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(Methyl)ammonium Transport in the Nitrogen-Fixing Bacterium *Azospirillum brasilense*

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An ammonium transporter of *Azospirillum brasilense* was characterized. In contrast to most previously reported putative prokaryotic NH_4^+ transporter genes, *A. brasilense amtB* is not part of an operon with *glnB* or *glnZ* which, in *A. brasilense*, encode nitrogen regulatory proteins P_{II} and P_Z, respectively. Sequence analysis predicts the presence of 12 transmembrane domains in the deduced AmtB protein and classifies AmtB as an integral membrane protein. Nitrogen regulates the transcription of the *amtB* gene in *A. brasilense* by the Ntr system. *amtB* is the first gene identified in *A. brasilense* whose expression is regulated by NtrC. The observation that ammonium uptake is still possible in mutants lacking the AmtB protein suggests the presence of a second NH_4^+ transport mechanism. Growth of *amtB* mutants at low ammonium concentrations is reduced compared to that of the wild type. This suggests that AmtB has a role in scavenging ammonium at low concentrations.

Azospirillum species are nitrogen-fixing organisms (diazotrophs), capable of forming an associative relationship with the roots of several economically important cereals (68). Many studies have indicated that *Azospirillum* promotes plant growth, but the exact mechanism of growth promotion has not been fully characterized. Like most organisms, *Azospirillum* uses ammonium salts as a preferred nitrogen source (53). In the absence of combined nitrogen and under microaerobiosis conditions, the nitrogenase enzyme complex is synthesized and converts atmospheric N_2 to NH_4^+.

Unprotonated NH_3 is predicted to diffuse out of bacterial cells due to a concentration gradient across the plasma membrane (36). The pH gradient (generally slightly more alkaline inside the bacteria) enhances this process. Therefore, an active ammonium uptake system is required to retain the intracellular fixed nitrogen, acquired at high energy cost by the nitrogenase. Hartmann and Kleiner (24) have shown that ammonium uptake in *Azospirillum* spp. is energy dependent, follows the Michaelis-Menten kinetics, and is repressed by ammonium. No functional characterization of genetic components of this system has yet been reported.

Recently, genes encoding ammonium transporter proteins and putative ammonium transporter proteins have been reported for *Saccharomyces cerevisiae* (43), *Arabidopsis thaliana* (52), and *Lycopersicon esculentum* (tomato) (39). In *Bacillus subtilis* the *nrgA* gene, whose corresponding amino acid sequence is homologous to those of NH_4^+ transporter proteins, is part of the dicistronic *nrgAB* operon (76). *nrgB* possibly encodes a nitrogen regulatory protein homologous to P_{II} proteins, but the biochemical functions of the *nrgA* and *nrgB* gene products in *B. subtilis* have not been reported. The *nrgAB* operon is highly expressed during nitrogen-limited growth.

More recently, Siewe et al. (61) characterized the first reported prokaryotic NH_4^+ transporter gene (*amt*) in *Corynebacterium glutamicum*. However, in 1986, Jayakumar et al. (28) had already reported on an *amtA* gene in *Escherichia coli* which complemented a mutant with less than 10% of the parental $CH_3NH_3^+$ uptake activity. The complete *amtA* sequence was published by Fabiny et al. (19). An analysis of the deduced amino acid sequence of the product of *amtA*, AmtA, predicted that the protein was a cytoplasmic component of an ammonium transport system. In 1992, however, Neuwald et al. (51) reported that the *amtA* gene corresponded to *cysQ*, a gene needed for cysteine synthesis in *E. coli*. Later on, Van Heeswijk et al. (70) isolated an *amtB* gene in *E. coli* K-12. The *amtB* gene product is homologous to transmembrane NH_4^+ transporters, but its functional characterization has not yet been reported. The *E. coli amtB* gene is cotranscribed with *glnK*, located upstream of *amtB*. *glnK* encodes a second P_{II}-like protein (6, 70). The *glnK* gene product and the *glnB* gene product (P_{II}) are known to play a role in the reversible adenylylation of glutamine synthetase (GS) in response to the nitrogen status of the cells. In addition, P_{II} stimulates the kinase-phosphatase enzyme, NtrB, to dephosphorylate the phosphorylated transcriptional activator NtrC. Phosphorylated NtrC is necessary to activate transcription from several RpoN-dependent promoters (reviewed in reference 65; 41, 46).

Two P_{II} homologs have been identified in *Azospirillum brasilense* (14, 15). *glnB* is part of the nitrogen-regulated, but NtrC-independent, *glnBA* operon, and its product is required for nitrogen fixation. In contrast to what is found for other species, P_{II} (*glnB* gene product) is not involved in the ammonium control of GS activity by adenylylation. The level of *glnA* expression is, however, lower in *glnB* mutant strains than in the wild-type strain (14, 15). The second P_{II}-like protein of *A. brasilense*, P_Z, is not functionally equivalent to P_{II}. *glnB*-null mutants exhibit a Nif^- phenotype that is not complemented by structural gene *glnZ*. The two-component regulatory system NtrB-NtrC and the σ^{54} factor (RpoN) in *A. brasilense* have been characterized (40, 48). NtrC is involved in nitrate utilization (40) and (methyl)ammonium uptake (69). Notably, the *rpoN* mutant has a pleiotropic effect: nitrogen fixation, nitrate assimilation, ammonium uptake, and flagellar biosynthesis are impaired (48).

We report here the isolation and characterization of a nitrogen-regulated (methyl)ammonium transporter gene from *A. brasilense.*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
A. brasilense		
Sp7 (ATCC29145)	Wild-type strain, isolated from *Digitaria decumbens* rhizosphere soil, Brazil	66
FAJ301	*rpoN*::*kan* Km^r	48
FAJ310	*amtB*::*kan* Km^r	This work
7148	*ntrC*::Tn*5*-148 Km^r	40
7194	*ntrB*::Tn*5*-194 Km^r	40
7606	*glnB*::*kan* Km^r	15
E. coli		
DH5 α	*hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15)	Gibco BRL
HB101	*recA hsdR hsdM pro leu lacZ supE* F^- Str^r	5
Plasmids		
pUC18	Cloning vector; ColE1 replicon, Ap^r	77
pUC18-2	pUC18 with 1.8-kb Km^r cassette from Tn*5*, Ap^r Km^r	13
pSUP202	Mobilizable plasmid, suicide vector for *A. brasilense*; ColE1 replicon, Cm^r Tc^r Ap^r	62
pRK2013	Tra^+ helper plasmid	20
pKW117	pUC8 + *gusA* + *trpA* terminator, Ap^r	74
pLAFR1	IncP1 broad-host-range cosmid	21
pLAFR3	pLAFR1 derivative containing the pUC8 multiple cloning site	64
pFAJ302	pLAFR3 containing an *amtB*::*gusA* translational fusion	This work
pFAJ304	pUC19 containing an *amtB*::*gusA* translational fusion	This work
pFAJ308	pUC18 containing the *A. brasilense amtB* gene as a 1.9-kb *Eco*RI-*Nar*I fragment cloned in *Eco*RI-*Acc*I	This work
pFAJ309	pLAFR3 containing the *A. brasilense amtB* gene as a 1.9-kb *Eco*RI-*Hin*dIII fragment from pFAJ308, $amtB^+$	This work
pFAJ312	pUC19 containing the 1.6-kb *Eco*RI-*Sal*I fragment of *amtB*	This work
pFAJ314	pUC18 containing the 14-kb *Eco*RI fragment with the *A. brasilense amtB* gene	This work

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The *E. coli* and *A. brasilense* strains used are listed in Table 1. Plasmids mentioned in the text are also described in this table. Sequencing constructs and intermediate constructs are not given. A genomic library of *A. brasilense* Sp7 was constructed by ligation of fragments generated by partial *Eco*RI digestion of total DNA into cosmid pLAFR1. These constructs were packed into phage particles, transferred to *E. coli* HB101, and selected for isolation of tetracycline-resistant colonies. *E. coli* strains were grown in Luria-Bertani (LB) medium (57) at 37°C. *A. brasilense* was grown in LB medium supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ (LB* medium) at 30°C. For solid media, 15 g of agar per liter was added. Conjugal transfers of recombinant plasmids, derived either from pLAFR1 or pLAFR3, from *E. coli* to *A. brasilense* were performed on D plates (containing 8 g of Bacto nutrient broth [Difco], 0.25 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of KCl, and 0.01 g of $MnCl_2$ per liter). After conjugation, MMAB minimal medium (71) with 0.5% malate as the C source was used for selection of *A. brasilense* transconjugants. MMAB medium was also used in growth experiments and in [^{14}C]methylammonium uptake, nitrogenase activity, and β-glucuronidase assays. Growth rates in liquid minimal medium supplemented with 20 mM NH_4^+, 2 mM NH_4^+, 8 mM nitrate, or 10 mM aspartate as the nitrogen source were measured by monitoring the optical density at 595 nm (OD_{595}). Solid medium was used for growth experiments involving low NH_4^+ concentrations (0.7 to 0.1 mM). For RNA work, minimal K medium (18) supplemented with sodium lactate (5 g per liter) was used.

DNA methods. Standard methods, as described by Sambrook et al. (57), were used for plasmid isolation, chromosomal DNA preparation, restriction analysis, ligations, transformations, Southern blotting, and hybridization. DNA fragments were recovered from agarose gels with the Nucleotrap kit (Macherey-Nagel Filter Service, Eupen, Belgium). For Southern hybridization, DNA was transferred to a Hybond-N membrane (Amersham, Ghent, Belgium). Hybridization was done overnight at 68°C with a DNA probe labeled with digoxigenin-dUTP by a random-primed labeling kit (Boehringer Mannheim, Brussels, Belgium). The signal was detected with a chemiluminescence detection kit (Boehringer Mannheim).

PCR. To identify *A. brasilense* genes encoding NH_4^+ transporter proteins, degenerate PCR primers were designed based on conserved amino acid sequences of reported NH_4^+ transporter homologs (43, 45, 52, 76). Two of them (Fig. 1) (5′-GTGAATTCGGNGCNTTYGCNGARCGNATG-3′ and 5′-TCG AATTCRTTRAANCCRAACCANCCRAACCA-3′) yielded a single amplification product of the expected size (344 bases), which was cloned and sequenced (data not shown). The deduced amino acid sequence corresponding to this amplification product showed significant homology with those of reported NH_4^+ transporter proteins.

An *Eco*RI recognition site had been added to the 5′ end of each primer to facilitate the cloning of the amplified PCR product into a pUC18 vector. PCRs were performed in a thermocycler (TRIO-Thermoblock; Biometra, Göttingen, Germany) in a reaction volume of 25 μl containing 0.5 U of *Taq* DNA polymerase (Boehringer Mannheim) and each of the primers at a 1 μM concentration. A PCR cycle consisting of 1 min of denaturation at 94°C, 1 min of primer annealing at 50°C, and 1 min of primer extension at 72°C was applied 30 times. These 30 cycles were preceded by 5 min of denaturation (94°C) and were followed by 7 min of primer extension (72°C).

DNA sequencing and analysis. The chain-terminating dideoxynucleotide triphosphate method (58) was performed by using an automated sequencer (ALF; Pharmacia Biotech Benelux, Roosendaal, The Netherlands) with the Autoread sequencing kit (Pharmacia Biotech Benelux). The PALIGN, CLUSTAL, PROSITE, SOAP, NOVOTNY, BETATURN, HELIXMEM, and PSIGNAL programs of the P.C. Gene software package (Intelligenetics) were used to process and analyze the sequencing data. Potential coding regions were identi-

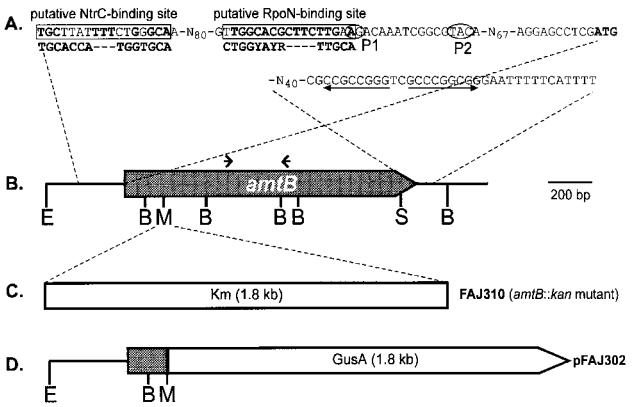


FIG. 1. Schematic representation of the *A. brasilense* Sp7 *amtB* gene and constructs used. (A) Promoter and terminator regions. A putative NtrC-binding sequence and a RpoN-binding sequence are boxed, and the corresponding consensus sequences (3, 41) are given below. The two transcription start sites, P1 and P2, are circled. The start codon (ATG) is in boldface. Arrows indicate the dyad symmetry of a putative Rho-independent terminator. (B) Physical map of the *amtB* gene. Arrows indicate the locations of the PCR primers used to amplify a 344-bp internal sequence. E, *Eco*RI; B, *Bgl*I; M, *Sma*I; S, *Sal*I. (C) Insertion site of the kanamycin resistance cartridge used to construct an *amtB*::*kan* mutant (FAJ310). (D) Construction of the *amtB*::*gusA* fusion (pFAJ302) in broad-host-range vector pLAFR3. Abbreviations are as defined for panel B.

fied with the GCWIND program (60). The Blast program (1) was used to search for related sequences.

RNA preparation and Northern blot analysis. RNA was prepared from wild-type and *glnB*::*kan* mutant cells grown under nitrogen fixation conditions (0.5% O_2) in minimal medium with 10 mM sodium aspartate or 20 mM NH_4Cl as the nitrogen source to an OD_{600} of 0.5. Total RNA was extracted with hot phenol according to the method of Gubler and Hennecke (23). Fifteen micrograms of RNA was separated on a 1.2% formaldehyde-agarose gel and transferred to a Hybond-N membrane (Amersham). The 1.6-kb *Eco*RI-*Sal*I DNA fragment containing the first 1,260 bp of *amtB* was, after purification from plasmid pFAJ312, labeled with [α-^{32}P]dCTP by using a random primer labeling kit (Amersham). Hybridization with the radiolabeled probe was performed for 6 h at 68°C in the presence of Rapid-hyb buffer from Amersham.

Primer extension. Primer extension was performed as described by Ausubel et al. (2) with two primers labeled with [γ-^{32}P]dATP: AMTB-15 (5′-CAGACCGG CCAAGCCCAGAATCGCCGCCAT-3′) and AMTB-17 (5′-GGCGGCGCTTT CCTGGGCGAGGGCGGC-3′). Ten picomoles of each primer added to 20 μg of total RNA was heated in the hybridization buffer with 80% formamide for 10 min at 85°C, and the mixture was subsequently incubated overnight at 30°C. The primer extension was performed for 90 min at 42°C with 50 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). The extension products were run on a sequencing gel adjacent to the DNA sequence obtained by using the same oligonucleotide as the primer.

Construction of an *amtB*::*gusA* fusion and β-glucuronidase assay. The 1.8-kb *Sma*I-*Hin*dIII cassette from pKW117 (74) containing the *gusA* gene was ligated behind the 525-bp *Eco*RI-*Sma*I fragment (Fig. 1) containing the 5′ region of the *amtB* gene. This was achieved by replacing the 1.1-kb *Sma*I-*Hin*dIII fragment of pFAJ312 with the 1.8-kb *Sma*I-*Hin*dIII cassette from pKW117, yielding plasmid pFAJ304. The construction was tested with the 22-bp oligonucleotide complementary to the *gusA* coding strand (29). The 2.3-kb *Eco*RI-*Hin*dIII fragment from pFAJ304, containing the *amtB* promoter region fused to the promoterless *gusA* gene, was cloned in broad-host-range vector pLAFR3 yielding plasmid pFAJ302.

β-Glucuronidase activity was measured as described by Vande Broek et al. (67). Cells were grown overnight in minimal MMAB medium containing the indicated nitrogen source. Nitrogen-fixing conditions were created in airtight 80-ml tubes with 3 ml of nitrogen-free MMAB medium by replacing the 77-ml air volume with N_2 plus 0.5% O_2 or in semisolid (0.07% agarose) nitrogen-free MMAB medium. Units of activity were calculated as described by Miller (49).

Uptake of [^{14}C]methylammonium. Cells were pregrown in minimal MMAB medium with 10 mM aspartate as the nitrogen source. When the cultures had reached a concentration of approximately 10^9 cells per ml they were centrifuged and resuspended in a double volume of the same medium without a nitrogen source. After 15 min of incubation at room temperature, [^{14}C]methylammonium (Amersham; 2.11 GBq/mmol) was added to a final concentration of 8.75 μM. Samples (100 μl) were filtered through a MultiScreen Durapore (type DV; 0.65-μm-pore-size) filtration plate, which was placed on a multiscreen filtration manifold. Filters were dried, and radioactivity was measured with a liquid scintillation counter (Wallac 1410; Pharmacia Biotech Benelux). Protein concentrations were determined by the bicinchoninic acid assay (63) after lysis of the cells in 1 N NaOH at 65°C.

Uptake of NH_4^+. Cells were pregrown as described for the [^{14}C]methylammonium uptake assay. Cell suspensions were centrifuged, washed once with the assay buffer containing 10 mM $MgSO_4$, 20 mM HEPES (pH 7) and 5 g of malate per liter, and resuspended in the same buffer. The original culture was concentrated 10 times in the assay buffer. After 15 min of incubation at room temperature, ammonium was added to a final concentration of 0.1 mM. The NH_4^+ concentration was measured with a selective ammonium electrode (F2322NH_4 AMMONIUM SELECTRODE; Radiometer, Copenhagen, Denmark). Values were stored every 30 s. The detection limit of the electrode was 10 μM; this value was reached after approximately 25 min.

Ammonium excretion. Ammonium excretion was measured in liquid MMAB medium containing 8 mM KNO_3 as the nitrogen source. At different growth stages, the presence of ammonium in the cell supernatants was assayed by the indophenol blue method as described by Chaney and Marbach (9).

Construction of an *amtB*::*kan* insertion mutant. A 1.8-kb *Bam*HI fragment containing the kanamycin resistance cassette from pUC18-2 was made blunt with T4 DNA polymerase and was ligated into the *Sma*I site of the 1.6-kb *Eco*RI-*Sal*I fragment (Fig. 1) containing the *amtB* gene. This 1.6-kb *Eco*RI-*Sal*I fragment had previously been ligated into suicide vector pSUP202 as an *Eco*RI-*Pst*I fragment. The resulting plasmid was conjugated to *A. brasilense* Sp7, with helper plasmid pRK2013 providing the *tra* genes. Transconjugants were selected on the basis of Km^r (for the presence of the cassette) and Tc^s (loss of the pSUP202 plasmid). Two PCR primers designed to amplify a 627-bp internal part of the *kan* gene were used to confirm the presence of the Km^r cassette, and the insertion of the Km^r cassette in the *amtB* gene was also checked by hybridization of mutant and wild-type total DNA with the 1.6-kb *Eco*RI-*Sal*I DNA fragment containing the first 1,260 bp of *amtB* (data not shown).

Nitrogenase assay. Strains were pregrown overnight in rich LB* medium. After being washed, cell suspensions were brought to equal OD_{595} values and 25 μl of these exponential-phase cells was inoculated into 5 ml of semisolid nitrogen-free MMAB medium (0.07% agarose). After overnight incubation at 30°C, acetylene was added (10% of the air volume), and ethylene production was measured after 5, 9, 12, and 25 h with a Hewlett-Packard 5890A gas chromatograph with a PLOT (porous layer open tubular) fused silica column. Propane was used as the internal standard.

Nucleotide sequence accession number. Sequence data has been submitted to the DDBJ/EMBL/GenBank database under accession no. AF005275.

RESULTS

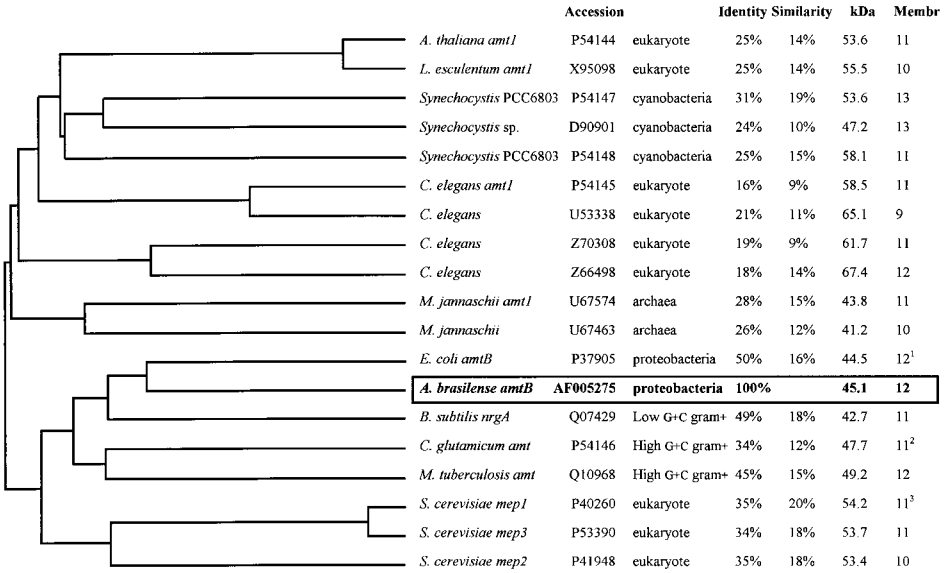
Cloning of an *A. brasilense* Sp7 gene encoding a putative NH_4^+ transporter. Degenerate PCR primers were used to amplify a 344-bp internal fragment of the putative NH_4^+ transporter gene (see Materials and Methods). This amplification product was used to screen a pLAFR1-derived genomic library of *A. brasilense* Sp7, made by cloning partial *Eco*RI-digested genomic DNA (see Materials and Methods). The 14-kb *Eco*RI fragment of a positively hybridizing cosmid clone was subcloned as a 1.6-kb *Eco*RI-*Sal*I fragment, and sequence analysis identified the first 1,249 bp of a putative *A. brasilense* NH_4^+ transporter gene. Cloning the original 14-kb *Eco*RI fragment in vector pUC18 (pFAJ314) permitted the sequencing of the remainder of the gene by using primer walking from the 3′-terminal part of the known sequence. Figure 1 shows the organization of the sequenced DNA region.

The G+C content of the entire open reading frame (ORF) is 67.2%. This is consistent with the high G+C content of *A. brasilense* DNA. The G+C content in the third position of the codons is 91.9%. The potential ATG start codon is preceded by a putative ribosome-binding site. Immediately downstream of the ORF there is a G+C-rich sequence with interrupted dyad symmetry ($\Delta G[25°] = -21.6$ kcal) followed by a T-rich region. This suggests the presence of a Rho-independent transcription terminator (56) (Fig. 1).

DNA sequence analysis did not reveal other ORFs in the 330-bp region immediately downstream of the putative NH_4^+ transporter gene or in the 350 bp preceding the ORF.

Analysis of the deduced amino acid sequence. A comparison of the deduced amino acid sequence of the product of the *A. brasilense amtB* gene with those of reported putative NH_4^+ transporter proteins revealed that the sequence of this protein showed the highest level of similarity (approximately 50%) to the deduced amino acid sequences of the products of the *E. coli amtB* gene and the *B. subtilis nrgA* gene (data not shown). In Fig. 2 a phylogenetic tree built by multiple sequence alignment of reported putative NH_4^+ transporter proteins is shown. Besides the proteins included in this figure, proteins belonging to the Mep/Amt family (44) have been reported for *Mycobacterium leprae* (accession no. L78818), *Rhodobacter capsulatus* (accession no., X12359), and *Methanobacterium thermautotrophicum* (accession no. AE000846). However, no NH_4^+ transporter homolog was found by examining the complete genomic sequences of *Haemophilus influenzae* and *Mycobacterium genitalium*, two bacteria whose natural environment is human tissues (44).

Analysis of the promoter region and the transcription of the *amtB* gene. To localize the transcription start site of *amtB*, primer extension analysis was performed. RNA was isolated from wild-type (Sp7) cells grown in the presence of different nitrogen sources. The complementary sequence of nucleotides between positions 379 and 408 was used as a primer (AMTB-15). Figure 3 shows that the P2 transcription start site, located 80 to 82 nucleotides upstream of the translation start site of *amtB*, is preferentially used when 10 mM aspartate is the nitrogen source. In conditions of ammonium excess, a weak but significant signal was obtained at the P2 transcription start site. Another weak signal also appeared on the autoradiogram at the P1 transcription start site (94 to 95 nucleotides upstream



	Accession		Identity	Similarity	kDa	Membr
A. thaliana amt1	P54144	eukaryote	25%	14%	53.6	11
L. esculentum amt1	X95098	eukaryote	25%	14%	55.5	10
Synechocystis PCC6803	P54147	cyanobacteria	31%	19%	53.6	13
Synechocystis sp.	D90901	cyanobacteria	24%	10%	47.2	13
Synechocystis PCC6803	P54148	cyanobacteria	25%	15%	58.1	11
C. elegans amt1	P54145	eukaryote	16%	9%	58.5	11
C. elegans	U53338	eukaryote	21%	11%	65.1	9
C. elegans	Z70308	eukaryote	19%	9%	61.7	11
C. elegans	Z66498	eukaryote	18%	14%	67.4	12
M. jannaschii amt1	U67574	archaea	28%	15%	43.8	11
M. jannaschii	U67463	archaea	26%	12%	41.2	10
E. coli amtB	P37905	proteobacteria	50%	16%	44.5	12[1]
A. brasilense amtB	**AF005275**	**proteobacteria**	**100%**		**45.1**	**12**
B. subtilis nrgA	Q07429	Low G+C gram+	49%	18%	42.7	11
C. glutamicum amt	P54146	High G+C gram+	34%	12%	47.7	11[2]
M. tuberculosis amt	Q10968	High G+C gram+	45%	15%	49.2	12
S. cerevisiae mep1	P40260	eukaryote	35%	20%	54.2	11[3]
S. cerevisiae mep3	P53390	eukaryote	34%	18%	53.7	11
S. cerevisiae mep2	P41948	eukaryote	35%	18%	53.4	10

FIG. 2. Dendrogram of the multiple sequence alignment of putative NH_4^+ transporter proteins according to the methods of Higgins and Sharp (25, 26). Genome sequencing of the archaebacterium *Methanococcus jannaschii* (7), the cyanobacterium *Synechocystis* (30, 31), and the nematode *Caenorhabditis elegans* (75) predicted several NH_4^+ transporter-coding genes. Percentages of similarity and identity to the *A. brasilense* protein sequence were calculated by the method of Myers and Miller (50). Predicted molecular masses are given in kilodaltons. The Membr column gives the predicted numbers of membrane-associated helices. Unless cited in the literature, these numbers were calculated by the method of Eisenberg et al. (17). Superscripts 1, 2, and 3 (Membr column) indicate that the data came from references 70, 61, and 43, respectively.

of the translational start site of *amtB*). Under conditions of nitrogen fixation the transcription of *amtB* starts at the P2 transcription start site, but less efficiently than in the presence of aspartate (data not shown). Using another oligonucleotide (AMTB-17; see Materials and Methods) allowed the detection of the same transcription start sites (P1 and P2) (data not shown). The results obtained under conditions of nitrogen fixation or with 10 mM aspartate as the nitrogen source were the same for a *glnB*-null mutant strain (7606) (data not shown).

An examination of the DNA region upstream from these transcription start sites reveals a putative RpoN-binding consensus sequence at positions −26 to −14 relative to the P2 transcription start site (Fig. 1). This sequence differs by two nucleotides from consensus sequence CTGGYAYR-N_4-TTGCA (four positions previously defined as invariable are underlined) (3) for RpoN-dependent promoters. The occurrence of A instead of C at position −14 is also found in the *Rhizobium leguminosarum nifH* promoter sequence (55) and in the *R. leguminosarum* biovar *phaseoli nifH1*, *nifH2*, and *nifH3* promoter regions (47). A partially conserved NtrC-binding consensus sequence is present at positions −128 through −112 (relative to the P2 transcription start site; the consensus sequence is TGCACCA-N_3-TGGTGCA) (41) (Fig. 1).

Northern blot analyses were performed with RNA extracted from wild-type Sp7 and *glnB*::*kan* mutant (7606) cells grown under different nitrogen conditions. Figure 4 shows the hybridization of total RNA with an *amtB* probe. A single transcript of 1.5 kb is detected under all physiological conditions, indicating that *amtB* is transcribed as a monocistronic unit. This is consistent with the absence of ORFs in the vicinity of *amtB* and the presence of a putative downstream Rho-independent terminator close to the 3′ end of *amtB* (Fig. 1).

As was also observed in the primer extension experiment, the level of the *amtB* mRNA signal is high in cells grown in aspartate-containing minimal medium, reduced in cells grown under conditions of nitrogen fixation, and very low in cells

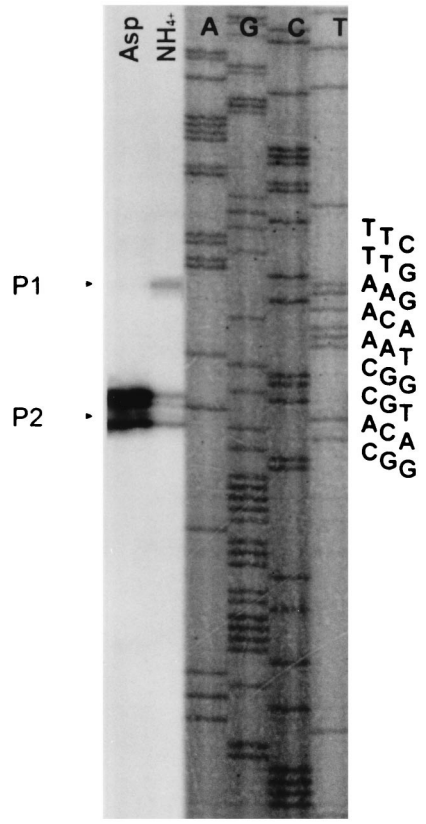


FIG. 3. Primer extension analysis of the *amtB* promoter region to identify the transcription initiation sites under different nitrogen conditions. RNA was prepared from wild-type Sp7 grown on minimal medium with 10 mM aspartate (Asp) or 20 mM NH_4^+ (NH_4^+) as the nitrogen source. The DNA sequence in the transcription start region is indicated next to the sequencing gel. Two transcription start sites, P1 and P2, are indicated by arrowheads, and the corresponding sequences are shown circled in Fig. 1.

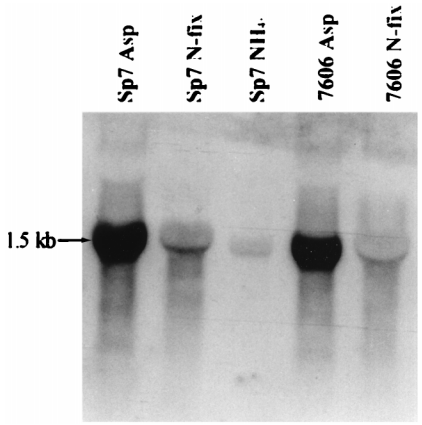


FIG. 4. Northern blot analysis of total RNA hybridized with the 1.6-kb *Eco*RI-*Sal*I *amtB* probe. Total RNA was prepared from Sp7 (wild-type) and 7606 (*glnB*::*kan* mutant) cells grown in minimal medium with 10 mM aspartate (Asp) or 20 mM ammonium (NH_4^+) as the nitrogen source or grown under nitrogen-fixing conditions (N-fix).

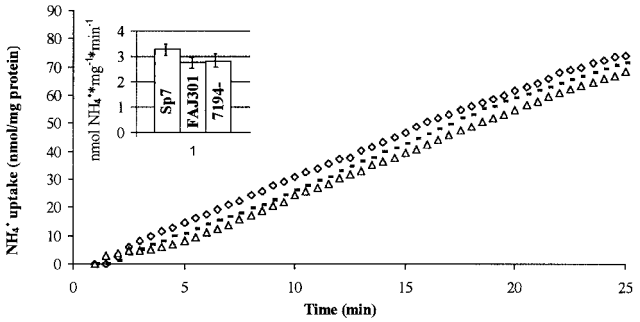


FIG. 5. Uptake of NH_4^+ in the wild-type *A. brasilense* Sp7 strain (◇), *rpoN*::*kan* mutant strain FAJ301 (△), and *ntrB*::Tn*5*-194 mutant strain 7194 (-). Uptake was measured as the disappearance of NH_4^+ in the assay buffer after the addition of ammonium to a final concentration of 0.1 mM at time zero. The detection limit of the selective ammonium electrode used was reached after 25 min. The inset gives a representation of the mean slope values for the wild-type *A. brasilense* Sp7 strain, *rpoN*::*kan* mutant strain FAJ301, and *ntrB*::Tn*5*-194 mutant strain 7194. The values shown are the means of two independent replicates. Standard deviations are indicated as vertical bars.

grown in the presence of 20 mM NH_4^+. This is in agreement with negative regulation by ammonium.

Northern blot analysis of a *glnB*::*kan* mutant strain shows that the absence of P_{II} does not affect the transcription of the *amtB* gene under conditions of nitrogen fixation or when 10 mM aspartate is the nitrogen source. Thus, in contrast to what is found for the regulation of *nif* gene expression (14), the transduction of the nitrogen signal for *amtB* gene expression does not require P_{II} under the conditions tested.

Expression of an *amtB-gusA* fusion in wild-type and regulatory mutant strains. To investigate the regulation of *amtB* transcription in *A. brasilense*, an *amtB*::*gusA* translational fusion was constructed in vector pLAFR3, resulting in pFAJ302 (Fig. 1D). As NH_4^+ transport is reported to be regulated by the Ntr system in *E. coli* (27, 59) and *Klebsiella pneumoniae* (35), the pFAJ302 vector was conjugated into wild-type *A. brasilense* and three different *A. brasilense ntr* mutants: 7194 (*ntrB*::Tn*5*-194), 7148 (*ntrC*::Tn*5*-148), and FAJ301 (*rpoN*::*kan*). Expression of the *amtB*::*gusA* fusion is maximal in the presence of 10 mM aspartate or 2 mM NH_4^+ and is reduced eightfold in the presence of 20 mM NH_4^+ (Table 2), in agreement with the results of the Northern blot analysis. This confirms that the nitrogen status of the cell influences *amtB* transcription. *amtB*::*gusA* expression levels were significantly lowered in the *ntr* mutants under all physiological conditions tested. This suggests that the Ntr system is involved in the nitrogen regulation of *amtB* transcription. The slightly higher *amtB*::*gusA* expression levels observed in the *ntr* mutants grown on 20 mM NH_4^+ could be explained by assuming that in these circumstances the first promoter, located upstream of the P2 transcription start site (Fig. 1 and 3), participates in the transcription of *amtB.*

TABLE 2. Expression of the *amtB*::*gusA* fusion in different genetic backgrounds

Strain[b]	β-Glucuronidase activity (U)[a] with indicated N source			
	10 mM Asp	2 mM NH_4^+	20 mM NH_4^+	N_2
FAJ301	3	2	9	0
7194	2	5	8	2
7148	1	4	6	0
Sp7	123	95	16	75

[a] β-Glucuronidase activity was measured after overnight incubation in minimal medium containing one of the four nitrogen sources. Units of activity were calculated as described by Miller (49). The values shown are the means of at least three independent replicates.

[b] For a description of the strains, see Table 1.

Ammonium uptake in *ntr* mutants. In line with the nearly absent expression of the *amtB-gusA* fusion in *ntr* mutants, these mutants do not show any [^{14}C]methylammonium uptake (48). In order to measure the transport of the natural substrate for the AmtB transporter protein, a selective ammonium electrode was used to measure the uptake of NH_4^+ added to a cell suspension of wild-type Sp7 cells, *rpoN*::*kan* mutant cells (FAJ301), and *ntrB*::Tn*5* mutant cells (7194). In contrast to the results of the [^{14}C]methylammonium uptake studies, no significant difference between wild-type cells and *ntr* mutant cells was observed (Fig. 5).

Construction and phenotypic characterization of an *A. brasilense amtB*::*kan* insertion mutant. An Sp7 mutant strain was constructed by inserting a kanamycin resistance gene (*kan*) into the structural *amtB* gene (Fig. 1C). This mutant strain is unable to take up [^{14}C]methylammonium (Fig. 6). It should be noted that although the activity of the AmtB carrier is measured with radioactively labeled [^{14}C]methylammonium, the affinity of this carrier for $CH_3NH_3^+$ is considerably less than its affinity for NH_4^+, and methylammonium cannot serve as a carbon or nitrogen source for *A. brasilense* (24).

Growth rates on rich medium or on minimal medium with 20 mM NH_4^+, 2 mM NH_4^+, 8 mM nitrate, or 10 mM aspartate were similar for both the wild-type and mutant strains. As with the wild-type strain, the *amtB*::*kan* mutant fixes nitrogen in nitrogen-free minimal medium and at low oxygen concentration. No ammonium excretion exceeding the minimum detection level of the assay method (30 mM) could be measured.

At low ammonium concentrations (0.1 mM) growth of the *amtB*::*kan* mutant cells was reduced compared to that of wild-type cells (Fig. 7).

Complementation of an *A. brasilense amtB*::*kan* insertion mutant and overexpression of *amtB* in wild-type cells. The *amtB* gene, expressed from its own promoter, was inserted in the broad-host-range vector pLAFR3 (resulting in pFAJ309). This construct was transferred to both wild-type cells and *amtB*::*kan* mutant cells. [^{14}C]methylammonium uptake was restored in the mutant and occurred at even a higher rate than that in wild-type cells (Fig. 6). This could be due to the presence of extra copies of the *amtB* gene carried on the low-copy-

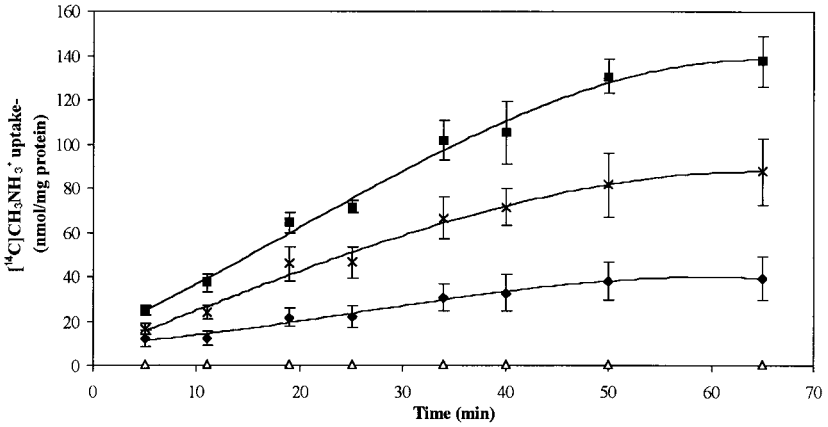


FIG. 6. Uptake of [^{14}C]methylammonium in the wild-type *A. brasilense* Sp7 strain (◆), the *amtB*::*kan* mutant (△), the *amtB*::*kan* mutant complemented with the *A. brasilense amtB* gene (pFAJ309) (×), and the wild-type Sp7 strain containing extra copies of the *A. brasilense amtB* gene (pFAJ309) (■). Values shown are the means of at least three independent replicates. Standard deviations are indicated as vertical bars. The standard deviation for the *amtB*::*kan* mutant (△) was less than 0.6 nmol/mg.

number pLAFR3 vector (4 to 7 copies per cell) (33). Another possibility is that the *lacZ* promoter adjacent to the multiple-cloning site in pLAFR3 is highly expressed in *A. brasilense*. Since the *amtB* gene is oriented in the direction of expression of the *lacZ* promoter, this could result in enhanced *amtB* expression. The enhancement of [^{14}C]methylammonium uptake in wild-type strain Sp7 when pFAJ309 was introduced is in line with these hypotheses.

DISCUSSION

When *Azospirillum* cells are grown under conditions of nitrogen fixation, no ammonium can be detected in the growth medium. It was postulated that bacteria have an active ammonium uptake system to compensate for the loss of NH_3 by diffusion through the plasma membrane (36). Here we report on the genetic and biochemical characterization of AmtB, a (methyl)ammonium transport protein of *A. brasilense.*

In contrast to that of *E. coli*, the *A. brasilense amtB* gene is not part of an operon with a *glnB*-like gene. The coexistence of two P_{II}-like proteins, encoded by *glnB* and *glnZ*, has been established in *A. brasilense* (15). Comparing physical maps of the DNA fragments containing the *A. brasilense glnB*, *glnZ*, and *amtB* genes confirms that they are located on different restriction fragments. In *E. coli*, *amtB* is cotranscribed with *glnK*, which encodes a nitrogen regulatory P_{II}-like protein (70). In *B. subtilis* the *nrgA* gene is also part of an operon with *nrgB*, encoding a P_{II}-like protein, but *nrgA* is located upstream of *nrgB*. In *M. jannaschii* two genes which encode putative NH_4^+ transporter proteins are located just next to a gene homologous to P_{II} (7). *C. glutamicum* is the only prokaryote for which it has also been reported that *amt* is not located in an operon structure with a gene encoding a P_{II}-like protein (61).

The synthesis of most prokaryotic NH_4^+ carriers is repressed by NH_4^+ (37). This is also the case for the yeast *S. cerevisiae* (16, 43), but *Arabidopsis thaliana* AMT1 activity is not lowered in cells grown in the presence of NH_4^+ (52). Transcription of the *E. coli glnKamtB* operon and the *C. glutamicum amt* gene is under nitrogen control in both organisms (61, 70). This is also observed for the *A. brasilense amtB* gene. The level of expression of an *amtB*::*gusA* fusion is significantly lowered in a *ntrC* mutant (Table 2). This indicates that the Ntr system is involved in the nitrogen-regulated transcription of *amtB.*

The NH_4^+ uptake rates of *ntr* mutant strains, as well as the observation that an *amtB*::*kan* mutant is still able to grow on ammonium concentrations as low as 0.1 mM (Fig. 7), suggest the existence of a second NH_4^+ transport mechanism. The existence of multiple NH_4^+ transporter proteins has been reported for other organisms. In yeast three genes encoding an NH_4^+ transporter (*MEP1*, *MEP2*, and *MEP3*) (43, 44) were isolated. Evidence also exists for the presence of two transporter systems in *Nostoc muscorum* (32), *Rhodobacter sphaeroides* (12), and *Anacystis nidulans* (4). In these three bacteria, one of the transport systems is constitutive and cannot transport methylammonium, while the other system is repressed in high NH_4^+ concentrations and is capable of transporting methylammonium.

The ammonium uptake profiles of *ntr* mutants, in K^+-free assay buffer (Fig. 5), indicate that in these conditions the second ammonium transport mechanism does not correspond to simple diffusion. Indeed, in this assay, the external ammonium concentration, as measured by the ammonium electrode, drops below 42 μM, which is the apparent K_m (Michaelis constant) of the GS enzyme of *A. brasilense* (54). The K_m value of the main ammonium-assimilating enzyme, which is the GS enzyme in *A. brasilense* (73), can be used to estimate the steady-state internal ammonium concentration (11). K^+ uptake systems

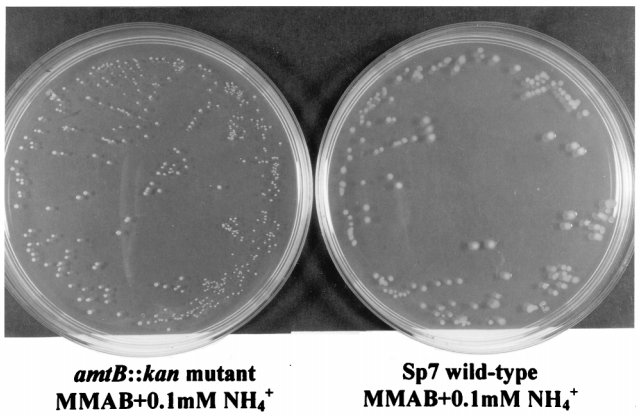


FIG. 7. Growth of the wild type and the *amtB* mutant on minimal medium with 0.1 mM NH_4^+ at pH 7. Cells were grown for 2 days at 30°C.

have been reported to support NH_4^+ uptake in *E. coli* (8), and in *Rhodobacter capsulatus* (22). Since NH_4^+ ions are similar both in charge and size to K^+ ions, the possibility that a K^+ transporter can mediate NH_4^+ uptake in K^+-free conditions cannot be excluded. Further investigation will be needed to establish the mode of ammonium transport in mutants lacking the nitrogen-regulated (methyl)ammonium transporter.

An analysis of the distribution of hydrophobic and hydrophilic amino acid residues shows that the AmtB amino acid sequence has highly hydrophobic stretches (38). The algorithm of Klein et al. (34) predicted 12 transmembrane domains and classified the protein as an integral membrane protein. The stretches separating the putative transmembrane regions are rich in charged amino acid residues and correspond to potential beta-turns (10). According to the positive-inside rule of von Heijne (72), the protein is oriented with its charged C and N termini exposed to the cytoplasm. These predictions are in agreement with the situation found for most bacterial carrier proteins (42).

In conclusion, we have characterized an *A. brasilense* (methyl)ammonium transporter. The corresponding gene, *amtB*, is the first *A. brasilense* gene known to require NtrC for expression. Indeed, this transcriptional activator is not required for the transcription of *nifA*, *glnA*, or *glnB* in *A. brasilense*. The AmtB NH_4^+ transporter is necessary for growth on low NH_4^+ concentrations. However, it appears not to be the sole mechanism for NH_4^+ uptake in *A. brasilense.*

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