

## Isolation and Characterization of Glutamate Synthase Mutants of *Azospirillum brasilense*

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Six mutants of *Azospirillum brasilense* Sp6 unable to fix nitrogen have been isolated and characterized. Analysis of the enzymes involved in nitrogen metabolism has shown that the mutants are deficient in glutamate synthase activity (*asm*). They also have a low activity of glutamine synthetase and no or very low nitrogenase activity (assayed by acetylene reduction). In addition, the mutants were unable to grow on various sources of combined nitrogen such as nitrate, nitrite, alanine, histidine, adenine and xanthine.

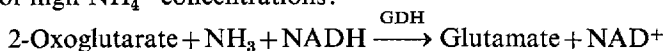
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### INTRODUCTION

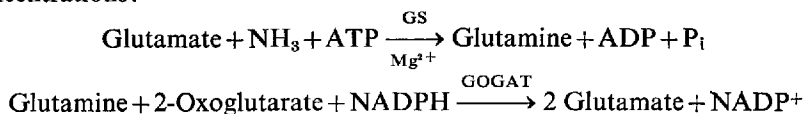
*Azospirillum brasilense* is a bacterium which is able to fix nitrogen in the free-living state (Becking, 1963). Recently, associative symbiosis of *A. brasilense* with tropical grass and cereal species has been reported (Day *et al.*, 1975; von Bülow & Döbereiner, 1975; Döbereiner & Day, 1975; Smith *et al.*, 1976). A number of papers have been published on the taxonomy, physiology and ecology of *A. brasilense* (Burris *et al.*, 1977; Okon *et al.*, 1976*a, b*, 1977; Krieg, 1977; Matin & Veldkamp, 1978). Mutants of *A. brasilense* altered in glutamine synthetase [GS; EC 6.3.1.2; L-glutamate:ammonia lyase (ADP-forming)] (Gauthier & Elmerich, 1977) and nitrate reductase and/or nitrite reductase (Magalhães *et al.*, 1978) have been described, and genetic transformation of *A. brasilense* has recently been reported (Mishra *et al.*, 1979).

According to the model based mainly on studies with *Klebsiella pneumoniae*, expression of the nitrogenase genes is positively controlled by the enzyme glutamine synthetase (Tubb, 1974; Streicher *et al.*, 1974). Ammonia, in many bacteria, is assimilated by two routes:

(1) via glutamate dehydrogenase [GDH; EC 1.4.1.2; L-glutamate:NAD<sup>+</sup> oxidoreductase], in the presence of high NH<sub>4</sub><sup>+</sup> concentrations:



(2) via glutamine synthetase (GS) and glutamate synthase [GOGAT; EC 1.4.1.13; L-glutamate:NADP<sup>+</sup> oxidoreductase (transaminating)], in N<sub>2</sub>-fixing conditions or in limiting NH<sub>4</sub><sup>+</sup> concentrations:



In the present work we describe six mutants of *A. brasilense* which are unable to fix nitrogen (Nif<sup>-</sup>) and which are altered in their glutamate synthase activity. The properties of the six mutants resembled those of the *asm* mutants described in *Klebsiella pneumoniae* by Nagatani *et al.* (1971) and Streicher *et al.* (1972).

## METHODS

**Bacterial strain.** *Azospirillum brasilense* strain Sp6, a prototroph, was used. Its DNA base composition and utilization of carbon and nitrogen sources (Mengoni *et al.*, 1980) are the same as for *A. brasilense* strain Sp7 (Döbereiner & Day, 1975); both strains belong to group I of Krieg (1977).

**Media.** Minimal medium (MSP) contained (g l<sup>-1</sup>): KH<sub>2</sub>PO<sub>4</sub>, 1.5; K<sub>2</sub>HPO<sub>4</sub>, 0.5; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.18; NaCl, 0.1; Na<sub>2</sub>MoO<sub>4</sub>, 0.01; CaCl<sub>2</sub>, 0.01; FeCl<sub>2</sub>, 0.01; sodium succinate, 5; NH<sub>4</sub>Cl, 1.3 (unless stated otherwise); pH 7. Complete medium (CM) was MSP plus yeast extract (5 g l<sup>-1</sup>) and acid hydrolysed casein (5 g l<sup>-1</sup>).

For the nitrogenase assays, the minimal medium was the salt base of Kalininskaja medium (Biggins & Postgate, 1969) supplemented with sodium succinate (5 g l<sup>-1</sup>) and yeast extract (5 mg l<sup>-1</sup>), and complete medium contained (g l<sup>-1</sup>): Bacto-nutrient broth (Difco), 8; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.25; KCl, 1; MnCl<sub>2</sub>, 0.001 (Gauthier & Elmerich, 1977).

All cultures were incubated at 35 °C.

**Enzyme assays.** Glutamine synthetase: exponential phase bacteria grown in MSP medium were resuspended in 2 mM-Tris/HCl buffer, pH 7, sonicated and centrifuged at 30000 g for 30 min; the supernatant was used for testing enzyme activity. Biosynthetic activity of GS was determined by the method of Rowe *et al.* (1970); the relative adenylation of GS was estimated by the ratio of absorbance values of transferase assays conducted in the presence or absence of 60 mM-MgCl<sub>2</sub> (Shapiro & Stadtman, 1970). Specific activities were expressed as  $\mu\text{mol } \gamma\text{-glutamyl hydroxamate produced min}^{-1} (\text{mg protein})^{-1}$ . Glutamate synthase and glutamate dehydrogenase: GDH and GOGAT were assayed as described by Meers *et al.* (1970) using crude extracts obtained from exponential phase cells grown in MSP medium and resuspended in 2 mM-Tris/HCl buffer plus 10 mM-2-mercaptoethanol, pH 7. Cells were broken by grinding in a mortar with alumina (type 305, Sigma) and then centrifuged at 6000 g for 10 min to remove the alumina; the supernatant was collected and centrifuged at 30000 g for 30 min. Specific activities were expressed as  $\mu\text{mol NADH (GDH) or NADPH (GOGAT) oxidized min}^{-1} (\text{mg protein})^{-1}$ . The three enzymes were assayed immediately after extract preparation.

**Nitrogenase:** nitrogenase activity was assayed by the acetylene reduction method (Postgate, 1972) on whole cells. Exponential phase cells from complete medium (Gauthier & Elmerich, 1977) were washed and diluted to about 10<sup>7</sup> cells ml<sup>-1</sup> in Kalininskaja medium. Portions (8 ml) of this cell suspension were incubated at room temperature in 80 ml test tubes filled with argon and 0.5% (v/v) O<sub>2</sub>. After 3 h incubation, 10% (v/v) acetylene was injected and the production of ethylene was followed for 3 h. In some experiments cells were kept in Kalininskaja medium plus aspartate (2 g l<sup>-1</sup>) for 8 h under argon and 0.5% (v/v) O<sub>2</sub> before injection of acetylene. The ethylene produced was measured with a Packard gas chromatograph with Porapak T column at 100 °C. Specific activity was expressed as nmol ethylene produced min<sup>-1</sup> (mg protein)<sup>-1</sup>.

**Protein determination.** Proteins were determined by the method of Lowry, using bovine serum albumin (Sigma) as standard.

**Utilization of nitrate and nitrite as electron acceptors.** Respiratory (dissimilatory) nitrate and nitrite reduction was assayed by estimating anaerobic growth in the presence of 25 mM-KNO<sub>3</sub> or 5 mM-KNO<sub>2</sub> in CM medium. Anaerobic growth was obtained in test tubes (160 × 15 mm) filled with CM medium containing 0.5% (w/v) purified agar (Difco) and sealed with rubber stoppers. The appearance of deep colonies was observed after 3 d.

**Isolation of mutants.** Mutants were obtained from a late-exponential phase culture of *A. brasilense* strain Sp6 treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Adelberg *et al.*, 1965). Appropriate dilutions of the mutagenized culture were plated on MSP medium containing 25 mM-NH<sub>4</sub>Cl; colonies were replicated on to MSP medium without NH<sub>4</sub>Cl. After 3 d incubation at 35 °C, the colonies which did not show growth on the replica plates were picked and purified.

## RESULTS

*Utilization of different nitrogen sources by six mutants with a Nif<sup>-</sup> phenotype*

Six mutants, isolated as described in Methods, were able to grow on MSP containing NH<sub>4</sub>Cl as sole nitrogen source but failed to grow with N<sub>2</sub> as sole nitrogen source. One of these mutants (SPF104) gave rise to small colonies on nitrogen-free minimal medium after 4 d incubation.

In *K. pneumoniae* mutations affecting the ability of the strain to grow on N<sub>2</sub> as sole nitrogen source may be either in one of the *nif* genes (and therefore specific for N<sub>2</sub> fixation) or in a gene concerned with general ammonia assimilation. The latter class can be recognized by their inability to utilize a wide range of different nitrogen compounds as sole

Table 1. Growth of mutants of *A. brasilense* on various compounds as nitrogen sources

Bacterial suspensions were streaked on MSP plates supplemented with different nitrogen sources (all 2 mg ml<sup>-1</sup> except KNO<sub>3</sub>, 0.4 mg ml<sup>-1</sup>). Growth was estimated visually after 5 d at 35 °C: +, growth; -, no growth; ±, reduced growth.

Nitrogen source	Sp6						
	(parental)	SPF101	SPF103	SPF104	SPF105	SPF109	SPF111
Ammonium chloride	+++	+++	+++	+++	+++	+++	+++
Glutamine	+++	+++	+++	+++	+++	+++	+++
Arginine	+++	++	++	++	++	++	++
Glutamate	+++	+	+	+	+	+	+
Asparagine	+++	+	+	++	+	+	+
Aspartate	+++	+	+	++	+	+	+
Proline	+++	+	+	++	±	±	±
Histidine	+++	-	-	+	-	-	-
Alanine	+++	-	-	+	-	-	-
Adenine	+++	-	-	+	-	-	-
Xanthine	+++	-	-	+	-	-	-
Nitrate (potassium)	+++	-	-	+	-	-	-
Nitrite (potassium)	+++	-	-	+	-	-	-
N <sub>2</sub> *	++	-	-	+	-	-	-

\* No combined nitrogen in the medium.

nitrogen source (Brill, 1975). All the six mutants described here were found to have such a pleiotropic phenotype (Table 1), a phenotype which was similar but not identical to that shown by *asm* mutants of *K. pneumoniae* (Nagatani *et al.*, 1971).

Owing to the inability of the mutants to assimilate nitrate and nitrite, they were also tested for respiratory utilization of nitrate and nitrite in anaerobic growth experiments, as described in Methods. All the mutants utilized nitrate and nitrite as electron acceptors (results not shown).

#### Enzymic characterization of the mutants

*Glutamine synthetase, glutamate dehydrogenase and glutamate synthase activities.* Extracts were prepared from all six mutants using bacteria grown in minimal medium with two different NH<sub>4</sub><sup>+</sup> concentrations. Five mutants had no detectable GOGAT activity (Table 2), while one (SPF104) retained about 25% of parental activity; this residual GOGAT activity would explain the ability of mutant SPF104 to give very small colonies in the presence of N<sub>2</sub> as sole nitrogen source. The GDH activity of the wild-type strain was similar at both high and low ammonia concentrations. Mutants SPF101, SPF103 and SPF104 had less than 50% of the parental GDH activity, but mutants SPF105, SPF109 and SPF111 were not affected. In comparison with the parental strain, all six mutants failed to derepress GS when subjected to ammonia limitation.

*Nitrogenase activity.* Five mutants (SPF101, SPF103, SPF105, SPF109 and SPF111) were unable to reduce acetylene and one (SPF104) showed only about 4% of parental activity [2 nmol ethylene produced min<sup>-1</sup> (mg protein)<sup>-1</sup> compared with 51 in the parental strain]. The mutants, with the exception of SPF104, did not show ethylene production even after 24 h incubation with acetylene. The same results were obtained when the mutants were grown in the presence of aspartate as sole nitrogen source (results not shown).

#### DISCUSSION

As reported by Tubb (1974), Nagatani *et al.* (1971) and Okon *et al.* (1976b), N<sub>2</sub>-fixing bacteria grown in the presence of excess NH<sub>4</sub><sup>+</sup> assimilate ammonia by GDH, and GS is present mainly in the inactive adenylylated form, while bacteria grown in NH<sub>4</sub><sup>+</sup>-limiting conditions or in NH<sub>4</sub><sup>+</sup>-free medium show higher levels of active unadenylylated GS, and GDH synthesis is repressed. The level of GOGAT activity is not necessarily affected by NH<sub>4</sub><sup>+</sup> concentration (Nagatani *et al.*, 1971).

Table 2. *Enzymic activities of extracts from A. brasilense mutants*

GOGAT activities are expressed as  $\mu\text{mol NADPH oxidized min}^{-1} (\text{mg protein})^{-1}$ , GDH activities as  $\mu\text{mol NADH oxidized min}^{-1} (\text{mg protein})^{-1}$ , and GS activities as  $\mu\text{mol } \gamma\text{-glutamyl hydroxamate formed min}^{-1} (\text{mg protein})^{-1}$  for the biosynthetic assay and for the transferase assay.

Strain	NH <sub>4</sub> <sup>+</sup> concn in culture medium (mM)	GOGAT	GDH	GS*			Unadenylylated form (%)
				Biosynthetic assay + Mg <sup>2+</sup>	Transferase assay		
				+ Mn <sup>2+</sup>	+ Mn <sup>2+</sup> + Mg <sup>2+</sup>		
Sp6 (parental)	26	0.024	0.139	0.040	6.535	0.385	5.9
	2.6	0.039	0.108	1.390	10.335	8.265	80.0
SPF101	26	ND	0.019	0.055	8.450	0.562	6.6
	2.6	ND	0.036	0.060	5.000	0.470	9.4
SPF103	26	ND	0.058	0.027	3.250	0.165	5.0
	2.6	ND	0.042	0.019	0.920	0.180	19.3
SPF104	26	0.007	0.046	0.050	0.675	0.067	10.0
	2.6	0.009	0.046	0.060	5.450	0.855	15.7
SPF105	26	ND	0.096	0.021	2.095	0.515	24.6
	2.6	ND	0.100	0.060	2.220	0.545	24.3
SPF109	26	ND	0.180	0.011	2.195	0.105	4.8
	2.6	ND	0.138	0.135	2.890	0.760	26.0
SPF111	26	ND	0.162	0.032	1.400	0.340	24.0
	2.6	ND	0.221	0.100	0.600	0.305	50.8

ND, Not detectable. \* Samples were incubated at 40 °C for 20 min.

Mutations causing a pleiotropic phenotype with respect to the utilization of a wide range of nitrogen sources have been described in a number of bacteria. They include mutations in the gene for glutamate synthase (GOGAT), referred to as *gltB* in *Escherichia coli* (Pahel *et al.*, 1978) and *asm* in *Klebsiella aerogenes* (Brenchley *et al.*, 1973) and *K. pneumoniae* (Nagatani *et al.*, 1971). Similar phenotypes are found in *glnG* mutants of *E. coli* (Pahel & Tyler, 1979) and *glnR* mutants of *Salmonella typhimurium* (Kustu *et al.*, 1979) and also in *nit* mutants of *S. typhimurium* (Broach *et al.*, 1976). However, all these mutations except *asm* (*gltB*) result in normal levels of GOGAT suggesting that the mutations described here are in the structural gene for glutamate synthase.

These *asm* mutants of *A. brasilense* failed to derepress GS when grown in conditions of ammonia limitation. A similar effect was observed by Brenchley *et al.* (1973) in *asm* mutants of *K. aerogenes* and these authors suggested that the inability to derepress GS was a consequence of GOGAT deficiency. The GOGAT-deficient strains could accumulate glutamine which could inhibit the activity and the deadenylation of GS. The low level of unadenylylated GS could, in turn, be correlated with the lack of nitrogenase activity in the mutants, if the synthesis of nitrogenase is, in fact, stimulated by unadenylylated GS as suggested by Tubb (1974) and Streicher *et al.* (1974).

Likewise, as GS has been reported to regulate a large number of enzymes involved in assimilation of poor nitrogen sources (Magasanik, 1977), the low GS activity in the mutants could result in failure to derepress many of these enzymes or failure to derepress certain transport systems as reported for some nitrogen regulatory mutants in *S. typhimurium* (Govons Kustu *et al.*, 1979). In the *A. brasilense* mutants, lack of assimilation of nitrate and nitrite was not due to failure of these substrates to enter the cell because they were easily utilized as electron acceptors in anaerobic respiration.

Nitrogenase activity was not found in *asm* mutants of *K. pneumoniae* unless they were grown on aspartate-supplemented medium (Streicher *et al.*, 1972; Shanmugam *et al.*, 1978); at variance with this result, we were unable to detect nitrogenase activity in the *A. brasilense*

mutants even in the presence of aspartate. However, we cannot exclude that our mutants would express nitrogenase activity in different assay conditions.

Whereas in *K. aerogenes* and *K. pneumoniae* GDH activities are significantly repressed at low concentrations of ammonia (Tyler, 1978), in *A. brasilense*, as in *E. coli* and *S. typhimurium* (Tyler, 1978), the levels of GDH are unaffected by ammonia limitation. GDH activity was not altered in three of the six mutants while the other three mutants, especially SPF101, showed a low GDH activity. It is, however, difficult to explain how GOGAT deficiency could affect GDH activity.

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