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NH_4^+-Excreting *Azospirillum brasilense* Mutants Enhance the Nitrogen Supply of a Wheat Host

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Spontaneous ethylenediamine-resistant mutants of *Azospirillum brasilense* were selected on the basis of their excretion of NH_4^+. Two mutants exhibited no repression of their nitrogenase enzyme systems in the presence of high (20 mM) concentrations of NH_4^+. The nitrogenase activities of these mutants on nitrogen-free minimal medium were two to three times higher than the nitrogenase activity of the wild type. The mutants excreted substantial amounts of ammonia when they were grown either under oxygen-limiting conditions (1 kPa of O_2) or aerobically on nitrate or glutamate. The mutants grew well on glutamate as a sole nitrogen source but only poorly on NH_4Cl. Both mutants failed to incorporate [^{14}C]methylamine. We demonstrated that nitrite ammonification occurs in the mutants. Wild-type *A. brasilense*, as well as the mutants, became established in the rhizospheres of axenically grown wheat plants at levels of $>10^7$ cells per g of root. The rhizosphere acetylene reduction activity was highest in the preparations containing the mutants. When plants were grown on a nitrogen-free nutritional medium, both mutants were responsible for significant increases in root and shoot dry matter compared with wild-type-treated plants or with noninoculated controls. Total plant nitrogen accumulation increased as well. When they were exposed to a $^{15}N_2$-enriched atmosphere, both *A. brasilense* mutants incorporated significantly higher amounts of ^{15}N inside root and shoot material than the wild type did. The results of our nitrogen balance and ^{15}N enrichment studies indicated that NH_4^+-excreting *A. brasilense* strains potentially support the nitrogen supply of the host plants.

Azospirillum spp. are known to colonize the rhizospheres of graminaceous plants, where they exhibit considerable rates of N_2 fixation (5, 36, 44). However, the amounts of nitrogen fixed by *Azospirillum* spp. which are transferred to their host plants appear to be small (3, 21, 29). Investigations in which the ^{15}N isotope dilution technique was used indicated that most of the fixed nitrogen remained below ground, probably still bound to bacterial cells, and contributed only very little to the upper plant parts (7, 34, 38). In experiments performed with wheat plants that were monoxenically associated with *Azospirillum brasilense*, only 1 to 2% of the shoot nitrogen originated from atmospheric sources (6a). The total nitrogen gain from nitrogen fixation by root-associated *A. brasilense* was calculated to be 2 to 3 kg of N per ha per year; by comparison, in intensive arable farming the chemical fertilizer input is up to 250 kg of N per ha per year.

The reason for the limited nitrogen supply from associative nitrogen fixation is probably that *Azospirillum* spp., like other free-living diazotrophs but in contrast to symbiotically living rhizobia, does not release fixed nitrogen to its environment. Kleiner (22, 24) postulated that intracellularly formed NH_4^+ does diffuse through cell membranes along with the pH gradient but that membrane-bound proteins with high affinities for ammonia immediately carry this released NH_4^+ back. Hartmann and Kleiner (19) and Kleiner (23) demonstrated that such an ammonia transport system is active in the cell membranes of *A. brasilense*. In order to stimulate the release of fixed nitrogen from *Azospirillum* cells, we tried to find *A. brasilense* mutants which lack this ammonia transport system. This was done by selecting for resistance to ethylenediamine, an inhibitor of bacterial glutamine synthetase. Ethylenediamine-resistant mutants of *A. brasilense* have been selected previously, yielding mutants that were unrepressed for nitrogenase activity at high concentrations of NH_4Cl (12). Recently, Pedrosa and co-workers have described ethylenediamine-resistant *A. brasilense* strains which release substantial amounts of ammonia into their environment (31). However, these authors did not report whether these mutants were active in the rhizospheres of plants and whether they supplied fixed nitrogen to host plants.

In this paper we describe two NH_4^+-excreting *A. brasilense* strains, the behavior of these strains in the rhizospheres of wheat plants, and the effect of these strains on growth of and nitrogen uptake by the plants. In addition to determining root colonization and rhizosphere acetylene reduction, we quantified the supply of fixed nitrogen to the host plants by using the ^{15}N enrichment technique. Previously, this technique was shown to be a suitable method for determining heterotrophic nitrogen fixation in grasses (9, 20). The advantage of this technique compared with the more widely used ^{15}N dilution technique (4, 14, 39, 42) is that it allows direct tracing of fixed nitrogen inside plant material; the ^{15}N dilution technique is only an indirect assay for determining N supply to plants by N_2 fixation activity (45).

MATERIALS AND METHODS

Bacterial strains. As the wild type we used *A. brasilense* Wa5, which originated from the rhizosphere of a greenhouse-grown summer wheat plant (6). *A. brasilense* Wa5 was grown overnight on Luria broth, centrifuged, and washed twice with a 0.85% NaCl solution. A 100-μl portion of the final cell suspension (approximately 10^7 cells) was plated onto nitrogen-free minimal malate medium (NFB medium) (36) supplemented with 0.05% (final concentration) ethylenediamine (12). Colonies that were resistant to ethylenediamine were picked, and cells were added to semisolid

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(0.2% agar) glycerol minimal medium containing (per liter of demineralized water) 15 ml of 87% (vol/vol) glycerol, 0.3 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.2 g of $MgSO_4$, 0.1 g of NaCl, 0.02 g of $CaCl_2$, 0.5 g of $FeSO_4$, and the trace elements described by Day and Döbereiner (8) (pH 6.8). This glycerol medium was chosen because it allows *Azospirillum* spp. to grow without affecting the pH of the medium. When *Azospirillum* spp. are grown on malic acid or succinic acid as a carbon source, the medium becomes strictly alkaline, which results in a loss of released ammonia. Bacterial NH_4^+ excretion was tested in medium containing either no nitrogen source, 8 mM KNO_3, or 3 mM glutamate. Selection was made on the basis of NH_4^+ accumulation in each medium within 3 days. NH_4^+-excreting strains were further tested for acetylene reduction in semisolid (0.2% agar) NFB medium in the presence of 10 mM NH_4Cl.

Bacterial assays. Bacterial growth rates were determined at 30°C in a 100-ml batch of minimal NFB medium (350 rpm) supplemented with either 3 mM NH_4Cl or 3 mM glutamate.

Bacterial acetylene reduction was measured in airtight vials containing semisolid (0.2% agar) glycerol minimal medium with 10% (vol/vol) acetylene added to the headspace. Acetylene reduction activity was determined both in N-free medium and in media containing different concentrations of ammonia, nitrate, or glumatate. The production of ethylene was assayed by gas chromatography (Varian model 1700 instrument; Porapack T column; flame ionization detector).

Total bacterial protein was analyzed after cell lysis in 1 N NaOH at 60°C by using the procedure of Lowry et al. (30) and bovine serum albumin as the standard.

In order to determine the rate of excretion of NH_4^+, bacteria were grown overnight in 40 ml of Luria broth, washed once with 0.85% NaCl, and resuspended in 100 ml of glycerol minimal medium containing either 8 mM nitrate or 3 mM glutamate as a sole nitrogen source or no nitrogen at all. The cell suspension was transferred to closed 1-liter serum bottles and incubated with intense shaking at 30°C. To reduce the oxygen concentration, the headspace air was replaced by pure N_2 and enough air was added to obtain a final O_2 concentration of 1 kPa. Sampling for NH_4^+ started after 4 h to allow the bacteria to develop an active nitrogenase system. The level of excreted NH_4^+ in the supernatant was determined after centrifugation of a cell suspension by using a modified Berthelot color reaction test (27). As glutamate interfered with color development, when glutamate-containing medium was used, the NH_4^+ assay was performed by using Nessler reagent (28). The pH of the medium was determined prior to each assay and at the end of the incubation time.

Transport of ammonia through cell membranes was determined by growing bacteria in [^{14}C]methylamine-enriched NFB minimal medium (19, 23). An overnight culture of *A. brasilense* was centrifuged, washed twice with a 0.85% NaCl solution, and resuspended in 100 ml of NFB medium containing 0.3 mM NH_4Cl and [^{14}C]methylamine at a final concentration of 1.6 μM. The ^{14}C activity was 34 kBq ml^{-1}. Samples (1 ml) were removed at 10-min intervals and centrifuged. The bacterial cells were washed once with 0.85% NaCl, resuspended in 1 N NaOH, and lysed for 1 h at 60°C. Intracellular radioactivity was measured by liquid scintillation counting with a Packard Tri-Carb model 4530 instrument.

Bacterial nitrate reductase activity was assayed by growing bacteria in semisolid (0.2% agar) NFB medium supplemented with 8 mM KNO_3. The nitrite formed was determined colorimetrically after 48 h by using the method of Neyra and Van Berkum (35).

Nitrite ammonification was measured in semisolid (0.2% agar) glycerol minimal medium supplemented with 3 mM $NaNO_2$. The NH_4^+ formed was determined after 24 h by using a modified Berthelot color reaction test (27).

Plants and growth conditions. We used *Triticum aestivum* 'Carasinho' (received from the Foundation for Agricultural Plant Breeding SVP, Wageningen, The Netherlands) as the host plant; this plant is an aluminum-tolerant cultivar that is known to develop an effective rhizosphere association with *A. brasilense* Wa5 (6a). Seeds were surface sterilized by soaking them in 1.5% sodium hypochlorite for 90 min. Plants were grown in glass tubes (length, 20 cm; diameter, 3 cm; two plants per tube) containing 2.5 g of sterilized perlite supplemented with 15 ml of a sterile nitrogen-free nutrient solution (11). The seeds were covered with a thin layer of sterile gravel to enable developing roots to penetrate the perlite.

Bacteria were inoculated by coating the seeds with a mixture of sterile gum arabic (40%) and a bacterial suspension containing 1.3×10^7 CFU ml^{-1}. The final bacterial count for each seed was approximately 2×10^6 CFU. Control plants were treated with the sterile gum arabic solution alone.

To trace the transfer of fixed nitrogen to host plant material, plants were exposed to a $^{15}N_2$-enriched atmosphere. The tubes were sealed airtight with a rubber seal 7 days after planting, after which 10 ml of the headspace gas was replaced with a 96.5%-enriched $^{15}N_2$ gas (VEB Technische Gase, Leipzig, Germany); this resulted in a final ^{15}N concentration of 7.302 atom% excess, which was maintained over the entire period of plant growth. Flushing of the headspace followed by $^{15}N_2$ injection was repeated at 14, 20, and 25 days after planting.

Plants were grown for 31 days with 12 h light per day at 20°C and 12 h of dark per day at 12°C with a relative humidity of 70%.

Plant assays. Acetylene reduction activity was determined at 30 days after planting by replacing 10% (vol/vol) of the tube headspace gas with acetylene. Then, the plants were incubated for 24 h in the light at a constant temperature of 25°C. The production of ethylene was determined by gas chromatography as described above. An ethylene standard was given to unplanted tubes. It was ensured previously that no ethylene was formed by the plants themselves.

Plants were harvested at 31 days after planting, and roots and shoots were sampled separately. Plant dry matter was measured after the material was dried at 80°C for 48 h. Nitrogen content was measured by using a Carlo Erba model NA 1500 automatic nitrogen analyzer, and ^{15}N enrichment was determined by mass spectroscopy (VG Sira model 10 instrument). The atoms percent values for ^{15}N from unexposed plants were used to calculate the values for ^{15}N excess. The amount of plant nitrogen gained from atmospherically fixed nitrogen was calculated by using the following equation: gained N = (^{15}N excess in plants × total N in plants)/^{15}N excess in headspace atmosphere. In addition to the ^{15}N enrichment, we calculated the nitrogen benefit obtained in a N balance study.

Bacterial root colonization was determined by carefully macerating 250-mg portions of fresh roots in homogenizing tubes and resuspending the root suspensions in 10-ml portions of 0.85% NaCl; 10-fold dilutions were made in 0.85% NaCl, and 100-μl portions of appropriate dilutions were plated onto tryptone soya agar (Oxoid).

TABLE 1. Acetylene reduction activities of wild-type *A. brasilense* Wa5 and NH_4^+-excreting mutants C3 and C5 grown on different nitrogen sources

Strain	Acetylene reduction (nmol of C_2H_4 produced per h per mg of protein)				
	Nitrogen-free medium	NH_4Cl		KNO_3 (8 mM)	Glutamate (3 mM)
		10 mM	20 mM		
Wild type	51 (100)[a]	0 (0)	0 (0)	0 (0)	32 (62.7)
C3	183 (100)	186 (101.6)	172 (93.9)	0 (0)	162 (88.5)
C5	101 (100)	29 (28.7)	11 (10.9)	0 (0)	88 (87.1)

[a] The values in parentheses are the percentages of nitrogenase activity determined by comparing the values obtained with the activities in nitrogen-free medium.

Statistical analysis. Bacterial acetylene reduction was measured by using three replicate cultures for each determination. Inoculation experiments were carried out by using a complete factorial design with four replicates per treatment. Bacterial counts in plant roots were determined by using two replicates. The standard error of the difference was calculated by performing analyses of variance (Genstat 5 Committee), and the Student *t* test was also performed. All significant differences reported below were at a level of $P = 0.05$ or at least $P = 0.01$.

RESULTS

Bacterial strains. Spontaneous ethylenediamine resistance in the bacteria which we studied occurred at a frequency of approximately 2×10^{-5} per cell. Of 120 colonies tested, 5 appeared to accumulate substantial amounts of NH_4^+ in all three media tested. Of these five, two mutants did not repress their nitrogenase systems in the presence of 10 mM NH_4Cl. The nitrogenase activities (C_2H_2 reduction) of both of these mutants were two to three times higher on nitrogen-free medium and on medium containing 3 mM glutamate than the nitrogenase activity of the wild type. The nitrogenase system of strain C5 was repressed in the presence of 10 mM NH_4Cl by approximately 70% and in the presence of 20 mM NH_4Cl by 90% compared with the nitrogenase activity of this strain in a nitrogen-free medium. In strain C3 the nitrogenase system was completely unrepressed even in the presence of 20 mM NH_4Cl (Table 1). Neither of the bacterial strains which we tested showed any nitrogenase activity in the presence of 8 mM KNO_3 (Table 1). The nitrate reductases were active in the wild type, as well as in the NH_4^+-excreting mutants of *A. brasilense* Wa5. Both mutants exhibited nitrite ammonification (Table 2).

TABLE 2. Nitrate reductase activities and nitrite ammonification by wild-type *A. brasilense* Wa5 and NH_4^+-excreting mutants C3 and C5

Strain	Nitrate reduction ($\mu M\ NO_2\ 48\ h^{-1}$)[a]	Nitrite ammonification ($\mu M\ NH_4^+\ 24\ h^{-1}$)[b]
Wild type	3.1	11
C3	1.7	293
C5	1.9	403

[a] Nitrate reduction was measured as nitrite accumulation in semisolid (0.2% agar) malate minimal medium (37) supplemented with 8 mM KNO_3.

[b] Nitrite ammonification was measured as NH_4^+ accumulation in semisolid (0.2% agar) glycerol minimal medium supplemented with 3 mM $NaNO_2$.

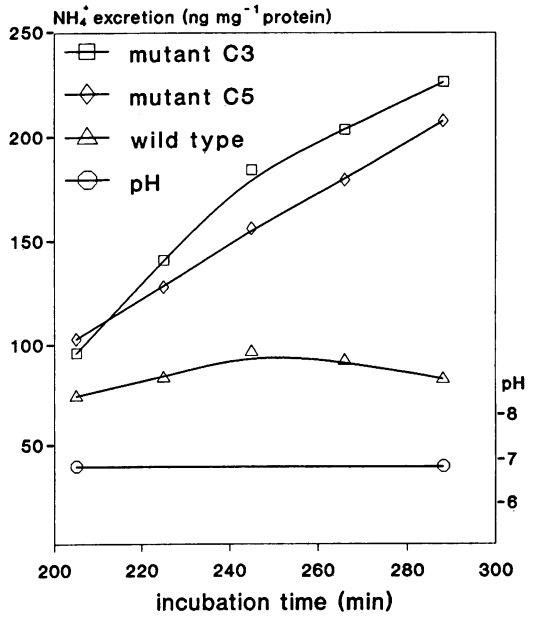

FIG. 1. NH_4^+ release by wild-type *A. brasilense* Wa5 and NH_4^+-excreting mutants C3 and C5 grown in nitrogen-free glycerol minimal medium at an O_2 tension of 1 kPa and 30°C. The pH of the medium was measured before and after incubation.

During incubation on glycerol minimal medium, the pH of the growth medium remained stable at pH 6.8 (Fig. 1). *A. brasilense* Wa5 released only very little NH_4^+ to its environment when it was grown on N-free medium or on nitrate. Only when cells were grown on 3 mM glutamate was some NH_4^+ excreted. Both mutant C3 and mutant C5 released large amounts of ammonia when they were grown at an O_2 concentration of 1 kPa on N-free medium, on nitrate-containing medium, and on glutamate-containing medium. NH_4^+ excretion was inhibited when the mutants were grown aerobically on N-free medium or on glutamate-containing medium but was not inhibited during aerobic growth on nitrate-containing medium. In all cases mutant C3 released the greatest amounts of NH_4^+ (Table 3).

In the presence of 3 mM NH_4Cl the growth of both mutants was slower than the growth of wild-type *A. brasilense* Wa5. Mutant C3 produced only a little protein on medium containing ammonia as the sole nitrogen source. However, the three bacterial strains had similar growth rates on medium containing 3 mM glutamate (Fig. 2).

TABLE 3. Ammonium excretion by wild-type *A. brasilense* Wa5 and NH_4^+-excreting mutants C3 and C5 grown on different nitrogen sources and at different oxygen tensions

Strain	NH_4^+ excretion (ng of NH_4^+ excreted per h per mg of protein)					
	1 kPa of O_2			20 kPa of O_2		
	Nitrogen-free medium	NO_3^- (8 mM)	Glutamate (3 mM)	Nitrogen-free medium	NO_3^- (8 mM)	Glutamate (3 mM)
Wild type	1.2	0.5	96	0	1.8	7
C3	156	146	322	15	149	155
C5	118	117	340	12	104	123

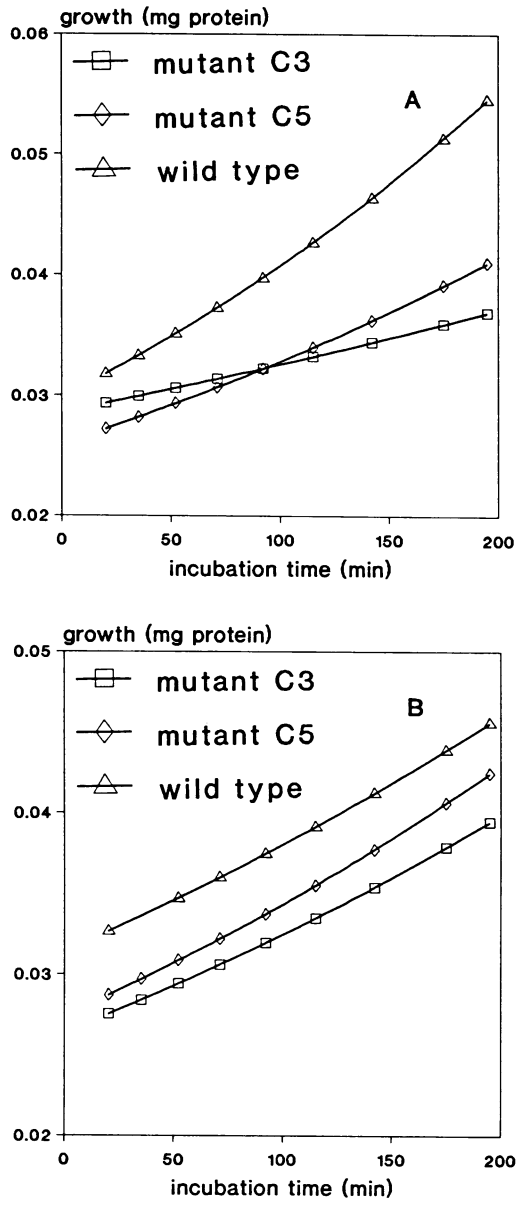

FIG. 2. Growth of wild-type *A. brasilense* Wa5 and NH_4^+-excreting mutants C3 and C5 in malate minimal medium (37) supplemented with 3 mM NH_4Cl (A) or 3 mM glutamate (B) at 30°C.

When grown on [^{14}C]methylamine-enriched NFB medium, wild-type *A. brasilense* Wa5 accumulated a considerable amount of radioactivity inside its cells. Neither mutant took up [^{14}C]methylamine during 140 min of incubation (Fig. 3).

Plant-bacterium interactions. When the bacteria were introduced to plants by seed coating, large numbers of both wild-type *A. brasilense* Wa5 and mutant C3 and C5 cells became established in the rhizospheres (Table 4). All of the bacterial strains strongly influenced root growth and morphology. The bacteria caused decreases in root elongation, and the plants developed increased numbers of short roots at the root bases; root branching was enhanced as well.

The nitrogenase activities (C_2H_2 reduction) in the rhizospheres were highest in the presence of mutants C3 and C5

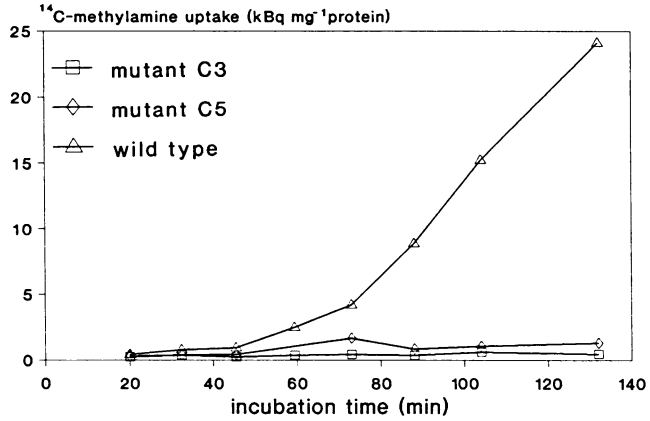

FIG. 3. [^{14}C]methylamine uptake by wild-type *A. brasilense* Wa5 and NH_4^+-excreting mutants C3 and C5 grown in malate minimal medium (37) supplemented with 0.3 mM NH_4Cl and 1.6 μM [^{14}C]methylamine (34 kBq/ml) at 30°C.

(Table 4). The absolute values for acetylene reduction activity appeared to be lower than the values observed previously in a similar system (6a).

The average nitrogen content of the wheat seeds was 0.71 mg of N per seed, so two seeds introduced a total of 1.42 mg of N into each tube. At harvest the noninoculated controls and the plants inoculated with wild-type *A. brasilense* Wa5 differed neither in dry matter production nor in nitrogen uptake. The amounts of dry matter and total nitrogen in roots and shoots were significantly greater in plants inoculated with strains C3 and C5, although the relative nitrogen concentrations were not affected by any bacterial inoculation (Table 5).

When exposed to a ^{15}N-enriched atmosphere, all of the plants, including noninoculated controls, incorporated ^{15}N to levels above the natural level; this observation could be attributed to ^{15}N adsorption, isotope exchange processes, or contamination of the $^{15}N_2$ gas added. The relative ^{15}N contents in the plant material were higher for plants inoculated with bacteria than for the controls, although these differences were not statistically significant ($P = 0.05$). The

TABLE 4. Root colonization and rhizosphere acetylene reduction activity in wheat inoculated with wild-type *A. brasilense* Wa5 or NH_4^+-excreting mutant C3 or C5[a]

Prepn or strain	No. of bacteria in roots (log CFU per g of dry roots)	Acetylene reduction activity (nmol of C_2H_4 produced per 24 h per plant)
Control[b]	ND[c]	0
Wild type	8.4	1.09
C3	7.6	1.83
C5	7.2	2.96

[a] Plants were grown axenically with nitrogen-free nutrition (10). Inoculation was by bacterial seed coating (2×10^6 CFU per plant). Acetylene reduction activity and root colonization were determined 31 days after planting. The standard error of the difference for the number of bacteria in roots was 0.7 log CFU per g of dry roots, which was determined from two replicates. The standard error of the difference for acetylene reduction activity was 0.81 nmol of C_2H_4 produced per 24 h per plant ($P < 0.05$), which was determined from four replicates.

[b] Noninoculated plants.

[c] ND, not determined.

TABLE 5. Dry matter production, nitrogen content, and total nitrogen accumulation in wheat inoculated with wild-type *A. brasilense* Wa5 or NH_4^+-excreting mutant C3 or C5[a]

Prepn or strain	Dry wt (mg)		N content (% of N)		Total N (μg of N)		
	Roots	Shoots	Roots	Shoots	Roots	Shoots	Total plants
Control[b]	22.0	34.3	1.30	3.64	290	1,240	1,530
Wild type	21.7	35.0	1.35	3.32	290	1,170	1,460
C3	35.7	46.0	1.36	3.49	480	1,600	2,080
C5	32.9	39.3	1.39	3.59	460	1,400	1,860

[a] Plants were grown axenically with nitrogen-free nutrition (10) and were harvested 31 days after planting. The standard errors of the difference were determined from four replicates and were as follows: root dry weight, 3.1 mg ($P < 0.01$); shoot dry weight, 2.8 mg ($P < 0.05$); root N content, 0.05%; shoot N content, 0.16%; root total N, 40 μg ($P < 0.01$); shoot total N, 90 μg ($P < 0.05$); and total plant total N, 108 μg ($P < 0.01$).

[b] Noninoculated plants.

total ^{15}N accumulation was more than twice as high in plants inoculated with NH_4^+-excreting mutants C3 and C5 than in controls. Total ^{15}N incorporation in plants inoculated with wild-type *A. brasilense* Wa5 was slightly increased as well (Table 6). On the basis of these ^{15}N enrichment data, we calculated that the potential nitrogen supply which was gained from atmospherically fixed nitrogen was 474 ng of nitrogen per plant for plants inoculated with wild-type *A. brasilense* Wa5, whereas strains C3 and C5 supplied 695 to 835 ng of N per host plant (Table 6).

DISCUSSION

A. brasilense mutants C3 and C5 failed to incorporate [^{14}C]methylamine (Fig. 3), which makes it very likely that these organisms lack the necessary enzyme systems to transport ammonia across cell membranes. Also, NH_4^+ repression of the nitrogenase system appeared to be affected in both mutants (Table 1). This observation fits the hypothesis that the biosynthesis of nitrogenase and the biosynthesis of the ammonia carrier proteins are controlled by the same regulation processes (25). Since mutants C3 and C5 grow well on glutamate but only poorly on NH_4Cl (Fig. 2), it is likely that the mutations of these mutants occur somewhere in the ammonia assimilation chain. Ammonia assimilation is controlled by the availability of NH_4^+ itself, and the regulation mechanisms have been reported to be commonly present in various diazotrophs, including *Azospirillum* spp. (1, 26, 43). The detailed mechanisms are not yet clear. Merrick (33) described a model for bacterial regulation of enzymes by NH_4^+ in which products of regulatory genes, such as *ntrA*, *ntrB*, and *ntrC*, repress or activate a promoter region for the controlled operons. In this model *ntrC* codes for an enzyme which can function either under the influence of the *ntrA* gene product as an activator or together with the *ntrB* gene product as a repressor for the transcription of the NH_4^+-controlled genes (10, 32). Bani et al. (2) and Gauthier and Elmerich (13) have provided evidence that a similar regulation mechanism is active in *Azospirillum* spp. Since in our *A. brasilense* Wa5 mutants the expression of *nif* (nitrogen fixation genes) was not the sole function affected, we suggest that the mutations probably did not occur at the promoter sites for the *nif* operon (*nifAL*) (15, 41), but rather occurred at *ntr*-like genes.

Wild-type *A. brasilense* Wa5 released some ammonia only when it was grown under oxygen-limiting conditions on glutamate (Table 3), which is consistent with the results of Hartmann et al. (18). Both mutants excreted large amounts of ammonia when they were grown on nitrate, although the bacteria did not develop nitrogenase activity (Table 1). This, together with the fact that the level of ammonia excretion under aerobic conditions was similar to the level of ammonia excretion under oxygen-limiting conditions (Table 3), makes it likely that the excreted NH_4^+ originated from the nitrate source. This is that the mutants retained their ability to take up and further reduce NO_3^- even if NH_4^+ uptake was inhibited. This was confirmed by the observation that both mutants had nitrate reductase activity and exhibited nitrite ammonification (Table 2).

After introduction to wheat roots, the numbers of rhizosphere-colonizing *A. brasilense* cells (both wild-type and mutant cells) were approximately 100 times higher than the numbers of cells observed previously in inoculation experiments performed with axenically grown summer wheat (6a). These relatively high cell densities in roots might explain the profound influence of all three bacterial strains on root development and morphology, which was probably due to bacterial production of the plant growth substance indoleacetic acid (16, 40).

Because the plants were grown on a nitrogen-free medium, the availability of mineral nitrogen was the limiting factor for plant growth. Plants inoculated with NH_4^+-excreting mutants of *A. brasilense* produced significantly more dry mass than either the controls or plants inoculated with the wild type (Table 5). As the numbers of rhizosphere-colonizing bacteria were similar for all three strains, these effects must be attributed to an additional supply of nitrogen rather than to bacterially produced plant growth substances. This hypothesis was confirmed by the accumulation of more total nitrogen in root and shoot material after inoculation with strains C3 and C5 than in the controls and after wild-type treatments (Table 5). The fact that the relative nitrogen concentrations in the plants were similar for all four treatments (including the uninoculated controls) was explained by the fact that in a nitrogen-limited plant growth system additional input of nitrogen leads to a proportional

TABLE 6. ^{15}N content, total ^{15}N accumulation, and amounts of N gained from atmospheric sources in wheat inoculated with wild-type *A. brasilense* Wa5 or NH_4^+-excreting mutant C3 or C5[a]

Prepn or strain	^{15}N content (atom% excess, 10^{-3})[b]		Total ^{15}N (ng of ^{15}N)		Amt of nitrogen gained (ng of N)		
	Roots	Shoots	Roots	Shoots	Roots	Shoots	Total plants
Control[c]	2.00	1.76	5.73	21.1	79	289	368
Wild type	2.67	2.33	7.77	26.8	107	368	474
C3	2.67	3.00	12.6	48.4	172	662	835
C5	2.44	2.83	11.0	39.8	151	545	695

[a] Plants were grown axenically with nitrogen-free nutrition (10) and were exposed to an atmosphere containing 7,302 atom% of $^{15}N_2$ excess. The amounts of nitrogen gained were calculated from the levels of ^{15}N enrichment in plant material. The standard errors of the difference were determined from four replicates and were as follows: root ^{15}N content, 0.35×10^{-3} atom% excess; shoot ^{15}N content, 0.52×10^{-3} atom% excess; root total ^{15}N, 1.16 ng ($P < 0.01$); shoot total ^{15}N, 7.04 ng ($P < 0.05$); root amount of nitrogen gained, 16 ng ($P < 0.01$); shoot amount of nitrogen gained, 96 ng ($P < 0.05$); and total plant amount of nitrogen gained, 96 ng ($P < 0.01$).

[b] ^{15}N excess values were calculated relative to the values for unexposed wheat plants.

[c] Noninoculated plants.

increase in plant dry matter and thus to dilution of the incorporated nitrogen.

The only mineral nitrogen input in our system was the introduction of wheat seeds (average input, 1.42 mg of N per tube). Thus, the amounts of nitrogen accumulated in plants in addition to the applied amounts as calculated by using the N balance were only 0.04 mg of N for the wild-type-treated plants and 0.66 and 0.44 mg of N for the plants inoculated with strains C3 and C5, respectively.

Wild-type *A. brasilense* Wa5 enhanced neither plant dry matter production nor total nitrogen accumulation, although the bacteria colonized the rhizospheres in large numbers and actively fixed nitrogen. This finding supports our hypothesis that a rhizosphere-associated *Azospirillum* sp. does not contribute fixed nitrogen directly to its host, but contributes nitrogen only in a late stage of plant development in the form of decomposed bacteria.

Both NH_4^+-excreting *A. brasilense* Wa5 mutants caused significantly greater accumulations of excess ^{15}N in roots and shoots than the wild-type strain did (Table 6). This additional nitrogen supply can only be attributed to biological nitrogen fixation. The values for total nitrogen gain, as calculated on the basis of the ^{15}N enrichment values (695 to 835 ng of N per plant), appeared to be much lower than the values calculated in the N balance study (0.44 to 0.66 mg of N per plant). This means that the *A. brasilense* Wa5 mutants transferred nitrogen to the system by means other than N_2 fixation alone. Perhaps recently fixed ^{15}N nitrogen is exchanged for intracellular bacterial ^{14}N, which is excreted unproportionally. This phenomenon has often been mentioned as the reason for artifacts observed when N uptake by plants has been studied by using ^{15}N-enriched fertilizer (17).

We demonstrated that NH_4^+-excreting mutants of *A. brasilense* are capable of becoming established in the rhizospheres of axenically grown wheat host plants to the same extent as the wild-type strain and of supplying larger amounts of nitrogen to the plant. However, much more research will be necessary to quantify the nitrogen benefits of such strains in plants grown over a longer period of time and under more natural conditions, where competitiveness with the natural microbial populations in the plant rhizospheres will be a factor.

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