Ammonium/Methylammonium Permeases of a Cyanobacterium

IDENTIFICATION AND ANALYSIS OF THREE NITROGEN-REGULATED amt GENES IN SYNECHOCYSTIS sp. PCC 6803*

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Ammonium is an important nitrogen source for many microorganisms and plants. Ammonium transporters whose activity can be probed with [14C]methylammonium have been described in several organisms including some cyanobacteria, and amt genes encoding ammonium/methylammonium permeases have been recently identified in yeast, Arabidopsis thaliana, and some bacteria. The unicellular cyanobacterium Synechocystis sp. PCC 6803 exhibited a [14C]methylammonium uptake activity that was inhibited by externally added ammonium. Three putative amt genes that are found in the recently published complete sequence of the chromosome of strain PCC 6803 were inactivated by insertion of antibiotic resistance-encoding gene-cassettes. The corresponding mutant strains were impaired in uptake of [14C]methylammonium. Open reading frame sll0108 (amt1) was responsible for a high affinity uptake activity (Kₘ for methylammonium, 2.7 μM), whereas open reading frames sll0117 (amt2) and sll0537 (amt3) made minor contributions to uptake at low substrate concentrations. Expression of the three amt genes was higher in nitrogen-starved cells than in cells incubated in the presence of a source of nitrogen (either ammonium or nitrate), but amt1 was expressed at higher levels than the other two amt genes. Transcription of amt1 was found to take place from a promoter bearing the structure of the cyanobacterial promoters activated by the nitrogen control transcription factor, NtcA.

Ammonium is a key compound in the assimilation of nitrogen in numerous biological systems because it is the inorganic form of nitrogen that is incorporated, usually via the glutamine synthetase/glutamate synthase cycle, into carbon skeletons. Ammonium transport is present in the environment and can be assimilated by many bacteria, yeast, fungi, algae, and higher plants. Ammonium solutions always contain ammonia (pKₐ [ammonium/ammonia], 9.25), which can diffuse through biological membranes (1, 2). Diffusion of ammonia followed by trapping of intracellular ammonium by glutamine synthetase can represent a significant process for nitrogen acquisition, especially in microorganisms like some bacteria, which can grow in alkaline media. This process would be less important in fungi that grow in acidic media. On the other hand, there is evidence for the presence of ammonium transport systems in numerous organisms (2). The first report of active ammonium uptake, by Hackette et al. (3), concerned the fungus Penicillium chrysogenum. These authors introduced the use of [14C]methylammonium as a probe for the activity of the ammonium permease, a technique that has proven useful to study ammonium transport in many other biological systems including bacteria (4) (the pKₐ of methylammonium/methylamine is 10.65). Bacterial ammonium/methylammonium permeases are commonly repressed by high concentrations of ammonium in a process that, in the enterobacteria, involves the nitrogen control Ntr system (Refs. 5 and 6; reviewed in Ref. 7).

A gene, amtA, putatively involved in ammonium transport has been described in Escherichia coli (8), but it rather appears to be involved in cysteine synthesis and has been renamed as cysQ (9). On the other hand, genes encoding ammonium/methylammonium permeases have been characterized in Saccharomyces cerevisiae (MEP genes (10, 11)) and Arabidopsis thaliana (AMTI (12)). Genes that would encode proteins homologous to the MEP and AMTI gene products are found in some bacteria like Bacillus subtilis (nrgA (13)), Rhodobacter capsulatus (14), Escherichia coli (15), Corynebacterium glutamicum (16), Azospirillum brasilense (17), and Azotobacter vinelandii (18). Inactivation of the putative amt genes of C. glutamicum, A. brasilense, A. vinelandii, and E. coli has confirmed that they encode permeases that can mediate methylammonium transport (16–19).

Cyanobacteria are organisms that belong to the bacteria (or eubacteria) group (20) and are characterized by their ability to perform oxygenic photosynthesis. Sources of nitrogen used by many cyanobacteria include nitrate, dinitrogen, urea, and ammonium (21). In cyanobacteria, incorporation of ammonium into carbon skeletons takes place mainly through the glutamine synthetase/glutamate synthase cycle (Ref. 22; reviewed in Ref. 21). The pH values of cyanobacterial growth media are usually above neutral (23). Diffusion of ammonia through cyanobacterial cytoplasmic membranes has been demonstrated (24) and can provide, pulled by glutamine synthetase, a mechanism for the net uptake of ammonium (see e.g. Ref. 25). Expression of an ammonium/methylammonium transport activity in some cyanobacterial strains, including Synechococcus sp. PCC 7942, Anabaena azollae, and Anabaena variabilis, has also been reported (26, 27). In Synechococcus sp. PCC 7942, the ammonium/methylammonium transport activity is repressed by growth in ammonium-containing medium (28) and requires an intact ntcA gene to be expressed (29). NtcA is a cyanobacterial transcriptional regulator, homologous to Crp from E. coli, that activates the expression of ammonium-repressible genes in the absence of ammonium (30, 31). The DNA target to which NtcA binds in the promoter of the regulated genes has been characterized in Synechococcus sp. PCC 7942 and contains the

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sequence signature GTAN8TAC located about 22 bp upstream from a −10, Pribnow box in the form TAN_{T}T (31). This promoter motif for NtcA-regulated genes is predicted to be valid for other cyanobacteria as well, because the putative helix-turn-helix motif for binding to DNA is identical in the NtcA polypeptides from different sources, including Synchocystis spp. PC6803 and Anabaena sp. PCC 7120 (32).

The whole sequence of the chromosome of the unicellular cyanobacterium Synchocystis sp. PC6803 has been determined (33)2 and shown to contain three ORFs, namely sll0108, sll0537, and sll1017, that would encode polypeptides homologous to the AMT1 and MEP gene products cited above. Another ORF (sll0895) described by Kaneko et al. (33) as ammonium transport protein would actually encode a homologue of CysQ (see above). In this work, we show that the three putative amt genes of Synchocystis sp. PC6803 actually provide the cells with the capability to take up [14C]methylammonium from the extracellular medium and are subjected to repression by ammonium.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions—Synchocystis sp. strain PC6803 was grown axenically in BG11 (nitrate-containing) medium (23) or in BG11C (nitrogen-free) medium supplemented with 5 mM NH\(_4\)NO\(_3\) and 30 mM MES-NaOH buffer (pH 7.5). For plates, the medium was solidified with 1%, separately autoclaved agar (Difco). Cultures were grown at 30 °C in the light (600-800 μmol photons m\(^{-2}\) s\(^{-1}\)) with, when necessary, 50 mM sodium bicarbonate-bicarbonate buffer. After incubation for the time periods indicated in the absence of any nitrogen source for 6 h were harvested by centrifugation and resuspended in BG11C medium. Cultures used for RNA isolation and for metabolic studies were incubated in the dark at 30 °C in the light (100-150 μmol photons m\(^{-2}\) s\(^{-1}\)) with, when necessary, 50 mM sodium bicarbonate-bicarbonate buffer. After a preincubation at 30 °C in the light (100 watt ml\(^{-2}\)) with, when necessary, 50 mM sodium bicarbonate-bicarbonate buffer. After a preincubation at 30 °C in the light (100 watt ml\(^{-2}\)) with, when necessary, 50 mM sodium bicarbonate-bicarbonate buffer. After a preincubation at 30 °C in the light (100 watt ml\(^{-2}\)) with, when necessary, 50 mM sodium bicarbonate-bicarbonate buffer.

**DNA and RNA Isolation and Manipulation—Isolation of genomic DNA from cyanobacteria was carried out as described previously (38), except for strain UTEX 1829 cells, which were frozen with liquid air and broken by grinding with glass beads in a mortar. Isolation of total RNA from Synchocystis sp. PC6803 was made as described previously (39). Other molecular biology manipulations were carried out by standard procedures (40).**

**Inactivation of ORFs sll0108, sll1017, and sll0537—DNA fragments corresponding to strain PC6803 ORFs sll0108, sll1017, and sll0537 (33) were amplified by PCR as described previously (41). The primers used (see Fig. 4 below) were: Tr15, 5′-GCCATTTCGAGAAGATCGCCATCCGTTCTTACAAGGAATCACGAGAATTGATAGTATAGGGCCTG-3′; Tr16, 5′-TGACTAAGGCTG-CCA ACCCGGC-3′; Tr3a, 5′-GGTTTCTGCTACTTATGTTGCGGCG-3′; and Tr3b, 5′-TCACGGTTGGCCGCGTTCAAGG-3′.**

**PCR products were cloned in pGEM-T vector (Promega). Plasmids containing PCR products were generated with primers Tr18-Tr19, Tr2a-Tr2b, and Tr3a-Tr3b were named pCSX23, pCSX53, and pCSX52, respectively. Plasmid pCSX23 was digested with ApcI and PstI to isolate the insert that was then cloned between the ApoI and PstI sites of Bluestrip SK(+), generating plasmid pCSX46. The 1.1-kb Km-gene cassette C3 (34) excised with SmaI was inserted into an unique MseI site of pCSX53 and into the unique MseI site of pCSX52 (these MseI sites are present in the strain PC6803 derived-inserts of the plasmids), rendering plasmids pCSX57 and pCSX56, respectively. The orientation of C3 in the inserts of pCSX56 and pCSX57 was not determined. Sequences of the inserts of pCSX23, pCSX53, and pCSX52 were verified by using a 5′-Sequencing kit (Amersham Pharmacia Biotech; the sequencing was performed by Hybrid-DNA Labo, Tokyo).**

**Transformation of Synchocystis sp. PC6803 with plasmids pCSX47a, pCSX47b, pCSX56, or pCSX57 as well as transformation of insertion mutant strain CSX47a with pCSX56 or pCSX57 was carried out as described previously (41). Transformants were selected in BG11 solid medium supplemented with 30 pg of Km·ml\(^{-1}\) or 10 pg of Cm·ml\(^{-1}\). Km′ and Cm′ transformants were then grown in medium supplemented with 20 pg of Km·ml\(^{-1}\) or 20–80 pg of Cm·ml\(^{-1}\).**

**Methylammonium Uptake Assays—Wild-type or mutant Synchocystis cells grown in nitrate- or ammonium-containing medium or incubated in the absence of any nitrogen source for 6 h were harvested by low speed centrifugation at room temperature, washed with 20 mM KH\(_2\)PO\(_4\), 10 mM NaHCO\(_3\)-NaOH buffer (pH 7.1) and resuspended in the same buffer. After a preincubation at 30 °C in the light (100 watt ml\(^{-2}\) s\(^{-1}\), white light) for 5 to 30 min, the assays were started by mixing the suspension of cells (4 to 15 μg of Chl·ml\(^{-1}\)) with a solution of \[^{14}C\]CH\(_3\)NH\(_2\)-HCl (50 μCi·μmol\(^{-1}\); ICN Pharmaceuticals, Inc.) in phosphate-bicarbonate buffer. After incubation for the time periods indicated in each experiment, 0.1- to 1 ml samples were filtered (0.45-μm pore size Millipore HA filters were used) and washed with 2 to 5 ml of phosphate-bicarbonate buffer. The filters carrying the cells were then immersed in scintillation mixture, and their radioactivity was measured. Retention of radioactivity by boiled cells was used as a blank. In some experiments, as indicated, 1 mm 1-methionine-1,1-d,1-sulfonamide was added to the cell suspension 25 min before the assay was started.

1 The abbreviations used are: bp, base pair(s); Ap, ampicillin; Chl, chlorophyll a; Cm, chloramphenicol; Km, kanamycin; ORF, open reading frame; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; tsp, transcription start point; PCR, polymerase chain reaction; kb, kilobase pair(s).

2 The www address of the sequence reported in Ref. 33 is: http://www.kazusa.or.jp/cyano/cyano.html.
agarose gels) with total cyanobacterial RNA (42), 15 μg of RNA from strain PCC 6803 was partially hydrolyzed by incubation in 125 mM NaOH for 30 min at 0 to 4 °C and labeled with T4 polynucleotide kinase (Boehringer Mannheim) and γ-32P-ADP (43). Hybridization was performed at 65 °C in a solution containing: 50 mM Tris-HCl, pH 8.0, 0.2% bovine serum albumin, 0.2% Ficoll, 0.1% sodium pyrophosphate, 1% SDS, 1 mM NaCl, and 100 μg/ml yeast tRNA (Boehringer) (44). Filters were washed twice at 65 °C for 30 min with 1× SSC and 1% SDS and once at room temperature for 15 min with SSC 0.2× (1× SSC is 0.15 M NaCl and 15 mM sodium citrate (pH 7.0)). Radioactivity of bands in filters was quantified with an InstantImager scanner.

**Purification of NtcA Protein**—A 525-bp fragment putatively containing the promoter region of ORF sll0108 was amplified using primers Am1 (5'-GGGAGCACTAATGGCAGG-3'), corresponds to positions –236 to –215 with respect to the translational start of ORF sll0108). Am2 (5'-CGCGTTCAATTACCGTAGGC-3') complementary to nucleotides +289 to +268 with respect to the translational start of ORF sll0108). The PCR product was cloned in pGEM-T vector, rendering plasmid pCSX49. This plasmid was used to generate dideoxy-sequencing ladders for primer extension analysis. Oligonucleotides used for primer extension were Am2 (see above) and Am3 (5'-GGGACACGGCCCAACCGAGGAG-3'); complementary to nucleotides +146 to +125 with respect to the translational start of ORF sll0108). Oligonucleotides were end-labeled with T4 polynucleotide kinase (Boehringer Mannheim) and γ-32P-ATP (43). Hybridization was performed at 65 °C in a solution containing; 50 mM Tris-HCl, pH 8.0, 0.2% bovine serum albumin, 0.2% Ficoll, 0.1% sodium pyrophosphate, 1% SDS, 1 mM NaCl, and 100 μg/ml yeast tRNA (Boehringer) (44). Filters were washed twice at 65 °C for 30 min with 1× SSC and 1% SDS and once at room temperature for 15 min with SSC 0.2× (1× SSC is 0.15 M NaCl and 15 mM sodium citrate (pH 7.0)). Radioactivity of bands in filters was quantified with an InstantImager scanner.

**RESULTS**

**Transport of Labeled Methylammonium**—The cellular activity level of uptake of [14C]methylammonium in *Synechocystis* sp. PCC 6803 is higher in cells starved for nitrogen than in nonstarved cells (grown with either nitrate or ammonium). Additionally, growth in CO2-enriched air allowed higher [14C]methylammonium uptake activities (Fig. 1). Determination of the rate of [14C]methylammonium uptake in 1-min assays using a range of substrate concentrations from 1 to 75 μM showed one kinetic component with the following parameters: Km, 2.7 μM; Vmax, 169 nmol·min⁻¹·(mg of Chl)⁻¹. Methylammonium does not serve as a nitrogen source in strain PCC 6803 (not shown), and as is the case in some other cyanobacteria (26, 27), intracellular radioactivity derived from [14C]methylammonium was distributed into two metabolites (Fig. 2). One of these metabolites was identified as methylammonium itself by co-chromatography with authentic [14C]methylammonium. Production of the second metabolite was hampered (96.4 and 97.5% inhibition in two independent experiments, respectively) by treatment of the cells with 1-methionine-d₅-l-sulfoximine, an inhibitor of glutamine synthetase, putatively identifying that metabolite as γ-glutamylmethylylalnine (47, 48). In experiments where [14C]methylammonium was supplied at 1 μM, intracellular methylammonium concentrations of about 50 μM, which represented, at the time of sampling, an intracellular to extracellular methylammonium ratio of close to 60, were detected. The process of [14C]methylammonium uptake was completely inhibited by the addition of ammonium (Fig. 3). Inhibition by ammonium was reversible, and the lag period before [14C]methylammonium uptake commenced was almost proportional to the added concentration of ammonium. Assuming that [14C]methylammonium uptake started when ammonium was exhausted from the incubation medium, an ammonium uptake rate of 780 nmol·min⁻¹·(mg of Chl)⁻¹ can be calculated that is similar to the figures obtained in direct determinations of ammonium uptake (not shown).

**Inactivation of Putative amin Genes**—Internal fragments from strain PCC 6803 ORFs sll0108, sll0537, and sll1017 were amplified by PCR and cloned. Gene-cassettes C.K3 (sll0108) or C.C1 (sll0537 and sll1017) were inserted into the cloned DNA fragments, and the resulting constructions (pCSX47a, pCSX47b, pCSX56, and pCSX57; see Fig. 4) were transferred to strain PCC 6803 by transformation. Km' and Cm' transformants were selected and further grown in antibiotic-containing medium, and the presence of mutant and wild-type chromosomes in these cells was studied by hybridization and PCR amplification. Clones that were homologous for the mutated chromosomes were chosen and named CSX47a (sll0108::C.K3, that carries the gene-cassette in the same orientation as sll0108), CSX47b (sll0108::C.K3, that carries the gene-cassette in the orientation opposite to sll0108), CSX56 (sll0537::C.C1), and CSX57 (sll1017::C.C1). Double mutants were then generated by transformation of strain CSX47a with pCSX56 or pCSX57, producing strains CSX200 (sll0108::C.K3, 3 A. M. Murro-Pastor, A. Herrero, and E. Flores, unpublished data.

![Fig. 1. Time course of [14C]methylammonium uptake in *Synechocystis* sp. PCC 6803. Cells grown in shaken cultures (open symbols) or in cultures bubbled with CO₂-enriched air (closed symbols) with ammonium (circles) or nitrate (triangles) or starved for nitrogen for 6 h (squares) were used in uptake assays with 6 μM [14C]methylammonium as a substrate.](image-url)
Glutamine synthetase and the capability to take up 1 μM [14C]methylammonium were determined in cells incubated for 6 h in BG110 medium for each of the mutants described above and the wild-type strain PCC 6803. Similar glutamine synthetase activity levels, 40 to 50 units (mg of Chl)⁻¹, were found in all the strains. On the other hand, inactivation of ORF sll0108 abolished [14C]methylammonium uptake by about 97%, whereas inactivation of sll0537 or sll1017 had only minor effects (Table I). Nonetheless, time course assays carried out with low substrate concentrations (5 to 270 nM) confirmed that inactivation of sll0537 or sll1017 further impaired [14C]methylammonium uptake in the sll0108-inactivated mutant (see data for 50 nM [14C]methylammonium in Fig. 5). The three investigated ORFs appear therefore to encode permeases that mediate methylammonium uptake. We have named them as amt1 (sll0108), amt2 (sll1017), and amt3 (sll0537), respectively.

The effect of the concentration of [14C]methylammonium on the rate of uptake was studied in mutant strains CSX47a,
genes were hybridized to 32P-labeled total RNA. The RNA was bound, PCR-generated DNA fragments from the three
rate this observation, we performed experiments where filter-
shown)).

Am3). (A band of about 2.6 kb was

porter mediated by Amt2 or Amt3, thus impeding the determi-

A single mRNA of about 2 kb was detected for
A

The expression of

Analysis of Expression of the amt Genes—The expression of
each amt gene was studied by Northern analysis using total RNA isolated from cells of strain PCC 6803 grown with nitrate or ammonium as the nitrogen source or grown with nitrate and incubated in the absence of any source of nitrogen for 6 h. As a probe, a DNA fragment generated by PCR as described above (see also Fig. 4) was used for each amt gene.

A relative levels of this transcript in the tested nitrogen regimes were 1:1.76:30 (ammonium:nitrate:minus nitrogen). An mRNA of about 1.5 kb was observed for amt2 only in nitrogen-starved cells (Fig. 6B). (A band of about 2.6 kb was also detected with the amt2 probe and RNA from the three different nitrogen regimes. It is possible, however, that this band represents nonspecific hybridization with some rRNA.) Although hardly visible, a 2.6-kb mRNA was detected only in nitrogen-starved cells for amt3 (Fig. 6C). These results showed that the three amt genes of strain PCC 6803 are expressed and that, in the three cases, expression is activated under nitrogen stress. Indeed, activation of expression took place quickly in response to nitrogen starvation, as shown in Fig. 7 for amt1 (similar results were obtained with an amt2 probe, whereas the amt3 transcript was hardly visible at any incubation time (not shown)).

Because the amt1 transcript was more readily detectable than the amt2 or amt3 transcripts, amt1 appears to be expressed at higher levels than the other two genes. To corroborate this observation, we performed experiments where filter-bound, PCR-generated DNA fragments from the three amt genes were hybridized to 32P-labeled total RNA. The RNA was isolated from cells of strain PCC 6803 grown with ammonium or nitrate as the nitrogen source or grown with nitrate and starved for nitrogen for 6 h. With RNA isolated from nitrogen-starved cells, hybridization was detected for the three genes, and the relative level of labeling was 1:6:54 (amt3:amt2:amt1) (Table II). With RNA isolated from ammonium- or nitrate-grown cells, only hybridization to DNA corresponding to the amt1 gene was detected.

Possible NtcA-dependent Transcription Start Point for the amt1 Gene—The possible tsp of amt1 was investigated by primer extension analysis. Two oligonucleotides, Am2 and Am3, complementary to sequences located close to the 5' end of the amt1 gene (Fig. 8A) were used as primers. An extension product whose 3' end corresponded to a T doublet located 142 nucleotides upstream from the amt1 start codon was detected with the Am3 primer. This extension product was much more efficiently obtained with RNA isolated from nitrogen-starved cells than from cells grown with nitrate and more with RNA from nitrate- than from ammonium-grown cells (Fig. 8B). The possible tsp of the amt1 gene can thus be localized to the A doublet indicated in Fig. 8A that is preceded by a putative −10 box in the form TTGAA. Seventeen nucleotides upstream from this box a TACAGA hexamer is found that might be considered a poor −35 promoter box. However, 20 nucleotides upstream from the −10 box a nucleotide sequence, AAAAGTAN8TAC, is found that would represent a perfect NtcA-binding site (Fig. 8C). See “Experimental Procedures” for conditions of hybridization and for generation of DNA probes. Sizes in kilobases are indicated on the left.

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of NtcA and a 138-bp DNA fragment corresponding to the first 138 nucleotides shown in Fig. 8A. Binding to a 244-bp fragment containing the putative NtcA-binding site that is located immediately upstream of the \textit{amt1} gene was also tested (see Fig. 8A). In both cases, a nonrelated, control DNA was included in the assay. As shown in Fig. 8C, retardation was only observed with the 138-bp DNA fragment containing the putative \textit{amt1} promoter. On the other hand, no retardation was observed in binding assays carried out with extracts of an \textit{E. coli} strain bearing expression vector pQE9 (not shown). These results suggest that the 138-bp DNA fragment bears a real NtcA-binding site, whereas the significance, if any, of the putative NtcA-binding site present in the 244-bp DNA fragment remains to be investigated.

**Occurrence of \textit{amt} Homologous Sequences in Other Cyanobacteria**—The same DNA fragments corresponding to the three \textit{amt} genes used to probe Northern blots were used in Southern blot analyses to investigate the presence of putative \textit{amt} homologues in some other cyanobacteria (not shown). Hybridization with the \textit{amt1} probe was observed for every cyanobacterium tested, including strains of taxonomic sections I (\textit{Synechococcus} sp. PCC 7942), II (\textit{Pseudanabaena} sp. PCC 6903), IV (\textit{Anabaena} sp. PCC 7120, \textit{Calothrix} sp. PCC 7601, and \textit{Nostoc} sp. strains PCC 7107 and PCC 7413), and V (\textit{F.}
muscicola UTEX 1829). Appreciable hybridization with the
amt2 probe was observed with DNA from all the strains but
UTEX 1829 and PCC 7120. A clear, though weak, hybridization
signal with the amt3 probe was only observed with DNA from
UTEX 1829.

DISCUSSION

Synechocystis sp. PCC 6803 shows an activity of [14C]methylammonium uptake that exhibits characteristics similar to
those of methylammonium uptake in other cyanobacteria in-
cluding Synechococcus sp. PCC 7942 and A. variabilis ATCC
29413 (26, 27). Thus, [14C]methylammonium uptake would
result from an initial transport of methylammonium followed
by further transport and metabolism via glutamine synthetase.
Under our experimental conditions, an accumulation of [14C]m-
ylammonium within the cells representing an intracellular
to extracellular ratio of methylammonium of up to about 60
was observed. Ratio values of up to 200 or 40 have been re-
ported for strains PCC 7942 and ATCC 29413, respectively (26,
27). An intracellular to extracellular ratio of 60 would corre-
spond, under the incubation conditions used in this work, to
a free energy change for [14C]methylammonium transport equiva-
 lent to $+107 \text{mV}$. Because the membrane potential of photo-
synthetically active cyanobacterial cells is known to be in the
range of $-110$ to $-130 \text{mV}$ (49, 50), the observed accumulation of
methylammonium is close to that which would be permitted
by the membrane potential. The effects of some metabolic in-
hibitors on methylammonium uptake have also been inter-
preted in terms of methylammonium transport being depend-
ent on the membrane potential of the cells (1, 16, 26, 27).
Interestingly, methylammonium influx has been shown to de-
crease the membrane potential in some algae (51, 52). It should
be noted, however, that the observed accumulation of [14C]m-
ylammonium may represent an underestimate of the methylammonium transport activity of the cells, because some
of the [14C]methylammonium taken up may be leaking out
from the cells by diffusion as [14C]methylamine (24).

Inactivation of each of the three putative amt genes of Syn-
echocystis sp. PCC 6803, namely sll1018 (amt1), sll0537 (amt3),
and sll1017 (amt2), impairs [14C]methylammonium uptake (Table I, Fig. 5). Tight inhibition by ammonium of [14C]methyl-
ammonium uptake (see Fig. 3 and Refs. 3, 12, 16, 26, 53, 54)
suggests that ammonium is the natural substrate for the permease(s) taking up methylammonium, especially in organisms
for which methylammonium is not a nutrient. The affinity of
those permeases for ammonium would be higher than for methyl-
ammonium ($K_m$ can be 40 to 100 times lower for ammonium
than for methylammonium (Refs. 3, 11, 53)). The Amt1 perme-
ase, which appears to be responsible for the $K_m$ methylammonium uptake kinetic component exhibited by strain PCC 6803, would thus represent a transport system with
a very high affinity for ammonium and would therefore be able
to mediate the uptake of ammonium that may be found at very
low concentrations in some natural habitats. Amt2 and Amt3,
on the other hand, make only a limited contribution to uptake of
methylammonium in the $\mu$m range and could therefore rep-
resent permeases with a lower affinity for ammonium/methyl-
ammonium than Amt1. The Synechocystis amt mutants iso-
lated in this work, including strains that bear only one func-
tional amt gene (strain CSX201, anmt1$, amt2$, amt3$; strain
CSX201; amt1$, amt2$, amt3$), are however still able to
grow using ammonium as a nitrogen source (not shown). It is
currently unknown whether diffusion of ammonia or transport
of ammonium via Amt2 or Amt3 is responsible for ammonium-
dependent growth of those mutants. An impairment in ammox-
nium-dependent growth has only been reported in a strain of S.
cerevisiae lacking the three MEP genes present in this orga-
nism (11) and in an E. coli amtB mutant when the cells were
incubated at pH values below 7 (19).

The regulatory pattern of expression of the amt genes sug-
gests a major role for the Amt permeases in uptake of ammox-
nium present at low concentrations in the extracellular me-
dium. [14C]Methylammonium uptake activity, representing
transport plus metabolism via glutamine synthetase, is re-
pressed by ammonium and is maximal in cells starved for
nitrogen in CO2-enriched air (Fig. 1). Consistently, the three
amt genes are preferentially expressed in nitrogen-starved
cells (Fig. 6). Expression of amt1, however, is much higher than
expression of amt2 or amt3 under any of the tested nitrogen
regimes. This resembles the situation in S. cerevisiae where the
gene encoding the permease with highest affinity for ammo-
nium, MEP2, is expressed at a much higher level than the
MEP1 and MEP3 genes encoding lower affinity permeases (11).

We have further studied the expression of amt1 and have
found that transcription of this gene takes place from a pro-
moter that shows the structure of the cyanobacterial NtcA-
activated promoters (31). Additionally, a DNA fragment carry-
ings this promoter binds NtcA in vitro (Fig. 8). Like some other
genes characterized in Synechococcus sp. PCC 7942 and
Anaerena sp. PCC 7120 that are subject to repression by
ammonium (29, 42, 55–58), amt1 appears to belong to the NtcA
regulon. A Synechocystis sp. PCC 6803 ntcA mutant is not yet
available to confirm NtcA-dependent gene expression in this
cyanobacterium. Nonetheless, NtcA-mediated nitrogen control
can be important also in Synechocystis sp. PCC 6803, because
a number of genes of this strain have been shown to be tran-
scribed from NtcA-type promoters that bind NtcA in vitro.
These include, in addition to amt1, icd coding for isocitrate
dehydrogenase (59), glnA for glutamine synthetase (60), and
glnB for the cellular nitrogen status signaling protein $P_{gln}$ (39).

The sizes of the transcripts for the three Synechocystis amt
genes (Fig. 6) were analyzed in relation to the DNA sequences
surrounding these genes (Ref. 33; see also Fig. 4). The amt1
gene, which is composed of 1521 bp, would be transcribed as
a monocistronic mRNA (observed transcript size, 2 kb). For
amt2, a gene consisting of 1326 bp, the observed 1.5-kb hybridi-
zation band would also correspond to a monocistronic tran-
script. The amt3 gene, which is composed of 1623 bp, is found
downstream of an ORF (sll0536) with which it shows a 4-nu-
cleotide overlap. A transcript containing both ORFs (amt3 and
sll0536) would have a size of, at least, 2693 nucleotides, which
roughly corresponds to the size of the mRNA detected with the
amt3 probe (2.6 kb). Interestingly, sll0536 would encode a
polypeptide that shares homology with a putative potassium
channel protein of E. coli (accession number P31069). Whether
this implies a coordinated function of Amt3 and a potassium
channel remains to be investigated.

The Synechocystis Amt proteins as well as their homologues
from other biological sources are highly hydrophilic polypep-
tides that show 10 to 12 putative membrane-spanning regions.
They appear to constitute monocistronic permeases whose
activity would not depend on ATPase subunits or periplas-
mic-binding proteins. Consistently, the methylammonium uptake
activity of Synechococcus sp. PCC 7942 is preserved in spher-
oplasts (26). As deduced from data found in currently available
data banks, the Amt family would comprise proteins from very
diverse biological groups. In addition to the three Amt per-
meases from Synechocystis sp. PCC 6803, some other members
of this protein family are the three MEP proteins from yeast
(accession numbers P40260, P41948, and P53390, respective-
ly), AMT1 from A. thaliana (P54144), Amt from C. glutamicum
(P54146), A. brasilense (AF005275), A. vinelandii (U91902),
and E. coli (P37905), and putative ammonium permeases of B.
subtilis (NrgA; Q07429), Mycobacterium tuberculosis (Q10968),
Methanococcus jannaschii (Q58739 and Q60366), and Caenorhabditis elegans (P54145). Phylogenetic analyses of these proteins can be found elsewhere (11, 17). Interestingly, the three Synechocystis Amt proteins show a higher identity degree to each other (40 to 43% identity) than to any of their homologues from other organisms listed above (27 to 37% identity).

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