Influence of Amino Acids on Nitrogen Fixation Ability and Growth of *Azospirillum* spp.

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The utilization of amino acids for growth and their effects on nitrogen fixation differ greatly among the several strains of each species of *Azospirillum* spp. that were examined. *A. brasiliense* grew poorly or not at all on glutamate, aspartate, serine, or histidine as the sole nitrogen and carbon sources. Nitrogen fixation by most *A. brasiliense* strains was inhibited only slightly even by 10 mM concentrations of these amino acids. In contrast, *A. lipoferum* and *A. amazonense* grew very well on glutamate, aspartate, serine, or histidine as the sole nitrogen and carbon sources; nitrogen fixation, which was measured in the presence of malate or sucrose, was severely inhibited by these amino acids. It was concluded that growth on histidine as the sole source of nitrogen, carbon, and energy may be used for the taxonomic characterization of *Azospirillum* spp. and for the selective isolation of *A. lipoferum*. The different utilization of various amino acids by *Azospirillum* spp. may be important for their establishment in the rhizosphere and for their associative nitrogen fixation with plants. The physiological basis for the different utilization of glutamate by *Azospirillum* spp. was investigated further. *A. brasiliense* and *A. lipoferum* exhibited a high affinity for glutamate uptake (K_m values for uptake were 8 and 40 µM, respectively); the V_{max} was 6 times higher in *A. lipoferum* than in *A. brasiliense*. At high substrate concentrations (10 mM), the nonsaturable component of glutamate uptake was most active in *A. lipoferum* and *A. amazonense*. The glutamate dehydrogenase activity of *A. lipoferum* was 7 times higher and the glutamate oxalacetate transaminase activities were 3 to 5 times higher in *A. lipoferum* and *A. amazonense* than in *A. brasiliense*. Glutamate-grown *A. brasiliense* had high glutamine synthetase and ammonium uptake activities, whereas these activities were low in glutamate-grown *A. lipoferum*. Thus, *Azospirillum* spp. differ quantitatively and qualitatively in their amino acid metabolism.

Azospirillum spp. are frequent inhabitants of the rhizosphere of a wide variety of plants with three-carbon, four-carbon, and crassulacean acid metabolism-type photosynthesis in diverse climatic regions of the world (23, 36). Results of physiological and genetic studies have led to the description of two species, *A. lipoferum* and *A. brasiliense* (43). A third species, *A. amazonense*, was isolated in an acidic enrichment medium from surface-sterilized roots of several grasses in Brazil (24). *A. amazonense* isolates form a taxonomic cluster that is clearly distinct from that of the other two species (6). The nitrogen fixation and plant growth-promoting abilities of *Azospirillum* spp. have aroused interest in its use as a bacterial fertilizer (5, 32, 33). *Azospirillum* spp. are also suitable model organisms for the study of plant-bacteria interactions.

Root exudates, which primarily contain organic acids, sugars, and amino acids, are a major source of nutrients for the microflora in the rhizosphere (4). Organic acids support the vigorous growth and nitrogen fixation of all *Azospirillum* spp., but the organisms have very different capabilities for the utilization of sugars (23). Whereas *A. brasiliense* does not grow and fix nitrogen with most sugars, *A. lipoferum* effectively utilizes glucose and *A. amazonense* utilizes sucrose (26). It has been suggested (39) that chemotaxis to organic acids, sugars, and amino acids is important for the establishment of root-associated growth of azospirilla in their ecological niche.

The regulation of nitrogen fixation and nitrogen assimilation has basic and practical importance for the azospirillum-plant association. Genetic regulation of nitrogenase synthesis probably is mediated by a *nifA* and *nifL*-type regulation system, such as that in *Klebsiella pneumoniae* (34, 35). In *A. lipoferum* and *A. brasiliense*, nitrogenase activity itself is inhibited by covalent modification of the dinitrogenase reductase after the addition of ammonium (13) or under anaerobic conditions (12). In *A. amazonense*, a noncovalent, less complete inhibition occurs (13, 42). Ammonium, the first product of nitrogen fixation, is assimilated through the glutamine synthetase (GS)-glutamate synthase (GOGAT) pathway (2, 30). GS is regulated by the nitrogen status of the cell through the adenylylation cascade (10, 30). Most studies on nitrogen assimilation have been performed with *A. brasiliense* Sp7, and there are few data that compare *A. brasiliense* with other species. Transaminase reactions have not been examined in *Azospirillum* spp.

The effects of amino acids on nitrogen fixation by *Azospirillum* spp. have not been studied in detail (9, 36). It has been well documented with other nitrogen-fixing microorganisms, however, that amino acids influence nitrogen fixation activity (29, 41, 44, 46). In slow-growing rhizobia, glutamate has been used to differentiate asymbiotically grown rhizobia that are capable and incapable of reducing acetylene (20). As constituents of plant cells and root exudates (4), amino acids may influence the nitrogenase activity of the azospirillum-root association. In this report we present a comparison of the patterns of amino acid utilization and the nitrogen assimilatory metabolism of *A. brasiliense*, *A. lipoferum*, and *A. amazonense*.

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(A preliminary account of this investigation was presented at the Third *Azospirillum* Workshop in Bayreuth, Federal Republic of Germany [14].)

MATERIALS AND METHODS

Organisms and culture conditions. *Azospirillum* strains were obtained from the American Type Culture Collection or from the Deutsche Sammlung für Mikroorganismen (DSM) (see Table 1). *A. amazonense* Y1 was kindly provided by N. R. Krieg and *A. brasiliense* Sp245 was provided by J. Döbereiner. *A. brasiliense* and *A. lipoferum* were grown in liquid or semisolid minimal salt medium by the method described by Albrecht and Okon (1). Trace elements were included, but yeast extract was not. *A. amazonense* was grown in the medium described by Magalhaes et al. (25), but 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 6.0) was added to stabilize the pH. Cells were grown on N_2 in a liquid, nitrogen-free medium under 0.2 to 0.5 kPa of oxygen pressure (microaerobic), as controlled by an oxystat (13, 31).

Assay for nitrogen fixation. Whole-cell nitrogenase activity was measured by the acetylene reduction method. At the time of assay, 21-ml bottles were stoppered and acetylene was injected into the gas phase (air) of the cultures on a semisolid medium to give 10 kPa of acetylene pressure. The cultures then were incubated for 2 h at 30°C, and the ethylene produced was analyzed by gas chromatography (3).

Assays for ammonium assimilatory enzymes. For the in vitro assays for ammonium assimilatory enzymes, cultures were grown aerobically in liquid minimal medium and harvested in the late-exponential growth phase (A_{580}, 1.0 to 1.5). The cells were washed twice with 20 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5) buffer and finally were suspended in about 1 ml of 100 mM HEPES buffer (pH 7.5) containing 10 mM dithiothreitol. Cell extracts were prepared by sonication (350 Ultrasonic cell disrupter with 50% duty cycle; Heat Systems) twice for 1 min each time with intermittent cooling in ice. The sonicated suspensions were ultracentrifuged at 200,000 × *g* for 1 h to remove membrane particles because they produce a high NADH oxidation background in some spectrophotometric enzyme assays. Finally, the clear extract was passed through a Sephadex G-10 column (6.0 by 1.3 cm [diameter]; equilibrated with HEPES buffer containing dithiothreitol) to remove the low-molecular-weight solutes from the enzymatic fraction. The enzyme assays were performed immediately after the extract was prepared.

The synthetic activity of GS was determined by γ-glutamylhydroxamate formation by the method described by Kleinschmidt and Kleiner (22). The activity obtained in the presence of Mg^{2+} was a suitable measure of the active form of GS, as it disappeared rapidly when ammonium chloride was added to the culture. Concomitantly, the Mn^2-dependent activity that represented the inactive form of GS increased (A. Hartmann, unpublished data). GOGAT activity was measured spectrophotometrically (28). Glutamate dehydrogenase (GDH) activity was determined by its reductive amination (NADH oxidation) of oxoglutarate (7). Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities were measured by previously described methods (7, 40).

Transport assays. Ammonium transport was determined with [^{14}C]methylammonium as described previously (15). [^{14}C]glutamate uptake was measured in the concentration range of 10 μM to 10 mM. At intervals, 50-μl samples of the

cell suspension, which was incubated with shaking at 30°C, were quickly filtered through polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.). The radioactivity taken up by the cells remained on the filter; the filter was then placed in scintillation fluid, and the radioactivity was measured.

Other methods. Glutamate was determined by high-performance liquid chromatographic analysis, as described previously (12). Ammonia was measured by the phenol hypochlorite method described by Fawcett and Scott (8). Protein was determined by the microbiuret method described by Goa (11).

Chemicals. All chemicals used in this study were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, Mo.); U.S. Biochemical Corp.; Aldrich Chemical Co., Inc. (Milwaukee, Wis.); and Bio-Rad Laboratories (Richmond, Calif.). The radioactive substances were purchased from New England Nuclear Corp. (Boston, Mass.).

RESULTS

Influence of amino acids on growth and nitrogen fixation. *A. brasiliense*, *A. lipoferum*, and *A. amazonense* strains showed a species-specific pattern of response toward glutamate, histidine, alanine, and serine (Table 1). *A. lipoferum* strains grew very well on these amino acids as the sole nitrogen and carbon sources and excreted high amounts of ammonium into the medium (Table 1). These amino acids abolished nitrogenase activity in the presence of malate. *A. amazonense* strains grew best with glutamate and alanine (Table 1). All amino acids that were tested inhibited the nitrogen fixation ability of *A. amazonense* at high concentrations. In contrast, *A. brasiliense* strains did not grow as well on glutamate and serine as did *A. lipoferum* and *A. amazonense*. *A. brasiliense* could not grow on histidine as the sole carbon and nitrogen source (Table 1). Nitrogen fixation was reduced a little or not at all by serine and glutamate, but was inhibited by alanine. In the presence of histidine, the specific nitrogen fixation activity of some *A. brasiliense* strains was regularly enhanced slightly (Table 2). A comparison of the effects of 11 amino acids on the nitrogen fixation activity of three representative *Azospirillum* strains is shown in Table 3. Glutamine, asparagine, arginine, and alanine effectively inhibited nitrogen fixation in all strains. Glycine and leucine did not affect the nitrogenase activity of *A. lipoferum*, but they inhibited it somewhat in *A. brasiliense* and strongly in *A. amazonense*.

Utilization of glutamate. In semisolid, NH_4^+-free, malate medium, glutamate (1 to 20 mM) inhibited the nitrogen fixation ability of *A. brasiliense* Sp7 only slightly (Fig. 1), but the nitrogen fixation ability of *A. lipoferum* SpRG20a was effectively inhibited by low concentrations of glutamate (Fig. 1A, 2-h assay). Low glutamate concentrations appeared to enhance the nitrogen fixation ability of *A. amazonense* Y1, and high concentrations partially inhibited nitrogen fixation (Fig. 1A). It was interesting that low levels of ammonium were detected in cultures of *A. brasiliense* and *A. amazonense* that were supplied with medium levels of glutamate (Fig. 1B); the cultures fixed nitrogen under these conditions (Fig. 1A).

In NH_4^+-free, semisolid malate medium, glutamate increased the final crop of *A. lipoferum* and *A. amazonense* up to 3.5 and 6 times, respectively, whereas it only doubled the final crop of *A. brasiliense* (Fig. 2). Thus, *A. brasiliense* was less effective in the uptake and utilization of glutamate than were *A. lipoferum* and *A. amazonense*.

A. lipoferum and *A. amazonense* grew equally well with glutamate or ammonium as the nitrogen source in liquid

TABLE 1. Influence of various amino acids on growth and nitrogen fixation by *Azospirillum* spp.

Species and strain(s) (source)	Growth increase (ammonium concn [mM]) on medium containing[a]:			
	Glutamate	Histidine	Alanine	Serine
A. brasiliense				
Sp7 (ATCC 29145)	4.9 (1.2)	0.8 (0)	4.3 (2.4)	1.3 (0.9)
SpCd (DSM 1843)	4.3 (1.0)	0.8 (0)	2.7 (1.4)	1.8 (1.1)
JM6A2 (DSM 1858)	12.5 (2.7)	0.9 (0)	8.0 (3.3)	1.6 (0.8)
SpP2 (DSM 2296)	5.2 (1.5)	0.8 (0)	4.9 (2.3)	1.5 (1.0)
SpT60 (DSM 2298)	12.4 (5.3)	1.0 (0)	8.3 (5.9)	1.5 (0.9)
Sp245 (J. Döbereiner)	10.2 (2.8)	1.1 (0)	8.1 (5.0)	1.4 (0.8)
A. lipoferum				
Sp59b (DSM 1691)	24.7 (5.6)	17.8 (16.2)	17.1 (6.4)	16.8 (6.9)
SpRG20a (ATCC 29708)	28.8 (4.9)	15.7 (11.8)	14.7 (6.5)	4.7 (3.3)
SpBr17 (ATCC 29709)	20.0 (5.1)	15.7 (15.5)	14.7 (6.6)	3.9 (2.5)
SpA3a (DSM 1838)	14.0 (4.7)	13.0 (14.4)	13.0 (6.3)	13.9 (6.5)
SpCo13 (DSM 2294)	13.2 (5.6)	10.2 (14.5)	11.4 (7.3)	10.0 (8.4)
Azospirillum sp. strain A1-3 (DSM 1726)	16.9 (5.6)	8.8 (17.0)	15.0 (7.6)	12.4 (6.9)
A. amazonense				
Y1 (N. R. Krieg)	17.2 (4.1)	4.5 (2.3)	10.9 (4.8)	2.2 (1.4)
Y2 (DSM 2788)	16.2 (4.8)	4.0 (3.2)	7.3 (4.3)	2.1 (1.6)
Y6 (DSM 2789)	24.3 (4.4)	2.7 (0.1)	10.2 (3.6)	3.9 (1.8)

[a] Growth and ammonium release in *Azospirillum* spp. with amino acids (10 mM) as the sole carbon, nitrogen, and energy sources. Semisolid minimal medium (without malate or sucrose and ammonium) was inoculated with 2% (vol/vol) stationary-phase cultures grown overnight (with NH_4^+) and incubated for 3 days at 30°C before growth (A_{580}), and the ammonium (in millimolar) in the medium was measured. Growth increase was calculated as the A_{580} in the presence of amino acids divided by the A_{580} without amino acids.

minimal medium (Table 4). In contrast, *A. brasiliense* grew much more slowly with glutamate than with ammonium as its nitrogen source. When 10 mM glutamate was added to the medium, *A. lipoferum* consumed almost all of it, whereas *A. brasiliense* used only a small portion of it (Table 4).

Comparison of glutamate uptake and metabolism in *Azospirillum* spp. The uptake of [^{14}C]glutamate supplied at 10 μM

to 10 mM was tested. Glutamate uptake was not saturated at 10 mM (Fig. 3); *A. lipoferum* and *A. amazonense* had the highest rates of uptake. At low concentrations, glutamate uptake appeared to be to levels of saturation in *A. brasiliense* and *A. lipoferum* but not in *A. amazonense*; however, further increases in glutamate concentrations supported increased uptake by all the organisms. The affinity for gluta-

TABLE 2. Influence of various amino acids on growth and ammonium release by *Azospirillum* spp.

Species and strain(s) (source)	Nitrogenase activity (nmol of ethylene/h per ml of culture [A_{580} = 1]) with[a]:				
	No amino acids	Glutamate	Histidine	Alanine	Serine
A. brasiliense					
Sp7 (ATCC 29145)	127	97	150	7	129
SpCd (DSM 1843)	109	98	137	2	101
JM6A2 (DSM 1858)	109	24	92	0	111
SpP2 (DSM 2296)	113	34	112	23	87
SpT60 (DSM 2298)	88	2	34	0	1
Sp245 (J. Döbereiner)	195	131	224	67	182
A. lipoferum					
Sp59b (DSM 1691)	81	0	0	0	0
SpRG20a (ATCC 29708)	105	0	0	0	14
SpBr17 (ATCC 29709)	94	0	0	0	71
SpA3a (DSM 1838)	97	0	0	0	0
SpCo13 (DSM 2294)	41	0	0	2	0
Azospirillum sp. strain A1-3 (DSM 1726)	147	0	0	0	12
A. amazonense					
Y1 (N. R. Krieg)	38	6	0	5	0.5
Y2 (DSM 2788)	23	4	0	3	1
Y6 (DSM 2789)	22	2	0	4	2

[a] Effects of amino acids (10 mM) on nitrogenase activity of *Azospirillum* spp. were measured in the presence of 0.5% malate or sucrose (*A. amazonense*). Semisolid minimal medium (with 0.5% malate or sucrose) was inoculated with 5% (vol/vol) stationary-phase cultures grown overnight (with NH_4^+) and incubated for 24 h at 30°C before the acetylene reduction assay was performed.

TABLE 3. Effects of amino acids on nitrogenase activity
and growth of *Azospirillum* spp.[a]

Amino acid (10 mM)	% Nitrogenase activity (% growth) of:		
	A. brasiliense Sp7	*A. lipoferum* SpBr17	*A. amazonense* Y1
Glutamine	0.5 (139)	0.6 (198)	0 (387)
Asparagine	0 (132)	0.5 (232)	0 (306)
Arginine	2.3 (89)	12 (35)	0.4 (298)
Alanine	5.3 (143)	0 (168)	4.8 (276)
Glutamate	77 (98)	0.7 (206)	15 (353)
Aspartate	103 (86)	0.5 (226)	12 (338)
Histidine	118 (125)	0.2 (439)	0 (329)
Serine	102 (98)	75[b] (271)	0.7 (138)
Leucine	27 (150)	126 (260)	0 (243)
Proline	34 (126)	45 (262)	3.2 (307)
Glycine	48 (18)	84 (48)	21 (94)

[a] Semisolid minimal medium (with 0.5% malate or sucrose) was inoculated with 5% (vol/vol) stationary-phase cultures grown overnight (with NH_4^+) and incubated for 24 h at 30°C before the acetylene reduction assay was started. Nitrogenase activity and growth in the absence of amino acids was 100% without amino acids added; the following acetylene reduction activities (nanomoles per hour per milliliter [$A_{580} = 1$]) were measured: Sp7, 89; SpBr17, 127; Y1: 46.

[b] This high nitrogenase activity of *A. lipoferum* SpBr17 with serine is exceptional for *A. lipoferum* (Table 1).

mate was higher in *A. brasiliense* (apparent K_m, 8 μM) than in *A. lipoferum* (apparent K_m, 40 μM), but the observed V_{max} was about 6 times as high for *A. lipoferum* (16 nmol/min per ml) as for *A. brasiliense*. Cultures grown with N_2 in the absence of glutamate showed similar levels of glutamate uptake (data not shown).

The activities of assimilatory enzymes were also tested. In *A. brasiliense*, GDH and GOT activities were low. However, glutamate-grown cells had a highly active GS and an active ammonium carrier (Table 5). In *A. lipoferum* and *A. amazonense*, activities of GDH, GOT, GTP, or all three were much higher. When grown on glutamate, the GS activity, of *A. lipoferum* (Mg^{2+}, active form) was low, and the ammonium carrier was not demonstrable in *A. lipoferum* or *A. amazonense* (Table 5). This indicates that only *A. brasiliense* experienced nitrogen-limiting conditions when grown in the malate-glutamate medium.

In liquid microaerobic cultures without glutamate (N_2-fixing conditions), GDH, GOT, GTP, or all three were also much more active in *A. lipoferum* and *A. amazonense* than in *A. brasiliense* (data not shown).

DISCUSSION

Azospirillum spp. differ not only in the utilization of various carbon sources (23, 26) but they also have different abilities to use certain amino acids as their sole nitrogen, carbon, and energy sources. Although there was some variation among strains, the degree of utilization of glutamate, aspartate, histidine, and serine appeared to be characteristic for each organism at the species level. In general, *A. lipoferum* and *A. amazonense* used amino acids more readily than did *A. brasiliense*. Nitrogen fixation, which was measured in the presence of malate or sucrose, was abolished by those amino acids that were utilized effectively (Tables 1 to 3). *A. lipoferum* and *A. amazonense* may develop a more saprophytic life-style in the rhizosphere than does *A. brasiliense*. Since growth of *A. lipoferum* and *A. amazonense* was enhanced even by low concentrations of glutamate (Fig. 2), glutamate or other amino acids may help

these strains to become established in the rhizosphere. If the supply of amino acids is not too high relative to the supply of organic acids and sugars, this may lead to an N_2-fixing association. For example, in the pea-*Rhizobium leguminosarum* system, the exudation of homoserine by roots is reported to stimulate the development of *Rhizobium* species of the right cross-inoculation group (45).

On the basis of their different utilization of amino acids, *Azospirillum* spp. should be clearly distinguishable (Tables 1 to 3). With histidine as the sole carbon, nitrogen, and energy source, it also should be possible to isolate *A. lipoferum* selectively in a semisolid medium (pH 7). According to Krieg and Döbereiner (23), *Azospirillum* sp. strain 1726 possesses properties that are typical of *A. lipoferum* and *A. brasiliense* and might be a variant of *A. lipoferum* (25). Our results concerning amino acid utilization (Tables 1 and 2) strongly indicate that strain 1726 is a strain of *A. lipoferum*.

The physiological basis for the different uptake and utilization of glutamate by *Azospirillum* spp. was studied. The apparently nonsaturable component of glutamate uptake was highest in *A. lipoferum* and *A. amazonense*. *A. brasiliense* and *A. lipoferum* exhibited both passive and active gluta-
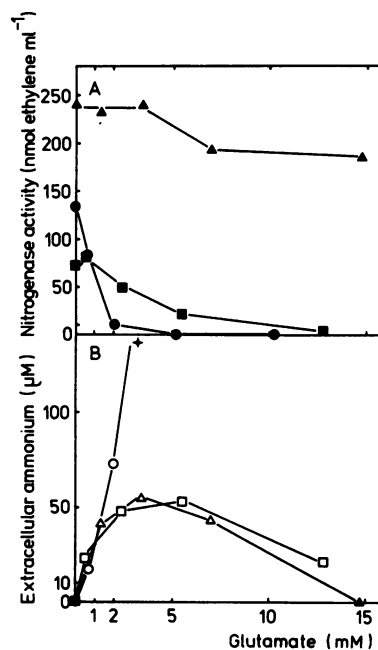

FIG. 1. Effect of glutamate on the specific nitrogenase activities (nanomoles of ethylene per milliliter of culture [$A_{580} = 1$]; closed symbols) (A) and ammonium release (micromolar; open symbols) (B) of *A. brasiliense* Sp7 (▲, △), *A. lipoferum* SpRG20a (●, ○), and *A. amazonense* Y1 (■, □). Nitrogen-free semisolid minimal medium, with 0.5% malate or sucrose (strain Y1) as the carbon source, was inoculated with 5% (vol/vol) stationary-phase cultures grown overnight in NH_4^+ minimal medium. After incubation at 30°C for 24 h, the vials were stoppered and acetylene reduction was measured for 2 h. At 24 h, ammonium release also was determined in the supernatants of the cultures. Growth was measured turbidimetrically (A_{580}) in appropriate dilutions; uninoculated semisolid medium was used as a blank. The glutamate concentrations were measured in the supernatants of the cultures by high-performance liquid chromatographic analysis at the time (24 h) that the nitrogenase and ammonium assays were done. The initial glutamate concentrations were 2, 5, 10, or 20 mM. In panel B, the star indicates that the ammonium released by *A. lipoferum* with 5 and 10 mM glutamate in the medium was 290 and 250 μM, respectively.

mate uptake (Fig. 3); this was similar to that shown for several metabolite uptake processes by *Rhizobium japonicum* (38). The maximal velocity that we measured for active, saturable glutamate uptake was much higher in *A. lipoferum* than in *A. brasiliense*. Interestingly, *A. amazonense* does not appear to possess an active, saturable component of glutamate uptake. This could reflect an adaptation to high glutamate concentrations, which *A. amazonense* commonly may have experienced in the endorhizosphere from which it was isolated (24). In contrast, we observed that *A. brasiliense* isolated from rhizosphere soil took up glutamate with a very high substrate affinity.

The effective uptake of glutamate and the high assimilatory enzyme activity permit *A. lipoferum* and, to a lesser extent, *A. amazonense* to use glutamate very well. In contrast, *A. brasiliense* was more like *Rhodospirillum rubrum*, which uses glutamate poorly as a nitrogen source and allows the expression of nitrogenase in the presence of malate (19). *A. brasiliense* (Mg^{2+}, active form) had highly active glutamine synthetase and ammonium permease activities, together with high nitrogenase activity, in the presence of glutamate (Fig. 1 and Table 5). Pedrosa and Yates (34) have reported the successful growth of N_2-fixing *A. brasiliense* in liquid cultures or on plates under air in the presence of glutamate. By increasing growth and thereby reducing the oxygen concentration to levels that are compatible with nitrogenase derepression and function, glutamate supported the growth of *A. brasiliense* under nitrogen-limiting conditions. However, this procedure cannot be used for *A. lipoferum* and *A. amazonense*, because glutamate represses nitrogen fixation in these species. Therefore, we suggest that N_2-fixing cultures of *Azospirillum* spp. should be grown with a small initial amount of ammonium (about 1 mM) as a starter for growth, and that the dissolved oxygen concentration should be controlled to ensure microaerobic conditions for optimal nitrogen fixation (13, 31, 42).

In *A. lipoferum* and *A. amazonense*, GDH, GOT, and GTP activities were much higher than those in *A. brasiliense* (Table 5). A high transaminase activity has been shown to support good growth with alanine in *Rhodopseudomonas acidophila* (17). High GDH activity also may be important for the effective use of glutamate by *A. lipoferum* (Table 5); the oxidative deamination reaction yields ammonium and oxoglutarate. This reaction was not very active and had a low affinity for glutamate (K_m, 250 mM; A. Hartmann,
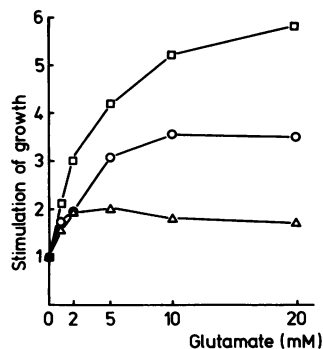
TABLE 4. Comparison of growth rates and consumption of nitrogen by *Azospirillum* spp. in liquid minimal medium with ammonium or glutamate (10 mM initially)

Strain	Growth on:			
	NH₄Cl		Glutamate	
	Doubling time (h)	NH₄⁺ concn (mM) after 22 h	Doubling time (h)	Glutamate concn (mM) after 22 h
A. brasiliense Sp7	2.5[a]	0.02	5.3	8.50
A. lipoferum SpRG20a	2.5	0.77	2.5	0.10
A. amazonense Y1	3.3	NT[b]	3.3	NT

[a] Two experiments gave doubling times of 2.5 and 3.5 h.
[b] NT, Not tested.

unpublished data). However, glutamate uptake yielded intracellular concentrations that were high enough for the deamination reaction to occur. In the presence of glutamate, *A. lipoferum* was not nitrogen limited. This was reflected by the low GS and ammonium uptake activities (Table 5), the fast growth rate with glutamate as the nitrogen source, and the rapid consumption of glutamate in the medium (Table 4). Glutamate uptake and metabolism to other nitrogen-containing compounds was adequate to signal nitrogen sufficiency. Nitrogenase activity also might be inhibited by an increase of organic acid intermediates, such as oxoglutarate, as occurs in *Rhodospirillum rubrum* (18).

A low level of ammonium was detected in the cultures when concentrations of glutamate were furnished in the medium and nitrogen fixation was operative at a reduced rate (Fig. 1). In *A. brasiliense* and *A. amazonense*, less ammonium was found at high glutamate concentrations. Glutamate, or a metabolite derived from it, might decrease the activities of ammonium assimilatory enzymes. It would be of considerable interest to find N_2-fixing strains that release substantial amounts of ammonium in the presence of an external effector. The release of ammonium from carbon-limited *Azospirillum* cultures has been reported (16), and this
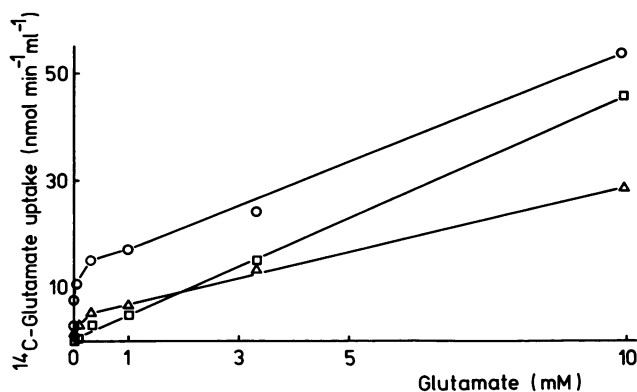

FIG. 2. Effect of glutamate on growth of *A. brasiliense* Sp7 ($\triangle$), *A. lipoferum* SpRG20a (○), and *A. amazonense* Y1 (□). Nitrogen-free semisolid minimal medium, with 0.5% malate or sucrose (strain Y1) as the carbon source, was inoculated with 5% semisolid culture (nitrogen source, 1 mM glutamate). Growth was measured after incubation at 30°C for 24 h.

FIG. 3. [^{14}C]glutamate uptake in *A. brasiliense* Sp7 ($\triangle$), *A. lipoferum* SpRG20a (○), and *A. amazonense* Y1 (□). Liquid minimal medium cultures with 10 mM glutamate were grown to the late-logarithmic growth phase (A_{580} = 1.0 to 1.5). Cells were spun down and washed twice with fresh nitrogen-free minimal medium. The transport assays were performed in the presence of 100 μg of chloramphenicol per ml. The amounts of [^{14}C]glutamate taken up 2 min after the start of the uptake assay (linear phase) were used to calculate the velocity of glutamate uptake (nanomoles per minute per milliliter of culture [A_{580} = 1]).

TABLE 5. Comparison of nitrogen assimilatory enzyme activities in glutamate-grown *Azospirillum* cultures

| Strain | mU[a] | | | | | | | Uptake (nmol/min per ml) of: | | |
| | GDH | GOT | GPT | GS (mU) | | | GOGAT | NH_4^+ | Glutamate at substrate concn of: | |
				MN	Mg	Mn/Mg			30 μM	10 mM
A. brasiliense Sp7	42	58	1	34	76	0.45	17	0.24	2.0	28.5
A. lipoferum SpRG20a	285	182	24	28	11	2.55	19	0.03	7.5	53.5
A. amazonense Y1	44	281	28	59	67	0.88	35	0.03	0.15	45.5

[a] mU is 10^{-3} unit. One unit of enzyme activity is equal to 1 μmol of NADH (GOT, GPT, GDH) or NADPH (GOGAT) oxidized per min per mg of protein. One unit of GS activity is equal to 1 μmol of γ-glutamylhydroxamate formed per min per mg of protein.

ammonium leakage was reversed by the addition of a carbon source. Release occurs after nitrogenase and ammonium permease activities cease as a result of carbon and energy limitations (A. Hartmann, unpublished data). A better understanding of the regulation of ammonium assimilation in *Azospirillum* spp. requires a detailed knowledge of the kinetic and regulatory properties of the enzymes that are involved. GDH and GOGAT of *A. brasiliense* have been obtained in pure form; some of their properties have been studied, and some data on the metabolite control of these enzymes has been reported (27, 37). The regulatory properties of GS, GOGAT, and ammonium permease (21) in strains with an effective associative nitrogen fixation remain to be studied.

Because the quantity and composition of amino acids in plant root exudates vary among plant species and growth stages (4), the utilization of specific amino acids by *Azospirillum* spp. may be important for their establishment in the rhizosphere of a particular plant and for their actual nitrogen fixation activity in association with roots. In this respect, data about the occurrence of amino acids and amino acid derivatives in root exudates and root tissues are needed for further evaluation of the potential of associative nitrogen fixation.

ACKNOWLEDGMENTS

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