes a protein more similar to the bacterial AmtB proteins than to any other characterized cyanobacterial Amt protein. The expression of *amtB* took place in response to nitrogen step-down, required the NtcA transcription factor, and occurred parallel to the expression of *amt1*. However, the transcript levels of *amtB* measured after 2 h of nitrogen deprivation were about 100-fold lower than those of *amt1*. An *S. elongatus* *amtB* insertional mutant exhibited an activity for uptake of [14C]methylammonium that was about 55% of that observed in the wild type, but inactivation of *amtB* had no noticeable effect on the uptake of ammonium when it was supplied at a concentration of 100 μM or more. Because an *S. elongatus* *amt1* mutant is essentially devoid of [14C]methylammonium uptake activity, the mature Amt transporter is functional in the absence of AmtB subunits but not in the absence of AmtB subunits. However, the *S. elongatus* *amtB* mutant could not concentrate [14C]methylammonium within the cells to the same extent as the wild type. Therefore, AmtB is necessary for full methylammonium uptake activity in *S. elongatus*.

The Amt proteins are transmembrane proteins that are ubiquitous in living organisms. These proteins are known as Amt proteins in higher plants and as MEP proteins in fungi, and the mammalian Rh proteins also belong to this protein family. A demonstrated function of these proteins is ammonia translocation across biological membranes. However, whether they mediate transport of the ammonium ion or translocation of the nonionic species ammonia (pK_a [NH_4^+]/[NH_3], 9.25) has been the subject of debate (36, 45). It has recently been shown that plant Amt proteins mediate electrogenic transport of ammonium, whereas the Rh proteins appear to mediate a nonelectrogenic translocation of ammonia (33). The crystal structures of the Amt proteins from *Escherichia coli* (23, 50) and *Archaeoglobus fulgidus* (1) have been determined. Inspection of these structures indicates that these proteins have a channel through which only ammonia can pass; however, the proteins initially bind ammonium, which is deprotonated before translocation. Coordinated transport of a proton could take place in some systems but not in others (25).

The available protein structures also show that the quaternary structure of Amt is a trimer in which each monomer provides a translocation pore. Many organisms carry more than one *amt* gene, and the formation of heterooligomers as well as of homooligomers has been shown for the yeast and plant proteins (26, 31). Interactions between the different polypeptides in the trimer could be deduced, but analysis of Saccharomyces cerevisiae mutants has shown that each MEP protein exhibits a different substrate affinity (30).

In many microbes, the *amt* genes are subjected to nitrogen control and are expressed mainly under nitrogen deficiency conditions. The Amt proteins appear, therefore, to have a role in uptake of ammonium when it is present at very low concentrations in the extracellular medium (30, 45). The Amt/MEP proteins are also responsible for a methylammonium uptake activity that was originally described in *Penicillium chrysogenum* (19) and is exhibited by many organisms. Uptake of [14C]methylammonium can be used to probe the activity of these proteins, although not all Amt/MEP proteins may recognize methylammonium as a substrate (34, 44). In many organisms, methylammonium is converted into methylglutamine by glutamine synthetase, and therefore measurements of [14C]methylammonium uptake involve both transport and metabolism of the substrate (5, 7).

The cyanobacteria are oxygenic photoautotrophs that can utilize for growth diverse inorganic sources of nitrogen, including nitrate and ammonium (14). Ammonium is a preferred nitrogen source that promotes repression of many nitrogen assimilation genes. When cyanobacterial cells are incubated in the absence of ammonium and with an adequate supply of CO_2_, the expression of these genes is activated by the transcriptional regulator NtcA (20). NtcA binds to a conserved sequence in the regulated promoters, GTAN_5TAC (20, 27), and binding is most efficient in the presence of 2-oxoglutarate (48). The unicellular cyanobacterium *Synechocystis* sp. strain...
TABLE 1. Some deoxyoligonucleotide primers used in this work

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>amtB-7942-1</td>
<td>5’-TCA GGC ATG TTC GAC AAA CG 3’</td>
</tr>
<tr>
<td>amtB-7942-2</td>
<td>5’-CCT AGC GTG CTT TGT AGG C 3’</td>
</tr>
<tr>
<td>glnB-qPCR-1</td>
<td>5’-GGG CGG CTC CTT CAT GGT C 3’</td>
</tr>
<tr>
<td>glnB-qPCR-2</td>
<td>5’-TCC ATC CGG TGT CCG CAA ATA 3’</td>
</tr>
<tr>
<td>glnA-qPCR-1</td>
<td>5’-GTC GCC CGC GCA CAA TG 3’</td>
</tr>
<tr>
<td>glnA-qPCR-2</td>
<td>5’-ACC GCC GGC CAG TGT GAG 3’</td>
</tr>
<tr>
<td>glbA-qPCR-1</td>
<td>5’-GCG GGG GCT GAG AGT GCT AAG 3’</td>
</tr>
<tr>
<td>glbA-qPCR-2</td>
<td>5’-CGT AAG CCC ACC GAA ATG AA 3’</td>
</tr>
<tr>
<td>glnA-qPCR-1</td>
<td>5’-ACC GGC GGC CAG TGT GAG 3’</td>
</tr>
<tr>
<td>glnA-qPCR-2</td>
<td>5’-GCG CGG GTG GTT TTT CTC G 3’</td>
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<tr>
<td>glbA-qPCR-1</td>
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</tr>
<tr>
<td>glbA-qPCR-2</td>
<td>5’-ACC GCC GGC CAG TGT GAG 3’</td>
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<tr>
<td>rpoD1-qPCR-1</td>
<td>5’-CAG AAG TGC GCG GGT TTT GGT C 3’</td>
</tr>
<tr>
<td>rpoD1-qPCR-2</td>
<td>5’-GTC GCC GCC GCA CAA TG 3’</td>
</tr>
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<td>rpoD1-qPCR-3</td>
<td>5’-CCT AGC GCT CTT TGC TGT AGG C 3’</td>
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<tr>
<td>rpoD1-qPCR-4</td>
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</tr>
<tr>
<td>rpoD1-qPCR-5</td>
<td>5’-GTC GCC GCC GCA CAA TG 3’</td>
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PCN 6803 has been shown to express, mainly under nitrogen deficiency conditions, three different amt genes, of which the amt1 gene is responsible for a major fraction of the methyl-ammonium uptake activity (35). An amt1 gene has also been characterized in Synchococcus elongatus strain PCN 7942 (49). Its expression is NtcA dependent, and the encoded protein, Amt1, is essential for methylammomium uptake, for growth in the presence of low concentrations of ammonium, and for recapture of ammonium leaked out from the cells (49). In this work, a second S. elongatus amt gene is characterized, and the mechanism of ammonium uptake in this cyanobacterium is discussed.

MATERIALS AND METHODS

Strains and growth conditions. S. elongatus strain PCN 7942 was grown axenically at 30°C in the light (85 microeinstes m⁻² s⁻¹) in BG11 (nitrate-containing) medium (41) or in BG11a (BG11 medium lacking NaNO₃) supplemented with 4 mM NH₄Cl and 8 mM NaVO₃ using the enzyme (Ecogen S.R.L.). The program used for amplification was 95°C for 2 min, followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 61.4°C, and elongation for 1 min at 72°C. The thermal cycling program consisted of an initial preheating step of 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 61.4°C, and 30 s at 72°C.

For Northern blotting, 20 μg of RNA was loaded in each lane and electrophoresed in denaturing 1% agarose formaldehyde gels. DNA probes for S. elongatus genes were generated by PCR using plasmids carrying the corresponding genes as templates and oligonucleotide primers that produced the following gene fragments: for amt1, from bp 13 to bp 345 with respect to the start of the gene; and for amt1, from bp 7 to bp 280 with respect to the start of the gene to the end of the gene. Hybridization was performed at 65°C in 1× EDTA-0.3 M sodium phosphate buffer (pH 6.8) containing 7% sodium dodecyl sulfate (SDS), and the filters were washed at 65°C successively with 2× SSC-0.1% SDS, 1× SSC-0.1% SDS, and 0.5× SSC-0.1% SDS (1× SSC is 150 mM NaCl and 15 mM sodium citrate dihydrate). As a control for RNA loading and transfer efficiency, the filters were reprobed with a 0.57-kb XhoI fragment that contained the RNase P RNA gene (mphB) from strain PCN 7942 (49). Probes were labeled with a DNA labeling kit (Ready to Go; Amersham Pharmacia Biotech) and [γ-³²P]dCTP (3). Hybridization was performed at 65°C in a solution containing 50 mM Tris-HCl (pH 7.4), 0.2% bovine serum albumin, 0.2% Ficoll, 0.1% sodium pyrophosphate, 1% SDS, 1 M NaCl, and 100 μg ml⁻¹ yeast RNA (Roche) (11). Filters were washed twice at 65°C for 30 min with 1× SSC–1% SDS and once at room temperature for 15 min with 0.2× SSC (pH 7.0).

For quantitative RT-PCR, total RNA was isolated (17) from five independent cultures of wild-type strain PCN 7942 incubated without combined nitrogen for 2 h. Hybridization was performed, using SuperScript II reverse transcriptase (Inverogen), was carried out with 10 μg RNA and oligonucleotide primers amt1-qPCR-2, amtB-qPCR-2, glbA-qPCR-2, glbB-qPCR-2, and rpdo1-qPCR-2 (Table 1), which were designed from the corresponding DNA probes for S. elongatus genes (from bp 13 to bp 345 with respect to the start of the gene). Hybridization was performed at 65°C in a solution containing 50 mM Tris-HCl (pH 7.4), 0.2% bovine serum albumin, 0.2% Ficoll, 0.1% sodium pyrophosphate, 1% SDS, 1 M NaCl, and 100 μg ml⁻¹ yeast RNA (Roche) (11). Different filters were washed twice at 65°C for 30 min with 1× SSC–1% SDS and once at room temperature for 15 min with 0.2× SSC (pH 7.0).

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Methylammonium uptake assays, ammonium uptake, glutamine synthetase activity, and growth tests. S. elongatus wild-type and mutant strains were grown in ammonium-containing medium buffered with a mixture of CO₂ (1%, vol/vol) and air, and the cells were harvested at room temperature, washed twice with BG110C, resuspended in BG110C, and incubated under culture conditions with CO₂-enriched air. The cells were harvested at room temperature, washed with 0.5 M (or 20 M; see Fig. 7) KH₂PO₄–10 mM NaHCO₃–NaOH buffer (pH 7.1), and resuspended in the same buffer to obtain a cell density corresponding to 10⁶ Bq ml⁻¹. After preincubation at 30°C in the light (14°C, 50 μmol m⁻² s⁻¹) for 5 to 30 min, the assays were started by mixing the suspension of cells with a solution of 14NH₃·HCl (2.11 × 10⁻⁶ Bq μmol⁻¹; Amersham) in phosphate-bicarbonate buffer. Alternatively (see Fig. 5), the cell suspension in BG110C was directly supplemented with 14NH₃·HCl solution in water.
After incubation for the time periods indicated below, 1-ml samples were filtered with 0.45-μm-pore-size Millipore HA filters. Without any further washing (22), the filters carrying the cells were immersed in scintillation cocktail, and the radioactivity was measured. The retention of radioactivity by boiled cells was used as a blank. For chase experiments, the cells were supplemented with 1 mM NH4Cl at the indicated time points before filtering.

Ammonium uptake assays were performed with cells grown in ammonium-containing medium bubbled with a mixture of CO2 (1%, vol/vol) and air. The cells were harvested at room temperature, washed twice with BG11, resuspended in BG11/C (pH 8) to obtain a cell density corresponding to 2.5 μg Chl ml−1, and incubated for 1.5 h under culture conditions with CO2-enriched air. The experiments were started by addition of NH4Cl (100 μM) to cell suspensions. Ammonium disappearance was determined by estimating the concentration of ammonium in the cell suspension after rapid removal of the cells by filtration of 2.5-ml samples (with 0.45-μm-pore-size Millipore HA filters) after incubation for different time periods up to 40 min. Ammonium contents were determined with glutamate dehydrogenase (6).

Glutamine synthetase transferase activity was determined in cell extracts as described previously (9). Cell extracts were prepared from an amount of cells containing about 7.5 μg Chl. The cells were harvested by centrifugation (16,100 × g, 4°C, 5 min) and resuspended in 200 μl of a buffer containing 50 mM Tris-HCl (pH 7.4), 4 mM EDTA, 1 mM dithiothreitol, and 0.5 mM benzamidine, which was supplemented with about 150 mg of glass beads. After three cycles of 3 min of vortexing and incubation on ice, each suspension was centrifuged as described previously (9). The glass beads were resuspended for the next cycle. After three cycles of resuspension, the supernatant was used to assay the enzyme activity. One unit corresponded to the formation of 1 μmol of γ-glutamylhydroxamate per min.

The growth of the mutants on solid medium, plates of BG11, that were not supplemented or were supplemented with 100 μM or 2 mM NH4Cl and buffered with 5 mM TEA at pH 7.5 were prepared. Because of its ferric ammonium citrate content, BG11 contains an unspecified concentration of ammonium that may be as high as about 20 μM. Drops (10 μl) of cell suspensions of strains PCC 7942, CSF72, CSP11, and CSP12 at a concentration corresponding to 1 μg Chl ml−1 were spotted on the medium, and the plates were incubated under culture conditions for 2 weeks.

Calculations. To apply the Nernst equation, the intracellular concentration of methammonium was calculated by using an intracellular cell volume of 125 μl mg Chl−1 (21, 39). Passive influx of NH3 was calculated by applying Fick's diffusion equation, J = P · Δc (where J is the flux and Δc is the concentration gradient), using a conservative permeability coefficient for ammonia (P) of 10−5 m s−1 (2, 24) (for ammonia permeability coefficients in the literature, see reference 43). To compare the deduced diffusion values to observed ammonium uptake rates, it was assumed that an S. elongatus cell has a surface area of about 10−11 m2 and that 1 μg of Chl corresponds to about 5 × 107 Synechococcus cells (E. Flores, unpublished).

Phylogenetic analysis. Predicted polypeptide sequences were aligned with the program ClustalX 1.8 (47). Phylogenetic trees were visualized with the NJplot program (38).

RESULTS

Identification of putative genes. Inspection of the genomic sequence of S. elongatus strain PCC 7942 (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) showed the presence of two putative genes. Open reading frame Syn_pcc7942442 is the previously characterized gene (49). Open reading frame Syn_pcc79422279 encodes a protein that exhibits 50.9% identity with Am1. However, a phylogenetic analysis of Am1 proteins from a number of different biological sources placed the Syn_pcc79422279 protein in the branch where the Am1 proteins from E. coli and other organisms are also located rather than in the branch containing S. elongatus Am1 or Synechocystis Am1, Am2, or Am3 (Fig. 1). Therefore, the Syn_pcc79422279 protein appears to be more closely related to some bacterial Am1 proteins, of which Escherichia Am1 is the best-known example, than to other characterized cyanobacterial Am1 proteins. We refer to Syn_pcc79422279 here as the S. elongatus am1 gene.

Expression of am1. The expression of am1 was studied by Northern analysis. The level of expression was low in ammonium-grown cells of S. elongatus (Fig. 2a), and induction was observed after transfer of the cells to media with nitrate or no combined nitrogen. Expression was detected as soon as 30 min after withdrawal of ammonium but declined after 1 h of incubation, especially in nitrate-supplemented cultures. For comparison, the filter was rehybridized with an am1 probe. The expression of am1 and the expression of amt1 were parallel in both nitrate-containing medium and medium lacking any source of combined nitrogen (Fig. 2a and b), although the levels of expression of amt1 were higher than those of am1 (see below). The am1 gene was expressed as an approximately 2-kb transcript. Since am1 is 1,410 bp long and inspection of the S. elongatus genome showed no putative accompanying gene, am1 appears to be transcribed monocistronically.

As is the case for am1 (49), induction of am1 was not observed in an S. elongatus nica mutant in medium containing nitrate or in the absence of combined nitrogen (Fig. 2c). This shows that like am1, amt1 is subject to NtcA-mediated nitro-
FIG. 2. Expression of the amtB gene in S. elongatus. (a) Northern analysis performed with RNA isolated from cells of wild-type S. elongatus grown with ammonium (lane A) or grown with ammonium and incubated with nitrate (N) or with no combined nitrogen (−N). The filter was successively hybridized with the amtB probe (upper panel), the amt1 probe (middle panel), and an rnpB probe that was used as a loading and transfer control (lower panel). (b) Comparison of the time course patterns of expression of amtB and amt1. The amtB and amt1 signals in panel a were quantified and normalized with the rnpB signals. For each probe (amtB or amt1), the signal obtained after 1 h of incubation without combined nitrogen was defined as 100. Diamonds, amtB; squares, amt1; open symbols, without N; filled symbols, with nitrate. Note that the relative levels of expression of the genes with respect to each other cannot be deduced from these data because visualization of amtB required a longer exposure than visualization of amt1. (c) Northern analysis performed with RNA isolated from cells of the wild type (WT) or the S. elongatus ntcA mutant grown with ammonium (lanes A) or grown with ammonium and incubated for 2 h in the presence of nitrate (lanes N) or with no combined nitrogen (lanes −N), using the amtB probe (upper panel) or an rnpB probe (lower panel).

FIG. 3. Expression of amtB compared to expression of other nitrogen assimilation genes (amt1, glnA, and glnB). DNA fragments corresponding to the indicated genes of S. elongatus were bound to a membrane filter and hybridized with 32P-labeled RNA extracted from cells grown with ammonium or grown with ammonium and incubated with nitrate or without combined nitrogen (−N) for 2 h. The presence of the amtB probe in the filters was corroborated by Southern analysis (not shown). Note that hybridizations with the different labeled RNA preparations (isolated from cells subjected to the different nitrogen regimens) cannot be quantitatively compared to each other because they required different exposure times.

gen control. A putative NtcA binding site, GTAN₈TAC, is located 84 to 72 bp upstream from the translation start of the gene, which could be involved in NtcA-dependent regulation. Unfortunately, however, we were unable to determine the transcription start point of amtB, perhaps because of its low level of expression.

To compare the level of expression of amtB to that of other nitrogen assimilation genes, DNA probes of amt1, amtB, glnA, and glnB were fixed in a membrane filter and hybridized with 32P-labeled S. elongatus total RNA. RNA isolated from cultures incubated with ammonium, nitrate, or no combined nitrogen was used. The hybridization signal of the amtB probe was much lower than that of any of the other probes for the three RNA preparations (Fig. 3). These results indicate that amtB is expressed at lower levels than the other nitrogen assimilation genes tested under any of the nitrogen regimens used in our analysis. The large difference between expression of amtB and expression of amt1 is remarkable.

To quantify the differences in expression of the amtB, amt1, glnA, and glnB genes, quantitative RT-PCR was performed, as described in Materials and Methods, using RNA samples isolated from cultures of S. elongatus incubated for 2 h in the absence of combined nitrogen. The relative levels of expression determined with five independent RNA samples, using the RNA polymerase rpoD1 gene as a reference, were 0.10 ± 0.02 for amtB, 1 for rpoD1, 3.47 ± 2.27 for glnA, 5.82 ± 1.40 for glnB, and 10.32 ± 2.59 for amt1. Therefore, a difference of over 100-fold was found between the expression of amtB and the expression of amt1 after 2 h of nitrogen deprivation.

Inactivation of amtB. An interrupted amtB::C.S3 gene was transferred by transformation to wild-type strain PCC 7942 and mutant strain CSF72 (amt1::C.C2), generating mutant strains CSP11 (amtB::C.S3) and CSP12 (amt1::C.C2 amtB::C.S3), respectively. Growth tests in solid medium supplemented with different concentrations of ammonium as the sole nitrogen source at pH 7.5 were performed with four strains, PCC 7942, CSF72, CSP11, and CSP12. No differences in growth were observed when the ammonium concentration was 2 mM (Fig. 4). Impairment of ammonium-dependent growth of the amtB mutant (strain CSP11), indicated by yellow-green coloration, was barely observed only with the lowest ammonium concentration tested (ca. 20 μM). In contrast, the ammonium-dependent growth of the amt1 mutant (strain CSF72) was impaired with ammonium concentrations of 100 and 20 μM (49). The results for the amt1 amtB double mutant (strain CSP12) were similar to those for the amt1 mutant.

Uptake of methylammonium. The uptake of [14C]methylammonium was determined for the wild-type strain and the single
and double ant mutants in time course induction experiments. The results of a representative experiment in which ammonium-grown cells that had been incubated in the absence of nitrogen for 1, 3, or 13 h were used in uptake assays with 5 μM [14C]methylammonium are shown in Fig. 5. As previously described (49), the ant1 mutant showed a very low rate of methylammonium uptake. In contrast, the antB mutant, although its activity was impaired, still showed a substantial level of uptake. The results of eight different experiments using 5 μM [14C]methylammonium as the substrate showed that in the antB mutant the uptake activity was about 55% ± 9% of the activity in the wild type. Like the ant1 mutant, the ant1 antB double mutant showed about 5% of the activity of the wild type. Some of the data in Fig. 5 also reflect the two phases that have been distinguished for methylammonium uptake in S. elongatus: quick uptake essentially reflecting transport, followed by a steady phase corresponding to methylamine incorporation into methyl glutamine (7).

Because the steady phase of methylammonium uptake is dependent on glutamine synthetase, the activity of this enzyme was determined in the wild-type and mutant strains. The trans- ferase activity of glutamine synthetase was measured in cell extracts from ammonium-grown cells that had been incubated for 2 h in the absence of combined nitrogen. The activities obtained were 63.1 ± 3.1 U mg of Chl⁻¹ for strain PCC 7942, 64.4 ± 6.9 U mg of Chl⁻¹ for strain CSP11, 63.2 ± 3.5 U mg of Chl⁻¹ for strain CSP12, and 67.2 ± 1.2 U mg of Chl⁻¹ for strain CSF72 (means ± standard deviations for the results of three independent assays). Therefore, no significant differences in glutamine synthetase activity were evident.

In cyanobacteria, methylammonium uptake is a membrane potential-dependent process that permits accumulation of the substrate within the cell (7, 35, 40). The effect of inactivation of antB on the ability of S. elongatus to retain [14C]methylammonium was investigated in chase experiments with ammonium. As shown in Fig. 6, a fraction of the methylammonium taken up was displaced by ammonium. This fraction must correspond to the methylammonium accumulated within the cells, and the remaining radioactivity must correspond to [14C]methylglu- tamine. A calculation based on the Nernst equation indicated that the observed concentration gradient of [14C]methylammonium in the wild type corresponded to a free energy change of 115 mV, roughly corresponding to the accumulation permitted by the membrane potential of actively photosynthesizing Synechococcus cells, which is about −110 to −130 mV (42). A similar chase experiment performed with the antB mutant showed that the amount of methylammonium that this mutant could accumulate was only about 10% of the amount observed in the wild type (Fig. 6).

Using a different experimental approach, the uptake of very low concentrations of [14C]methylammonium was investigated. Figure 7 shows the results of a representative experiment in which the uptake of 0.12 μM [14C]methylammonium was tested. Whereas the two phases of [14C]methylammonium uptake were observed for the wild type, only the linear phase was observed for the antB mutant. The ant1 mutant and the ant1
The recently described \textit{amt1} gene (49) and the \textit{amtB} gene characterized in this work. These two genes are subjected to N control mediated by the NtcA transcriptional regulator, so that they are expressed in response to withdrawal of ammonium. For both genes, expression is higher in the absence of combined nitrogen than in the presence of nitrate, and the latter conditions lead to a significant decrease in expression soon after induction (Fig. 2a and b). This decrease in expression has also been observed in \textit{S. elongatus} for other nitrogen assimilation genes (37), and it might be related to a feedback effect of the ammonium resulting from nitrate assimilation. Expression of NtcA-activated genes in nitrogen-starved \textit{S. elongatus} cells has been shown, on the other hand, to be dependent on the signal transduction protein P$_{II}$ (the \textit{glnB} gene product [37]).

The results presented in this work show that whereas \textit{Amt1} is essential for [14C]methylammonium uptake, \textit{AmtB} appears to be needed only for maximum uptake levels. It is possible that the optimal Amt complex in the \textit{S. elongatus} cytoplasmic membrane consists of both Amt1 and AmtB subunits, but whereas an Amt1 homooligomer is functional, a complex consisting of only AmtB subunits is not. This would be consistent with the differences observed in the expression levels of \textit{amt} genes that support a scenario where Amt1 subunits would be more represented than AmtB subunits in Amt trimers. Nonetheless, given the much lower expression of \textit{amtB}, the contribution of AmtB to activity seems to be substantial. Additionally, AmtB is important for providing \textit{S. elongatus} cells with the ability to concentrate methylammonium and, hence, probably also ammonium. This can be especially important when the cells encounter very low external concentrations of ammonium, conditions under which accumulation of ammonium within the cells (to the level permitted by the membrane potential) can favor the functioning of glutamine synthetase. NtcA-dependent expression of the \textit{amt} genes under nitrogen deficiency conditions is consistent with a role for the Amt complex(es) in scavenging nitrogen from low ammonium concentrations by the \textit{S. elongatus} cytoplasmic membrane.

DISCUSSION

The \textit{S. elongatus} genome carries two \textit{amt} genes, the previously described \textit{amt1} gene (49) and the \textit{amtB} gene characterized in this work. These two genes are subjected to N control mediated by the NtcA transcriptional regulator, so that they are expressed in response to withdrawal of ammonium. For both genes, expression is higher in the absence of combined nitrogen than in the presence of nitrate, and the latter conditions lead to a significant decrease in expression soon after induction (Fig. 2a and b). This decrease in expression has also been observed in \textit{S. elongatus} for other nitrogen assimilation genes (37), and it might be related to a feedback effect of the ammonium resulting from nitrate assimilation. Expression of NtcA-activated genes in nitrogen-starved \textit{S. elongatus} cells has been shown, on the other hand, to be dependent on the signal transduction protein P$_{II}$ (the \textit{glnB} gene product [37]).

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The physiological studies described in this work do not permit derivation of mechanistic conclusions regarding the func-
tioning of the Amt proteins. They raise, however, novel ques-
tions. It is noteworthy that AmtB, which allows greater
efficiency in methylammonium uptake and permits accumu-
lation of methylammonium within the cells, cannot support this
activity alone (Fig. 6 and 7). Whether this protein has a struc-
tural role in Amt trimers or has an activity complementary to
that of the Amt1 proteins is unknown. Parallel expression of
the amtB and amt1 genes (Fig. 2) is consistent with possible
joint action of their corresponding protein products. Whatever
the mechanism, the possibility that AmtB is necessary for the
Amt complex to transport the methylammonium ion (espe-
cially when methylammonium is present at low concentrations
in the outer medium), whereas Amt1 is sufficient to make a
complex that can mediate translocation of methylamine, is
intriguing. In this scenario, Amt1-dependent uptake of methyl-
amine pulled by glutamine synthetase would take place, as
shown for the amtB mutant in Fig. 6 and 7.
A phylogenetic analysis of cyanobacterial Amt proteins re-
cently performed by Luque and Forchhammer (28) showed
that Amt1 is the Amt protein that is widespread in the cyano-
bacteria, consistent with its major role in methylammonium
uptake (35, 49). In contrast, proteins that group with S. elon-
gatus AmtB are found only in the heterocyst-forming cyanobac-
tera (28), which are not closely related to S. elongatus (46).
The amtB gene might have been imported into S. elongatus and
the heterocyst formers from noncyanobacterial sources by lat-
eral gene transfer, which is a recognized mechanism in cyano-
bacterial genome evolution (15). One possible scenario, con-
sistent with our experimental results, is that amtB is a re-
cently acquired secondary gene in the S. elongatus genome
whose protein product has been adapted to provide a function
complementary to the function of Amt1.
We have also considered the possibility that AmtB has a regu-
ulatory role, specifically mediating some regulatory effects of
ammonium on gene transcription and the activity of the
nitrate permease. The repressor effect of ammonium was
checked, using the glnA gene as a probe, in ammonium-grown
cells that had been incubated for 60 or 90 min in the absence
of combined nitrogen. Addition of 50 to 500 μM ammonium
inhibited glnA expression, and the effects were similar in the
wild type and the amtB mutant, as well as in the Amt1 mutant
and the amt1 amtB double mutant (results not shown). Inhi-
bition by ammonium of nitrate uptake (12) was also tested,
using 50 to 150 μM nitrate as a substrate and adding 100 μM
ammonium, but again no differences were found between the
wild type and the mutants (results not shown). These negative
results do not support any possible regulatory role of the Amt
proteins in S. elongatus.
With an ammonium concentration of 100 μM, a relatively
high rate of ammonium uptake was observed in this work for
the S. elongatus amt1 amtB double mutant, similar to that
observed for the wild type and the amt single mutants. As
mentioned above, diffusion of ammonia pulled by glutamine
synthetase can account for such an uptake rate. Therefore,
although the existence of ammonium uptake systems other
than Amt cannot be ruled out, growth of S. elongatus on high
ammonium concentrations can be explained based on ammno-
diffusion. For similar concentrations of ammonium
(around 100 μM), diffusion of ammonia also appears to be
sufficient to mediate regulatory effects such as the repression of
N-regulated genes and the inhibition of nitrate uptake. The
Amt complex might be needed, however, for regulation pro-
moted by more limiting concentrations of ammonium.

ACKNOWLEDGMENTS
We thank Vicente Mariscal for help with the quantitative RT-PCR
and Ignacio Luque for critical reading of the manuscript.
This work was supported by grant BFU2005-07672 from the Minis-
terio de Educación y Ciencia (Spain).

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