Phenotypic Changes Resulting from Distinct Point Mutations in the *Azospirillum brasilense glnA* Gene, Encoding Glutamine Synthetase

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Sequencing the *glnA* genes of two chemically induced *Azospirillum brasilense* glutamine synthetase mutants revealed an Arg→Cys mutation, corresponding to the glutamate binding site, in one mutant and an Asp→Asn mutation, corresponding to the ammonium binding site, in the second mutant. The phenotypic changes in these mutants are discussed in relation to their genotypes.

Azospirillum brasilense is a gram-negative nitrogen-fixing soil bacterium, well known for its ability to colonize plant roots and to increase plant productivity (16). The main ammonium-assimilating pathway in this bacterium is the glutamine synthetase (GS; EC 6.3.1.2), glutamate synthase (EC 1.4.4.13) pathway (25). GS, a key enzyme in the nitrogen metabolism of both prokaryotes and eukaryotes, catalyzes the condensation of ammonium and glutamate to yield glutamine. This ATP-consuming process is regulated both transcriptionally (7) and posttranslationally by adenylylation of the GS in nitrogen excess conditions (4, 15, 25). Presently, only one GS (GSI type) has been found in *A. brasilense*, and no inactivating insertion mutations of *glnA* (encoding GS) have yet been reported. Previously isolated nitrosoguanidine-induced mutants 7028 and 7029 were used in this study (9). Both mutants have reduced GS activity. Mutant 7029, which has less than 1.5% GS activity compared to the wild type, lacks nitrogenase activity, while 7028, which still has ~18% GS activity compared to the wild type, has constitutive nitrogenase activity (i.e., fixing nitrogen in the presence of ammonia) (9). [^{14}C]methylammonium uptake, which is used as a measure of the activity of the AmtB ammonium transporter, is impaired in both mutants (19). Plasmids carrying the wild-type *glnA* gene complement both mutants for glutamine auxotrophy, wild-type nitrogen fixation, and [^{14}C]methylammonium uptake (4; A. Van Dommelen, unpublished results).

Measuring ammonium excretion. Since release of combined nitrogen is a key trait for agronomically useful diazotrophic bacteria and since impairment of ammonium assimilation generally correlates with ammonium excretion (17, 20, 22), whether strains 7028 and 7029 excrete ammonium was tested. *A. brasilense* strains Sp7 (wild type) (18), 7028, and 7029 were grown in liquid MMAB medium (24) supplemented with 100 mg of glutamine/liter and containing 8 mM KNO_3 as the nitrogen source. The ammonium concentration in the supernatant of exponentially grown cells was measured as described by Chaney and Marbach (5). Concentrations up to 2 mM ammonium were observed for strain 7029. For strain 7028 a maximum concentration of 1 mM ammonium was measured, whereas, for the wild-type strain Sp7, no ammonium excretion was detected (detection limit of the assay: 25 μM). Complementing the GS mutants with plasmid pAB462, containing the wild-type *glnA* gene (4), resulted in loss of ammonium excretion (data not shown).

Identification of the *glnA* mutations in 7028 and 7029. Since the presence of functional *glnB* and *glnA* promoters in mutants 7028 and 7029 had been established previously (7), it was decided to determine the *glnA* coding sequence in both mutants. Two primers annealing outside the published Sp7 *glnA* sequence (GenBank accession no. M26107.1) (3) and two high-fidelity PCR polymerases (Vent [Biolabs] and High Fidelity [Roche]) were used to amplify the *glnA* regions of 7028 and 7029. Three independent amplification products from each mutant were sequenced. Primer 1 (5′GTGAATTCTTG GGAAAGGCATGACATAACG3′) anneals 80 bp upstream of the *glnA* coding region, and primer 2 (5′GTGAATTCGG GCGGACACCGGAATCCG3′) anneals 20 bp downstream of the *glnA* coding region. Both primers contained an *Eco*RI restriction site at their 5′ ends to facilitate cloning the amplified fragments. Four differences between the sequenced *glnA* coding regions and the published *A. brasilense* Sp7 *glnA* sequence were found (Table 1). The observed changes can be divided in two groups: differences found in both mutant strains and differences found in only one of the mutant strains. The two differences found in both GS mutants, compared to the M26107.1 *glnA* sequence, are also found in the *A. brasilense glnA* sequence submitted by Chen and coworkers (GenBank accession no. AF323964.1). Therefore, they probably reflect an inaccuracy in the published M26107.1 sequence. These changes occur in regions not known to be of importance in the GS reaction mechanism. The CG→GC substitution at positions 368 and 369 results in a Gly at position 123 of the amino acid sequence, which corresponds to the GS sequences of related bacteria, such as *Rhodospirillum rubrum*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, and other proteobacteria, such as *Azorhizobium caulinodans*, *Sinorhizobium meliloti*, *Rhizobium leguminosarum*, *Azotobacter vinelandii*, and *Pseudomonas aeruginosa*. The CTG insertion at position 1243 generates a Leu residue that is conserved in GS of prokaryotes (8).

The sequence difference found only in mutant 7028 (C964T) changes a large positively charged Arg, corresponding to a

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TABLE 1. Differences in nucleotide and deduced amino acid sequence between the published Sp7 *glnA* gene (M26107.1) and the *glnA* sequences of mutants 7028 and 7029

Nucleotide position(s)	Nucleotide(s) in:		Amino acid position	Amino acid in		Strain(s)
	Published sequence	This study		Published sequence	This study	
139	G	A	52	D	N	7029
368, 369	CG	GC	123	A	G	7028, 7029
964	C	T	322	R	C	7028
1243	—[a]	CTG	415	—	L	7028, 7029

[a] —, no nucleotide or amino acid at this position.

FIG. 2. Expression of a p*amtB*-*gusA* fusion (pFAJ302) in wild-type Sp7 (white) and *glnA* mutants 7028 (black) and 7029 (grey) grown in minimal medium supplemented with the indicated nitrogen source. MU, 4-methylumbelliferone. The data are means and standard deviations from four replicates.

conserved residue of the glutamate binding site, to a small, nonpolar Cys. It has been proposed that the guanidine group of the conserved Arg residue forms hydrogen bonds with the α-carboxylate group of glutamate (12).

In mutant 7029, the G139A mutation changes a conserved Asp into an uncharged Asn at a site corresponding to the proposed NH_4^+ binding site (1, 13). The negative charge of the conserved Asp is crucial in the GS catalytic mechanism since it facilitates the deprotonation of NH_4^+ in order to generate the active species NH_3, which will attack the γ-glutamyl phosphate formed in the GS biosynthetic reaction (11). This Asp residue also increases the affinity of GS for ammonium binding.

Resistance of GS mutant 7028 to GS inhibitor MetSox. Since the potent GS inhibitor methionine sulfoximine (Met-Sox) is known to bind at the same site as glutamate (8), the question of whether there was any effect on the sensitivity of mutant 7028, which has an altered glutamate binding site, to this antimetabolite was investigated. Wild-type Sp7 and GS mutant 7028 were grown in liquid MMAB medium (24) with 20 mM ammonium as the nitrogen source and different concentrations of MetSox. Strain 7028 contained plasmid pAB462, with the wild-type *glnA* gene (4), to ensure glutamine-independent growth. Growth was measured in a Bioscreen C growth analyzer (Labsystems) with a white band filter. Concentrations up to 100 μg of MetSox/ml did not significantly alter growth of strain 7028, while 5 μg of MetSox/ml was enough to completely inhibit wild-type growth (Fig. 1).
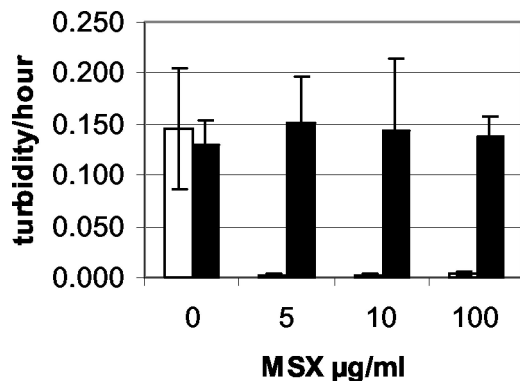

FIG. 1. Growth of wild-type Sp7 (white) and GS mutant 7028 (containing plasmid pAB462) (black) in minimal medium with 20 mM ammonium as the nitrogen source and different concentrations of MetSox (MSX). The data are means and standard errors from five replicates.
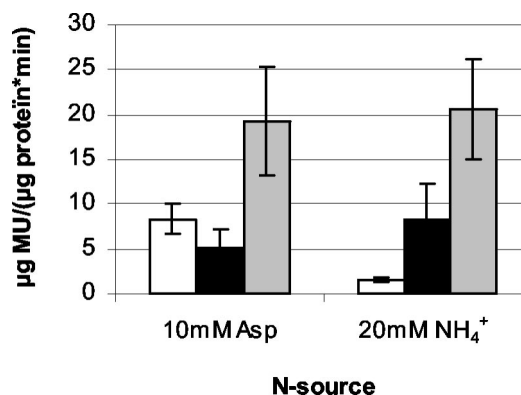
Measuring the GS biosynthetic reaction with hydroxylamine as a substrate. Although the GS biosynthetic reaction with ammonium as a substrate is strongly reduced (9) in both GS mutants, when the biosynthetic GS reaction was measured as described by Bender and coworkers (2), activity in mutant 7029 was found to be twice as high as wild-type activity (data not shown). In this reaction ammonium is replaced by hydroxylamine and the concentration of γ-glutamyl hydroxamate formed is determined spectrophotometrically according to the reaction

$$\text{Glutamate} + NH_2OH + ATP \rightarrow \text{γ-glutamyl hydroxamate} + NH_3 + ADP + P_i$$

The enhanced reaction with this alternative substrate most likely reflects a better binding of the uncharged hydroxylamine when the negatively charged Asp of the ammonium binding site is replaced by an uncharged Asn. Mutant 7028 was found to have 15% of the wild-type biosynthetic GS activity when the method of Bender and coworkers (2) was used. This corresponds to the activity measured by Gauthier and Elmerich (9) with ammonium as a substrate.

Influence of GS mutation on the expression of the Ntr-regulated *amtB* gene. The NtrB-NtrC two-component regulatory system has a central role in nitrogen regulation in many bacteria (14). The *A. brasilense amtB* gene, encoding an ammonium transporter, is activated by this two-component regulatory system in nitrogen-limiting conditions (21). To monitor *amtB* expression, pFAJ302 containing a p*amtB*-*gusA* translational fusion (21) was conjugated into the wild type and GS mutants. After an overnight preculture in rich medium, cells were grown for 4 h in MMAB medium supplemented with 100 mg of glutamine/liter and the nitrogen source indicated in Fig. 2. β-Glucuronidase activity was measured fluorometrically by monitoring the cleavage of 4-methylumbelliferyl-β-D-glucuronide (Sigma-Aldrich N.V.) to 4-methylumbelliferone and glucuronic acid (10, 23). Results are shown in Fig. 2. Both GS mutants fail to repress *amtB* transcription in the presence of 20 mM NH_4Cl. This could indicate that in the wild type the glutamine formed by GS signals a high nitrogen level in the nitrogen sensory cascade of *A. brasilense*. It has also been proposed that glutamine is the signaling molecule for the am-

monium switch-off of the *A. brasilense* nitrogenase (26). In this respect it is interesting that de Zamaroczy and coworkers (7) found that the *glnA* promoters of GS mutants 7028 and 7029 also fail to respond to high levels of ammonium. This change was not due to a change in the *glnA* promoter since de Zamaroczy and coworkers (7) found no difference in nucleotide sequence between the *glnA* promoters of both GS mutants and the wild type.

Although the GS mutants lack [^{14}C]methylammonium uptake, which reflects the activity of the *amtB* gene product (19), *amtB* is expressed in the conditions used for the [^{14}C]methyl-ammonium uptake assay (MMAB supplemented with 10 mM aspartate as the nitrogen source) (Fig. 2). Perhaps the lack of [^{14}C]methylammonium uptake that was observed (19) is due to the presence of excreted ammonium (which is taken up with a much higher affinity than [^{14}C]methylammonium) or a negative posttranslational regulation of the AmtB transporter. In *Escherichia coli* and *Azotobacter vinelandii*, the P_{II}-like signal transduction protein GlnK binds to AmtB and negatively regulates AmtB transport activity (6).

Conclusion. In this paper, alterations of the ammonium and glutamate binding site of GS were found in two ammonium-excreting *Azospirillum* mutants. Since in *Azospirillum* GS is not switched off during plant association, as it is in, e.g., *Rhizobium* (17), protein engineering of GS offers the possibility to generate ammonium-excreting *Azospirillum* strains, as exemplified by the two mutants analyzed in this study.

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