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The *amt* Gene Cluster of the Heterocyst-Forming Cyanobacterium *Anabaena* sp. Strain PCC 7120[▽]

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The genome of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 bears a gene cluster including three *amt* genes that, based on homology of their protein products, we designate *amt4*, *amt1*, and *amtB*. Expression of the three genes took place upon ammonium withdrawal in combined nitrogen-free medium and was NtcA dependent. The genes were transcribed independently, but an *amt4-amt1* dicistronic transcript was also produced, and expression was highest for the *amt1* gene. A mutant with the whole *amt* region removed could grow under laboratory conditions using ammonium, nitrate, or dinitrogen as the nitrogen source.

Cyanobacteria are a group of organisms characterized by the fact that they perform oxygenic photosynthesis. Filamentous cyanobacteria, such those of the genera *Anabaena*, *Nostoc*, and *Nodularia*, fix nitrogen in specialized cells called heterocysts that are distributed at semiregular distances between the vegetative cells in the filament. In diazotrophic filaments, oxygenic photosynthesis is restricted to vegetative cells, which transfer fixed (reduced) carbon, probably in the form of a sugar like sucrose, to the heterocysts (5, 37, 39). Heterocysts in turn transfer fixed nitrogen to the vegetative cells (38). The heterocysts can synthesize at least some amino acids, and it is thought that nitrogen is transferred to vegetative cells in the form of glutamine (33, 40) and/or other amino acids (29). However, the possibility that ammonium, the product of the reaction catalyzed by nitrogenase, is also transferred has not been unequivocally ruled out.

The bacterial Amt proteins are members of a family of permeases widely distributed in living organisms, and their demonstrated function is translocation of ammonia across biological membranes (14). The quaternary structure of Amt is that of a trimer in which each monomer provides a translocation pore. Many organisms carry more than one *amt* gene, and the Amt complexes can be hetero-oligomers as well as homo-oligomers (17, 20, 26). Although some exceptions appear to exist (23, 32), the Amt proteins can also transport methylamine, and therefore, their activity can be probed with radiolabeled [^{14}C]methylammonium (12). The question of whether the Amt proteins mediate the translocation of the charged or noncharged species (ammonium or ammonia in the case of the natural substrate; pK_a [NH_4^+/NH_3], 9.25) has been amply debated (e.g., see references 14 and 16). However, in cyanobacteria, the activity of the Amt proteins results in a net uptake of methylammonium by the cells (3, 24, 26, 30).

In cyanobacteria, expression of nitrogen assimilation genes under nitrogen deprivation requires the transcription factor NtcA (13). In the promoters of regulated genes, NtcA binds to a DNA site of conserved sequence, $GTAN_8TAC$, which is usually located about 22 bp upstream from a −10 promoter hexamer (13). The unicellular cyanobacteria *Synechococcus elongatus* strain PCC 7942 and *Synechocystis* sp. strain PCC 6803 have been shown to express, mainly under nitrogen deficiency, two (*amt1* and *amtB*) and three (*amt1*, *amt2*, and *amt3*) different *amt* genes, respectively (24, 26, 35). The *amt1* gene is responsible for a major fraction of the methylammonium uptake activity in both strains, and Amt1 has been shown to be essential for growth in the presence of low concentrations of ammonium and for recapture of ammonium leaked out from the cells in *Synechococcus elongatus* (35). In this work, we characterized the *amt* gene cluster of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 and tested whether the Amt proteins could have a role in the diazotrophic growth of this cyanobacterium.

Methods. *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 was grown axenically at 30°C in the light (85 $\mu E \cdot m^{-2} \cdot s^{-1}$) in a shaker. BG11 (nitrate-containing) medium (31), $BG11_0$ medium (BG11 medium lacking $NaNO_3$), or $BG11_0$ medium supplemented with 4 mM NH_4Cl and 8 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH buffer (pH 7.5) was used. For mutants, the medium was supplemented with 5 to 10 μg neomycin (Nm) · ml^{-1} (strain CSP19) or 2 to 5 μg spectinomycin (Sp) · ml^{-1} and 2 to 5 μg streptomycin (Sm) · ml^{-1} (strain CSE2). For plates, the medium was solidified with 1% agar (Difco) that had been sterilized separately. Cultures used for RNA isolation and for uptake assays were grown in $BG11_0C$ medium ($BG11_0$ medium supplemented with 10 mM $NaHCO_3$) supplemented with 8 mM NH_4Cl and 16 mM TES-NaOH buffer (pH 7.5) and bubbled with a mixture of CO_2 (1% [vol/vol]) and air (high supply of CO_2). When the cultures reached a cell density of 4 to 5 μg of chlorophyll *a* (Chl*a*) · ml^{-1}, the cells were harvested at room temperature, washed twice with $BG11_0C$ medium, resuspended in $BG11_0C$ medium, and incubated for the indicated times under culture conditions with CO_2-enriched air. Cyanobacterial cell mass was estimated by measuring the concentration of Chl*a* of the cultures, determined in methanolic extracts of the cells (19). *Escherichia coli* strain DH5α was grown

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TABLE 1. Deoxyoligonucleotide primers used in this work

Deoxyoligonucleotide primer	Sequence
41	5′-AGCGGTAGATACTCCTACCC-3′
42	5′-CAACCATTGGAGTTCCAAGCC-3′
11	5′-GACAATCTAACTCGCAAATCCA-3′
12	5′-ACCAGAGACAATTGTTGCCGC-3′
B1	5′-AGTCTTGCTGATGACACCAGG-3′
B2	5′-GAAACCCCAGAGCTAATATGAA-3′
amts-7120-1	5′-AGATCTGGAAAATAGACAATGGG-3′
amts-7120-2	5′-GTTTGGATCCATAATTGATGTTAG-3′
amts-7120-7	5′-ATGGATCCGTAAAAACTTAGTCG-3′
amts-7120-8	5′-AGATGAGATCTATAAGATGGTAG-3′
RT1	5′-TGGCGATTACGGTTTTCA-3′
RT2	5′-CCGGGCTGTTATCTACTCC-3′
RT3	5′-CCGCAGTAGGTGGTATGAC-3′
RT4	5′-GGGGCAAACGACAGACTA-3′

in LB medium to which antibiotics were added, when necessary, at the following concentrations: 50 μg ampicillin · ml^{-1}, 25 μg Sp · ml^{-1}, 25 μg Sm · ml^{-1}, and 30 μg chloramphenicol · ml^{-1}.

Isolation of genomic DNA from cyanobacteria was carried out as described previously (4). Isolation of total RNA from *Anabaena* sp. strain PCC 7120 was done as described previously for gram-negative bacteria (1). For Northern blots, 20 μg of RNA was loaded per lane and electrophoresed in denaturing 1% agarose formaldehyde gels. Hybridizations were performed as previously described (26). DNA probes were generated by PCR using *Anabaena* DNA and the oligonucleotide primers indicated in each case (deoxyoligonucleotide primer sequences shown in Table 1). For a control of RNA loading and transfer efficiency, the filters were hybridized with a probe of the RNase P RNA gene (*rnpB*) from *Anabaena* sp. strain PCC 7120 (36). Probes were labeled with a DNA labeling kit (Ready to Go; Amersham Pharmacia Biotech) and [α-^{32}P]dCTP. Radioactive areas in Northern blot hybridizations were visualized and quantified with a Cyclone storage phosphor system (Packard).

For reverse transcription-PCR (RT-PCR) experiments, 10 μg of *Anabaena* sp. strain PCC 7120 total RNA was mixed with 40 pmol of oligonucleotide RT2 or RT4 in the presence of 10 mM Tris-HCl (pH 8.0), 150 mM KCl, and 1 mM EDTA and heated first for 10 min at 85°C and then at 50°C for 1 h for annealing. The extension reactions were carried out at 47°C for 1 h in a final volume of 45 μl containing the whole annealing reaction mixture, 0.25 mM (each) deoxynucleoside triphosphate, 200 U of reverse transcriptase (Superscript II; Invitrogen), and the buffer recommended by the reverse transcriptase provider. To control for the presence of contaminating DNA, samples containing 10 μg of RNA, 40 pmol of oligonucleotides, and 1 μg of RNase A (DNase free; Roche) were incubated in a 45-μl reaction volume at 50°C for 15 min. PCR was carried out with 2 to 5 μl of retrotranscription mixture or RNase-treated sample as the template and oligonucleotides RT1 or RT3 and RT2 or RT4 as primers. Samples containing the same oligonucleotides and strain PCC 7120 genomic DNA as the template were run in parallel and used as a control. PCR was performed by standard procedures. Half of each sample was resolved by electrophoresis in a 0.7% agarose gel.

For deletion of the *amt* genomic region, DNA fragments were amplified by standard PCR using DNA from *Anabaena* sp. strain PCC 7120 and primer pairs amts-7120-1/amts-7120-2 and amts-7120-7/amts-7120-8. The PCR products were cloned in vector pGEM-T (Promega), and the plasmids generated were digested with BamHI and NaeI and ligated together, producing plasmid pCSP92, in which a 0.62-kb region upstream of *amt4* and a 0.54-kb region downstream of *amtB* are joined at the BamHI site. The 1.3-kb Nm^r gene cassette C.K3 (7) was inserted into this BamHI site, producing plasmid pCSP93, and the Δ*amt*::C.K3 construct was transferred into Sm^r/Sp^r and *sacB*-carrying mobilizable plasmid pRL277 (2), producing plasmid pCSP94. This plasmid was transferred by triparental mating to *Anabaena* sp. strain PCC 7120 as previously described (8) using pRL623 as a helper plasmid (6).

For [^{14}C]methylammonium uptake assays, *Anabaena* sp. strain PCC 7120 and mutant strain CSP19 were grown in ammonium-containing medium bubbled with a high supply of CO_2. The cells were harvested at room temperature, washed twice with $BG11_0C$ medium, resuspended in $BG11_0C$ medium, and incubated under culture conditions with a high supply of CO_2. The cells were harvested at room temperature, washed with 0.5 mM KH_2PO_4 and 10 mM $NaHCO_3$-NaOH buffer (pH 7.1), and resuspended in the same buffer to give a cell density corresponding to 10 μg Chl*a* · ml^{-1}. After a preincubation at 30°C in the light (85 μE · m^{-2} · s^{-1}) for 5 to 15 min, the assays were started by mixing the suspension of cells with a solution of [^{14}C]CH_3NH_2 · HCl (2.11×10^6 Bq · μmol^{-1}; Amersham) in phosphate-bicarbonate buffer. After incubation for the indicated time periods, 1-ml samples were filtered (0.45-μm-pore-size Millipore HA filters were used). Without any further washing, the filters carrying the cells were immersed in scintillation cocktail, and their radioactivity was measured. To determine nonspecific retention of radioactivity, samples of boiled cells were used as a blank.

To test the growth of mutants on liquid medium, cells of *Anabaena* sp. strain PCC 7120 and CSP19 that had been grown in BG11 medium (with Nm for the mutant) were harvested, washed with $BG11_0$ medium, and resuspended in $BG11_0$ medium, BG11 medium, or $BG11_0$ medium supplemented with 4 mM NH_4Cl and 8 mM TES-NaOH (pH 7.5) at 0.2 μg Chl*a* · ml^{-1}. After incubation for the indicated times, 0.2-ml samples were taken, and their protein content was determined (22). To test the growth of mutants on solid medium, 10-μl drops of cell suspensions of strains PCC 7120 and CSP19, at 1 μg Chl*a* · ml^{-1}, were spotted onto plates of $BG11_0$ medium, BG11 medium, or $BG11_0$ medium supplemented with 4 mM NH_4Cl and 8 mM TES-NaOH (pH 7.5), and the plates were incubated under culture conditions.

For phylogenetic analysis, predicted polypeptide sequences were aligned with the program ClustalX 1.8 (34). Phylogenetic trees were visualized with the NJplot program (28).

Cluster of three *amt* genes. The genome of *Anabaena* sp. strain PCC 7120 carries three open reading frames, which are clustered together, alr0990, alr0991, and alr0992, whose predicted protein products are homologues to the Amt proteins (15; see http://bacteria.kazusa.or.jp/cyano/). A phylogenetic analysis of Amt proteins shows that the Alr0991 protein is most similar to the Amt1 proteins of *Synechocystis* sp. strain PCC 6803 and *Synechococcus elongatus* strain PCC 7942 and that

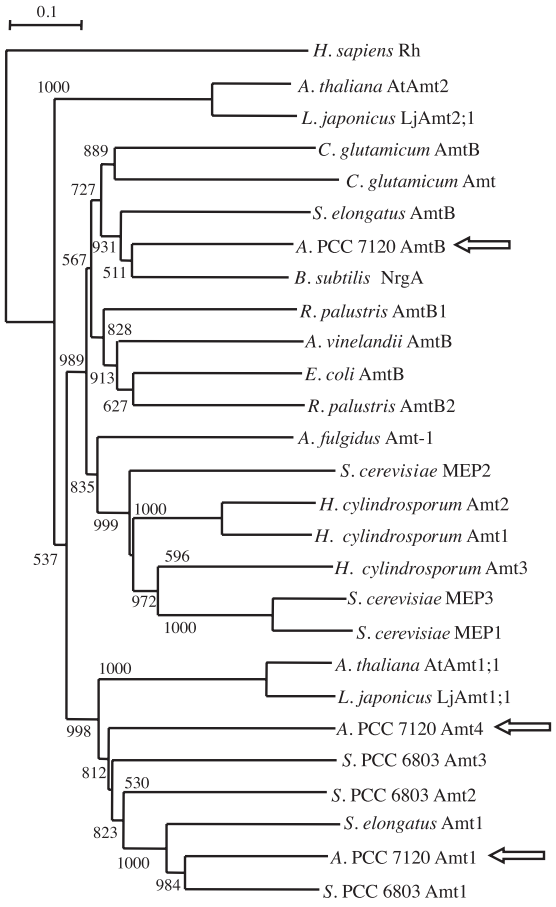

FIG. 1. Phylogenetic relationships of some Amt proteins (named MEP in *Saccharomyces cerevisiae* and NrgA in *Bacillus subtilis*). The analysis was restricted to some proteins that have been experimentally characterized, and a distantly related human (*Homo sapiens*) Rh protein was used as an outgroup. The Amt proteins were from the following species: *Arabidopsis thaliana*, *Lotus japonicus*, *Corynebacterium glutamicum*, *Synechococcus elongatus*, *Anabaena* sp. strain PCC 7120, *Bacillus subtilis*, *Rhodopseudomonas palustris*, *Azotobacter vinelandii*, *Escherichia coli*, *Archaeoglobus fulgidus*, *Saccharomyces cerevisiae*, *Hebeloma cylindrosporum*, and *Synechocystis* sp. strain PCC 6803. Bootstrap values based on 1,000 trials are indicated. The arrows point to the *Anabaena* sp. strain PCC 7120 (*A*. PCC 7120) Amt proteins. The bar represents 0.1 substitution per amino acid position. *S*. PCC 6803, *Synechocystis* sp. strain PCC 6803.

Alr0990 also clusters in the Amt1 branch of the phylogenetic tree, although its degree of similarity to Amt1 is lower than that of Alr0991 (Fig. 1). We designate the alr0991 gene *amt1*, and to distinguish it from the *Synechocystis amt2* and *amt3* genes, we designate the alr0990 gene *amt4*. On the other hand, Alr0992 clusters in the phylogenetic analysis with the bacterial AmtB proteins, including the *S. elongatus* AmtB protein (Fig. 1), and therefore, we designate the all0992 gene *amtB*. A similar cluster of *amt* genes, with homologous genes in the same order, is found in *Anabaena variabilis* (reviewed in reference 18). However, in the less closely related *Nostoc punctiforme*, only *amt4* and *amtB* are found, and only *amt1* is found in *Nodularia spumigena*.

Expression of the *amt* genes. To study whether the three *Anabaena amt* genes form an operon and the physiological conditions under which they are expressed, transcript levels were investigated by Northern (RNA-DNA hybridization) and RT-PCR analyses. For Northern analysis, RNA was isolated from filaments of *Anabaena* sp. grown with ammonium ($BG11_0C$ medium supplemented with NH_4Cl and TES buffer) or grown with ammonium, washed, and incubated in $BG11_0C$ medium that lacks any source of combined nitrogen for different times. Probes of the *amt* genes were generated by standard PCR with *Anabaena* DNA and the oligonucleotide primers indicated in Fig. 2A. With RNA from wild-type *Anabaena* sp. strain PCC 7120, the *amt4* probe hybridized to a band of 3.8 kb and to a smear of RNA molecules of different sizes up to about 1.7 kb, which were induced upon ammonium withdrawal (Fig. 2B). An *amt1* probe also marked transcripts of 3.8 and 1.7 kb, which were induced upon ammonium withdrawal; however, in this case, the 1.7-kb transcript was much more abundant than the 3.8-kb transcript (Fig. 2B). Finally, the *amtB* probe identified an inducible transcript of about 1.6 kb that, because it was difficult to detect, was likely expressed at a very low level (Fig. 2B). Induction of the *amt4*, *amtB*, and putative *amt4-amt1* transcripts was not observed with RNA from *Anabaena* sp. strain CSE2, a strain with a mutation of the *ntcA* regulatory gene (11). In this mutant, the expression of *amt1* was notably decreased, but an inducible transcript of about 1.7 kb was still observed (Fig. 2B).

For RT-PCR, RNA isolated from wild-type *Anabaena* filaments grown with ammonium and incubated for 2 h in $BG11_0C$ medium was used in retrotranscription reactions with oligonucleotide primers RT2 and RT4 (Fig. 2A), and the products of these reactions were subjected to PCR with the oligonucleotide primer pairs RT1/RT2, RT3/RT4, and RT1/RT4 (Fig. 2C). A strong PCR amplification product, clearly indicating cotranscription, was obtained only with the RT1/RT2 primer pair, whereas only a weak band was obtained with the RT3/RT4 primer pair and no amplification product was observed with RT1/RT4.

The results presented above are summarized in the gene expression scheme shown in Fig. 2A. Because the bands of 3.8 kb and 1.7 kb marked in the Northern analysis by the *amt4* probe are of similar intensities, we assume that a monocistronic *amt4* transcript and a bicistronic *amt4-amt1* transcript are similarly abundant in *Anabaena* sp. strain PCC 7120 in combined N-free medium. Cotranscription of *amt4* and *amt1* was corroborated by the RT-PCR analysis. However, the *amt1* probe marked a 1.7-kb band much more strongly than the 3.8-kb band. This suggests that, in addition to being expressed as an *amt4-amt1* transcript, *amt1* is independently expressed as a very abundant monocistronic transcript. Finally, although some cotranscription of *amt1* and *amtB* might occur, our results suggest that *amtB* is mainly expressed as a low-abundance monocistronic transcript. These results are consistent with those found with *Synechococcus* sp. strain PCC 7942, in which the level of expression of the *amt1* gene is about 100-fold that of *amtB* (26). Thus, *amt1*, which is the *amt* gene expressed at the highest levels in *Synechocystis* sp. strain PCC 6803 (24) and *Synechococcus* sp. strain PCC 7942 (26) and encodes the principal Amt polypeptide for methylammonium transport in both cyanobacteria (24, 35), is also the most highly expressed *amt* gene in *Anabaena* sp. strain PCC 7120.

Under the conditions we tested, expression takes place upon

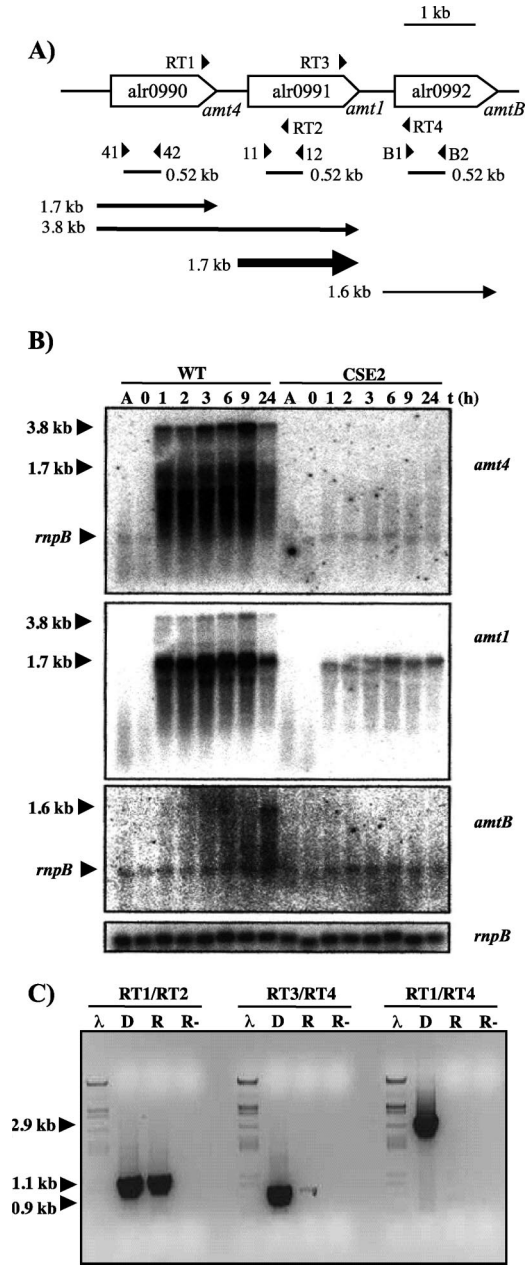

FIG. 2. Expression of the *amt* genes in *Anabaena* sp. strains PCC 7120 and CSE2 (*ntcA*::C.S3). (A) Scheme of the *Anabaena amt* genomic region (15). Arrowheads represent primers used in RT-PCR analysis (RT1, RT2, RT3, and RT4) and in Northern analysis to generate the *amt4* probe (primers 41 and 42), the *amt1* probe (primers 11 and 12), and the *amtB* probe (B1 and B2). The arrows at the bottom of the panel represent transcripts. (B) Northern analysis was performed with RNA isolated from cells of strain PCC 7120 (wild type [WT]) or CSE2 grown with ammonium (lanes A) or grown with ammonium and incubated for the indicated number of hours with no combined nitrogen. The filter was successively hybridized with the *amt4* probe, the *amt1* probe, an *rnpB* probe (that was used as a loading and transfer control), and the *amtB* probe. (C) RT-PCR analysis of cotranscription in the *amt* region. RNA isolated from cells of *Anabaena* sp. strain PCC 7120 grown with ammonium and incubated for 2 h without any source of combined nitrogen was used for retrotranscription with primer RT2 or RT4, and the products of these reactions were subjected to PCR with oligonucleotide RT1 (forward primer) and the same primer used for retrotranscription as reverse primer. The products of RT4 retrotranscription were also subjected to PCR with oligonucleotide RT3 (forward primer) and RT4. Amplification (lanes R) was observed only for the reaction with RT1 and RT2, which gave rise to a DNA fragment of the expected size, 1.1 kb. Control assays in which the RNA preparation was treated with RNase I (lanes R-) or in which the PCR was carried out with the same primers and strain PCC 7120 DNA (lanes D) are also presented. Lanes λ, phage lambda DNA digested with HindIII.

ammonium withdrawal in $BG11_0C$ medium in an NtcA-dependent process. Putative NtcA binding sites, $GTAN_8TAC$, are located 249 bp upstream of the translation start of *amt4* and 109 bp and 101 bp upstream of the translation start of *amtB*. Therefore, the *amt4*, *amt4-amt1*, and *amtB* transcripts could be produced from NtcA-dependent promoters. On the other hand, no standard NtcA binding site is found upstream of *amt1*. Primer extension analyses performed with RNA isolated from the wild type and the *ntcA* mutant strain CSE2 (not shown) identified a transcription start point located 199 or 200 nucleotides upstream from the translation start of *amt1* that could correspond to the start of the NtcA-independent *amt1* transcript observed in Fig. 2B. A putative σ^{70}-type promoter is found appropriately located upstream of this transcription start at chromosome coordinates 1,158,191 to 1,158,220. However, the promoter elements responsible for the prominent expression of *amt1* remain to be identified.

Isolation and characterization of an *amt* deletion mutant. Because we were interested in characterizing an *Anabaena* strain devoid of any Amt activity, a mutant with a deletion removing most of the DNA region covering the three *amt* genes was generated (Fig. 3A). DNA fragments from the 5′ region of *amt4* and from the 3′ region of *amtB* were amplified by PCR and cloned, separated by the C.K3 gene cassette, in plasmid pCSP94, which carries an Sm^r/Sp^r determinant and the *sacB* gene and can be mobilized by conjugation into *Anabaena* sp. strain PCC 7120. After conjugation, exconjugants resistant to Nm were selected in BG11 medium, which includes nitrate as a nitrogen source. A further screening for clones resistant to sucrose and sensitive to Sp/Sm identified clones that should have replaced the wild-type *amt* region with the Δ*amt*::C.K3 construct. PCR analysis with DNA isolated from a mutant clone, *Anabaena* sp. strain CSP19, and oligonucleotide primers amts-7120-1 and amts-7120-8, which are external to the *amt* genes, confirmed that this strain carries the Δ*amt*::C.K3 construct (not shown). PCR analysis performed with primers specific for each *amt* gene and for the *sacB* gene confirmed that this clone was missing all the *amt* genes and lacked the *sacB* gene (not shown), which indicates that no wild-type chromosomes remained and that the vector part of pCSP94 has been lost in strain CSP19.

Assays of uptake of 1 μM [^{14}C]methylammonium were carried out with *Anabaena* sp. strains PCC 7120 (wild type) and CSP19 (Δ*amt*::C.K3). Ammonium-grown filaments that had been incubated for 90 min in the absence of combined nitrogen (with a high supply of CO_2) to induce the *amt* genes were used in the assays. Whereas the wild-type strain showed a substantial activity of uptake, including the two phases characteristic of [^{14}C]methylammonium uptake (3), the mutant was hampered in this activity, confirming the identity of at least one of

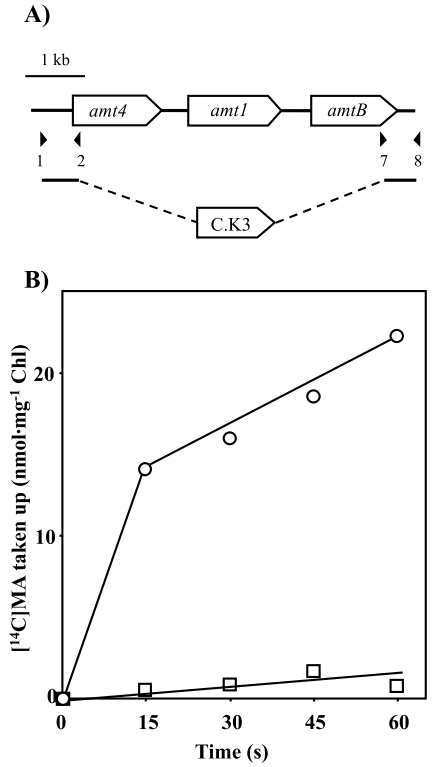

FIG. 3. Deletion of *amt* genes and uptake of [^{14}C]methylammonium by *Anabaena* sp. strain PCC 7120 and mutant CSP19 (Δ*amt*::C.K3). (A) Scheme showing the *Anabaena amt* region replaced by the C.K3 cassette in the generation of mutant strain CSP19 (see the text for details). The arrowheads labeled 1, 2, 7, and 8 indicate the approximate locations of deoxyoligonucleotide primers amts-7120-1, amts-7120-2, amts-7120-7, and amts-7120-8, respectively. (B) Ammonium-grown cells incubated for 90 min in BG11$_0$C medium with a high supply of CO_2 were used in uptake assays in phosphate-bicarbonate buffer with 1 μM [^{14}C]methylammonium ([^{14}C]MA). Uptake in wild-type strain PCC 7120 (circles) and mutant strain CSP19 (squares) is depicted.

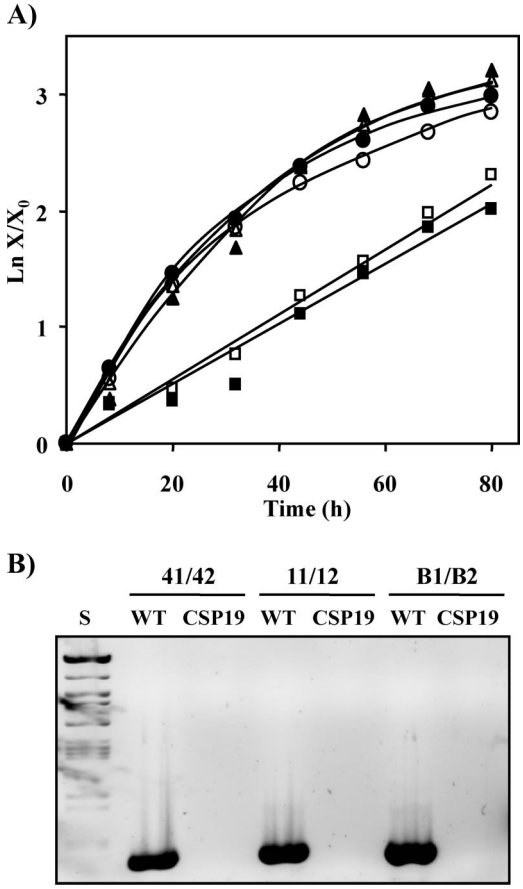

FIG. 4. Growth of *Anabaena* sp. strain PCC 7120 and mutant CSP19 with different nitrogen sources. (A) Cells of strain PCC 7120 (open symbols) and strain CSP19 (closed symbols) were incubated in liquid medium with ammonium (circles), nitrate (triangles), or dinitrogen (squares) as nitrogen source (see "Methods"). X_0, protein concentration at time zero; X, protein concentration at sample time. (B) PCR analysis carried out on DNA from strain PCC 7120 (wild type [WT]) or CSP19 grown with dinitrogen as a nitrogen source. The locations of primers used for PCR (primer pairs 41/42, 11/12, and B1/B2) are shown in Fig. 2A. Positive controls for PCR amplification with DNA from strain CSP19 were carried out with primers of two unrelated genes, all4294 and alr2394 (not shown). S, phage lambda DNA digested with HindIII used as a size standard.

the *amt* gene products as a (methyl)ammonium transporter (Fig. 3).

The growth performance of strain CSP19 (Δ*amt*::C.K3) compared to that of the wild type was determined using ammonium, nitrate, or dinitrogen as a nitrogen source. In liquid media, no growth defect was detected with the mutant (Fig. 4A). PCR analysis of diazotrophically grown cells showed that the mutant strain lacked all the *amt* genes, confirming that no wild-type chromosomes remained (Fig. 4B). These results indicate that none of the *amt* gene products is needed for growth under these laboratory conditions. Growth on solid medium using ammonium, nitrate, or dinitrogen as a nitrogen source was also not impaired by deletion of the *amt* genes (not shown). No indication of heterocyst development was observed in the cultures with 4 mM NH_4Cl. Thus, ammonium is utilized in these cultures and supports growth of the cyanobacterium lacking any Amt protein (see also reference 26). Diffusion of ammonia present in solutions at these ammonium concentrations appears to be sufficient to provide the nitrogen required for growth (26). As in the unicellular cyanobacteria (24, 26, 35), in *Anabaena* sp. strain PCC 7120, the Amt proteins might be involved in uptake of ammonium when it is present at very low concentrations in the extracellular medium.

A corollary of these observations is that the Amt proteins are not needed for the diazotrophic growth of *Anabaena* sp. strain PCC 7120. This is in contrast to the requirement of the amino acid transporters N-I and N-II for optimal diazotrophic growth of this cyanobacterium (27, 29). Recent advances in the knowledge of the structure of the *Anabaena* filament have shown two possible communication conduits between cells: a continuous periplasm (21) and cell-to-cell connecting structures located at the intercellular septa (10, 25). Molecular transfer through the periplasm would require permeases mediating membrane transport into and out of the cytoplasm (9). Because some amino acid transporters are required for optimal diazotrophic growth but the Amt proteins are not, if the periplasmic route were important, our results would be con-

sistent with the notion that heterocysts transfer fixed nitrogen to the vegetative cells as glutamine (33, 40) or other amino acids (29) rather than as ammonium itself.

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