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Coexistence of Two Structurally Similar but Functionally Different P_{II} Proteins in *Azospirillum brasilense*

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The coexistence of two different P_{II} proteins in *Azospirillum brasilense* was established by comparing proteins synthesized by the wild-type strain and two null mutants of the characterized *glnB* gene (encoding P_{II}) adjacent to *glnA*. Strains were grown under conditions of nitrogen limitation or nitrogen excess. The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or isoelectric focusing gel electrophoresis and revealed either by [^{32}P]phosphate or [^{3}H]uracil labeling or by cross-reaction with an anti-*A. brasilense* P_{II}-antiserum. After SDS-PAGE, a single band of 12.5 kDa revealed by the antiserum in all conditions tested was resolved by isoelectric focusing electrophoresis into two bands in the wild-type strain, one of which was absent in the *glnB* null mutant strains. The second P_{II} protein, named P_Z, was uridylylated under conditions of nitrogen limitation. The amino acid sequence deduced from the nucleotide sequence of the corresponding structural gene, called *glnZ*, is very similar to that of P_{II}. Null mutants in *glnB* were impaired in regulation of nitrogen fixation and in their swarming properties but not in glutamine synthetase adenylylation. No *glnZ* mutant is yet available, but it is clear that P_{II} and P_Z are not functionally equivalent, since *glnB* null mutant strains exhibit phenotypic characters. The two proteins are probably involved in different regulatory steps of the nitrogen metabolism in *A. brasilense*.

Azospirillum brasilense is a plant growth-promoting rhizobacterium that associates with the roots of grasses and fixes nitrogen under microaerobic conditions in the free-living state (33). As in all proteobacteria, the transcription of *nif* (nitrogen fixation) genes proceeds from σ^{54} (NtrA or RpoN)-dependent promoters with upstream activator sequences that are the binding target of the transcriptional activator NifA (reviewed in reference 10). In contrast, the regulation by nitrogen of both the expression and activity of NifA differs considerably from that described for other free-living diazotrophs. In *A. brasilense*, NifA is synthesized in an inactive form under conditions incompatible with nitrogen fixation (24), and NtrC is not essential for the regulation of *nifA* expression (23).

A. brasilense possesses a glutamine synthetase (GS) encoded by *glnA* (5). GS activity is modulated by reversible adenylylation as in enteric bacteria (8, 19). However, the regulation of *glnA* expression has some unusual features. The *glnA* gene is adjacent to *glnB*, the structural gene for the P_{II} protein. P_{II} plays an essential role in regulation of nitrogen metabolism in *Escherichia coli* by controlling the level and activity of GS (reviewed in references 27 and 32). The transcription of the *glnB-glnA* cluster in *A. brasilense* depends on three different, selectively used and nitrogen-regulated promoters (11) and does not involve the two-component regulatory system NtrB-NtrC (12). A *glnB* null mutant strain does not have impaired ammonia assimilation but is strictly Nif$^-$ (12, 24). It thus appears that *A. brasilense* is the only known species in which *glnB* is essential for nitrogen fixation. Furthermore, the P_{II} protein is required for modulating NifA activity in response to the changes in cellular nitrogen levels (N status) (12).

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In this paper, we describe the identification of a P_{II}-like protein named P_Z, encoded by a *glnB*-like gene named *glnZ*. The characteristics of the P_{II} protein led us to investigate the properties that differentiate the structurally similar P_{II} and P_Z proteins.

(A preliminary account of this work has previously been reported [10].)

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and GS assay. The wild-type strain of *A. brasilense* used was Sp7 (33); strain 7606 is a *glnB*::*kan* null mutant strain in which the kanamycin cartridge is inserted near the 3′ end of the *glnB* gene (12). A second *glnB*-disrupted strain, 7628, in which the kanamycin cartridge was inserted into the central *Sal*I site of the *glnB* coding sequence was constructed. The inactivation of *glnB* in strain 7628 was verified by PCR with the previously described oligonucleotides 1 and 3* (12), corresponding to sequences about 350 nucleotides upstream and downstream from *glnB*, respectively (data not shown). No phenotypic difference was observed between the two *glnB*-disrupted strains (see Results). pAB914 carries the *glnB* gene of *A. brasilense* (12) and complements both strains 7606 and 7628. In pAB914/10 (12) *glnB* is expressed from a *kan* promoter.

Rich or lactate-containing minimal medium for *A. brasilense* and growth under conditions of nitrogen limitation (nitrogen fixation) or nitrogen excess (ammonia assimilation) were as previously described (12). The GS activity (determined by a GS transferase assay) was measured by monitoring the production of γ-glutamyl hydroxamate, as described in reference 4.

Molecular biology techniques. Recombinant DNA methods were performed as previously described (29). PCR was performed as described elsewhere (12). The amino acid sequence of P_{II} proteins from *E. coli* (25), *Klebsiella pneumoniae* (21), and *A. brasilense* (11) and of the P_{II}-like protein from *E. coli* (2) were compared to identify the most conserved peptide motifs at the C and N termini. Two 30-mer degenerate oligonucleotides, corresponding to these consensuses, and with an *Eco*RI and a *Bam*HI restriction site, respectively (underlined), at the 5′ end were synthesized (Eurogentec), taking into account the high GC level in the third position of *A. brasilense* codons: 5′-CTGCGAATTCATCATYAAGC CGTTCAARCT-3′ and 5′-ACGCGGATCCCCGGTVCGRATVCGGAYVAC -3′.

The intervening 0.3-kb region in strain 7628 was amplified by PCR, digested with *Eco*RI and *Bam*HI, and inserted into pBluescript KS$^+$ to give pAB940. Two independent pAB940 isolates were sequenced to verify that they were identical. The pAB940 insert was used as a probe to screen by colony hybridization a gene

bank of partially digested *Sal*I DNA fragments of *A. brasilense*, cloned into the pVK100 vector (39). The plasmid pSP7107 was recovered from a clone hybridizing with the probe, and an ~5.2-kb *Sal*I fragment, carrying the *glnB*-like gene, was subcloned into pTZ19R to yield pAB947. The complete *glnZ* sequence and flanking regions up to 100 nucleotides at each side were determined on both strands by using suitable oligonucleotides derived from the nucleotide sequence of the pAB940 insert: 5′ CCCGAGCGACGTCAGCGCCTCGC 3′ (towards the 5′ end of *glnZ*) and 5′ TCGTTCTGGACATCGCCCAGG (towards the 3′ end of *glnZ*). Sequence data were analyzed with the Genetics Computer Group program, and similarity searches were performed with Blitz and Blast programs of the Institut Pasteur, National Center for Biotechnology Information, and City2 servers.

Nucleotide sequence accession number. The DNA sequence of *glnZ* has been submitted to the EMBL/Genbank/DDBJ nucleotide sequence databases under accession number X92496.

Immunological procedures. Two different oligopeptides of P_{II}, synthesized in vitro, were used as immunogens for the generation of rabbit antisera. They correspond to two short amino acid regions with the highest level of hydrophilicity. Oligopeptide 1 corresponds to the best-conserved 20 residues of P_{II} (positions 31 to 50): TEAKGFGRQKGHTELYRGAE, just upstream from residue Tyr-51, involved in uridylylation in enteric bacteria. This motif is located in the highly accessible 3-4 loop, containing the exposed Tyr-51 residue, according to the stereo plot of the C_α backbone trace of an *E. coli* P_{II} monomer (7). Oligopeptide 2 corresponds to the 12-amino acid sequence at the C terminus of P_{II}: RIRTGEKGGDAI. In *E. coli*, these two amino acid motifs belong to functionally important regions of P_{II}. Such hydrophilic regions are usually supposed to be exposed at the surface of the molecule and can be expected to have good antigenic properties.

Anti-*A. brasilense* P_{II}-antisera were obtained as follows. New Zealand White female rabbits were immunized by intradermal injection with each of two different oligopeptides of P_{II} which were synthesized in vitro and coupled to the keyhole limpet hemocyanin carrier protein (Neosystem, Strasbourg, France). The conjugated oligopeptide (250 μg) was mixed with an equal volume of Freund's complete adjuvant for the first injection or with Freund's incomplete adjuvant for the second, third, and fourth injections, 6, 12, and 15 weeks later, respectively. One month after the fourth injection, a final injection of 100 μg of the conjugate was given intravenously. P_{II} antisera were collected after 2.5 and 5 months. For Western immunoblot experiments (29), proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Hybond C; Amersham). Cross-reactive antibodies were revealed by an enhanced chemiluminescent (ECL kit; Amersham) antibody detection system (anti-rabbit immunoglobulin G peroxidase conjugate; Sigma).

In vivo [^{32}P]phosphate and [^{3}H]uracil labeling. Bacteria were grown in rich medium under air, harvested by centrifugation, washed twice with minimal medium (without phosphate in the case of phospholabeling), and resuspended to an optical density at 600 nm of 0.2 in the same medium. The minimal medium contained 20 mM or no ammonia, so as to create the nitrogen excess or nitrogen limitation conditions, respectively (12). Under nitrogen limitation conditions, the oxygen tension was adjusted to 0.5% and the samples (1 ml of cell suspension in a 10-ml bottle) were incubated with shaking at 30°C for 2 h. Thirty microcuries of carrier-free $^{32}P_i$ (or 30 μCi of 50-Ci/mmol [5,6-^{3}H]uracil; Amersham) was added to each sample. After 2 h of incubation at 30°C, cells were pelleted, washed twice with minimal medium (supplemented with 1 mg of uracil per ml in the case of uracil labeling), and lysed in 50 μl of denaturing Laemmli buffer by heating at 96°C for 5 min. Each sample was subjected to SDS-PAGE (linear acrylamide gradient from 7.5 to 15%) and then to semidry electroblotting (Prolabo) on nitrocellulose (Hybond C; Amersham). The membrane was washed for 10 min with distilled water to eliminate the unbound radioactivity and labeled DNA and RNA. Blots were stained with 0.1% red-Ponceau solution, to verify that the lanes contained similar protein loads, and were subsequently autoradiographed and/or subjected to immunodetection. When tritiated uracil was used, blots were impregnated with Amplify NAMP 100 (Amersham) before autoradiography. Samples were treated with 40 μg of RNase (DNase free; Boehringer) per ml to check that the radioactive signal was resistant to RNase.

N-terminal peptide sequence. Proteins extracted from the *glnB* null mutant 7628 were separated by SDS-PAGE and transferred onto Problott (Applied Biosystems) membranes. The 12.5-kDa band was recovered, and the N-terminal sequence was established on an ABI 473 A automatic sequencer.

Isoelectric focusing (IEF) gel electrophoresis. Bacteria were grown under conditions of nitrogen limitation or nitrogen excess and resuspended to an optical density at 600 nm of 0.2 in a solution of 0.5% Nonidet P-40. Samples were sonicated for 6 min with Sonifier 250 (Branson) and loaded onto a nondenaturing 1% agarose gel (0.4 mm thick and cast on GelBond PAG film), containing 12% sorbitol and 5% Ampholine (pH 3.5 to 9.5) (LKB). Gels were placed on a flat-bed cooling plate (15°C) for horizontal electrophoresis (Multiphor II; Pharmacia-LKB). Focusing was started at 300 V (1 W) for 10 min and continued for 65 min with maximum settings of 1,280 V, 3 mA, and 3 W for a total of 1,200 V · h. Gels were fast blotted by pressing on a CNBr-activated cellulose-nitrate membrane (Schleicher & Schuell). After transfer, P_{II} isoforms were immunodetected as described above, except that the cross-reacting antibody detection system was based on diaminobenzidine-imidazole staining.

Swarming motility test. Plates of semisolid (0.3% agar), minimal medium, dried at room temperature for 24 h, were inoculated with 5 μl of exponentially grown *A. brasilense* culture deposited on the surface. After 36 h of incubation at 33°C in a humid atmosphere, the diameters of wild-type and mutant strain growth zones were compared.

RESULTS

Identification of P_Z and its structural gene *glnZ*. (i) In vivo labeling of cell proteins by ^{32}P and by [^{3}H]uracil. Nothing is known about the mechanism of P_{II} modification in response to the N status of the cell and its association with the modulation of NifA activity in *A. brasilense*. Total proteins of the wild-type and mutant strains, grown under conditions of nitrogen excess or nitrogen limitation, were labeled in vivo with ^{32}P. After separation by SDS-PAGE, a band of about 12.5 kDa, in agreement with the molecular mass predicted from the *glnB* nucleotide sequence, was detected in the wild-type strain under all physiological conditions (Fig. 1A, lanes 5 and 6). Thus, it may represent the P_{II} polypeptide. However, it was also present in the *glnB* null mutant strain 7606 (Fig. 1A, lanes 7 and 8), suggesting the existence of a P_{II}-like protein, encoded by a *glnB*-like gene. It was then verified that the 12.5-kDa polypeptide was also present in strain 7628, which carries another insertion in the coding sequence of *glnB* (data not shown).

To analyze whether the modification of the 12.5-kDa band was due to uridylylation, proteins were labeled in vivo with [^{3}H]uracil. Indeed, a band corresponding to a uridylylated polypeptide of about 12.5 kDa was detected in both the wild-type and *glnB* null mutant strains, under conditions of nitrogen limitation (Fig. 1A, lanes 2 and 4, and B, lanes 3 and 4). This band was not detected in the presence of ammonia (Fig. 1A, lanes 1 and 3). The signal detected in the *glnB* null mutant strains evidences a uridylylation process affecting, under conditions of nitrogen limitation, a protein with the same molecular weight as P_{II}.

Interestingly, the intensity of the signal obtained by phosphorylation or uridylylation seemed to be higher in the *glnB* mutants than in the wild type (Fig. 1A, lanes 2, 4, and 5 to 7). This could reflect a possible role of P_{II} in a negative regulation of the synthesis or covalent modification of the P_{II}-like polypeptide, a hypothesis which requires further documentation.

(ii) Immunodetection of a P_{II}-like protein. To detect the presence of P_{II} and a possible P_{II}-like protein, Western blot analysis, with two anti-*A. brasilense* P_{II} antisera, was performed. A 12.5-kDa polypeptide was detected with anti-*A. brasilense* P_{II} antiserum 1 in the wild-type strain and in the *glnB* null mutant strain, grown under conditions of nitrogen limitation (Fig. 1B, lanes 1 and 2). This polypeptide comigrated with the uridylylated polypeptide (Fig. 1B, lanes 3 and 4) and the phosphorylated polypeptide (data not shown). The same polypeptide was immunodetected in both strains grown under conditions of nitrogen excess (Fig. 2). Similar results were obtained with anti-P_{II} antiserum 2 (data not shown). It thus appears that, in the *glnB*-disrupted mutant, a polypeptide with antigenic characteristics similar to those of P_{II} is present. This implies that in the wild-type strain, the 12.5-kDa polypeptide band is in fact a doublet, composed of the two comigrating P_{II} and P_{II}-like polypeptides. Apparently, the gene encoding the P_{II}-like polypeptide is expressed under all physiological conditions tested.

The N-terminal amino acid sequence of the P_{II}-like polypeptide was determined for 15 residues, from the 12.5-kDa band present in the *glnB* null mutant strain. The sequence obtained is MKLVMAIIKPFKLDE, which differs from that of the deduced *glnB* product, MKKIEAIIKPFKLDE.

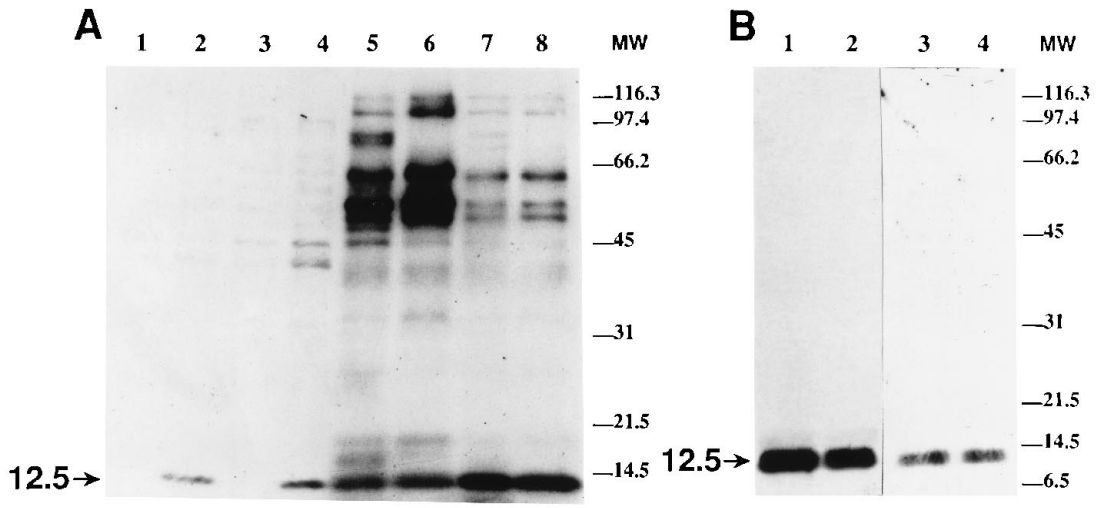

FIG. 1. (A) Autoradiograph of *A. brasilense* protein labeled in vivo and transferred onto nitrocellulose after separation by SDS-PAGE. Lanes 1 to 4, [^{3}H]uracil labeling; lanes 5 to 8, ^{32}P labeling; lanes 1, 2, 5, and 6, wild-type strain; lanes 3, 4, 7, and 8, *glnB* null mutant strain 7606; lanes 1, 3, 5, and 7, conditions of nitrogen excess; lanes 2, 4, 6, and 8, conditions of nitrogen limitation. (B) Immunoblot analysis and autoradiograph of [^{3}H]uracil-labeled proteins under conditions of nitrogen limitation. Lanes 1 and 2, immunodetection by anti-P_{II} antiserum 1; lanes 3 and 4, autoradiography; lanes 1 and 3, wild type strain; lanes 2 and 4, mutant strain 7606. MW, protein molecular weight standards (numbers are in thousands).

(iii) Separation of P_{II} and P_Z by IEF. The two comigrating polypeptides were separated by IEF gel electrophoresis with a pH gradient between 3 and 10. Anti-P_{II} antiserum 1 gave a single band at pI 5.7, in *glnB* null mutant extract (Fig. 3, lane 2), and two bands at pI 5.7 and at pI 6.3, in the wild-type strain extract (Fig. 3, lane 3). This established the existence of a P_{II}-like protein, in agreement with its partial amino acid sequence determination, that we propose to call P_Z. The P_Z protein appears to have not only primary but possibly secondary and tertiary protein structures similar to those of P_{II}, as anti-P_{II} antiserum 1 recognized the native forms of both P_{II} and P_Z.

(iv) Identification of the structural gene (*glnZ*) encoding P_Z. We tried to identify the P_Z-encoding locus by Southern hybridization with *A. brasilense* total restricted DNA. The probe used was the 0.11-kb *Sal*I-*Bgl*II intragenic fragment of *A. brasilense glnB* purified from pAB914. A strong signal was obtained with an 18-kb *Eco*RI fragment that carried the *glnB-glnA* cluster. In addition, a weak signal was observed with an *Eco*RI band of 8.2 kb. This second band was detected in total DNA of both the wild-type and the *glnB*-disrupted strains (data not shown). In spite of several attempts, the weakness of the signal prevented us from using this approach to clone the *glnB*-like gene, so we tried another strategy based on PCR amplification.

Degenerate oligonucleotides deduced from P_{II} sequences were used as primers. They were expected to amplify a fragment of 0.3 kb from *glnB* and thus by homology from the putative *glnB*-like gene. Because of the insertion of the kanamycin cartridge (1.3 kb), a fragment of 1.6 kb was expected with DNA of the *glnB* mutant. A single band of 0.3 kb was amplified from DNA of the wild-type strain and two bands of 0.3 and 1.6 kb from DNA of the *glnB* mutant strain 7628 (data not shown). This strongly suggests the existence of a *glnB*-like gene of about the same size as *glnB*.

The 0.3-kb DNA fragment obtained from strain 7628 by PCR amplification was purified and used as a hybridization probe with several digests of *A. brasilense* total DNA. As expected, a single strong signal was revealed at 8.2 kb with the *Eco*RI digest, confirming that the weak signal obtained with the 0.11-kb *Sal*I-*Bgl*II probe corresponded to the second copy (data not shown). The 0.3-kb *Eco*RI-*Bam*HI fragment was inserted into a pBluescript (KS^+) vector to yield pAB940. The nucleotide sequence of the cloned fragment was then determined.

The nucleotide sequence of the *glnB*-like gene and its flanking regions was completed with pAB947, which contains the entire *glnB*-like gene. It is indeed very similar to *A. brasilense glnB* (73.2% identical nucleotides) and was designated *glnZ*. *glnZ* is devoid of the central *Sal*I site in contrast to *glnB* (11). The deduced N-terminal part of *glnZ* matches that of P_Z. This clearly indicates that *glnZ* is the structural gene of P_Z. The sequence encodes a polypeptide of 112 amino acids, 66.1% identical (82.1% similar) with that of the *glnB* product (Fig. 4). It should be noted that higher similarities are observed by comparing P_Z with *E. coli* P_{II} (25) (85.7%) and with the *E. coli* P_{II}-like polypeptide (2) (88.4%) (data not shown). The Tyr residue at position 51, corresponding to the uridylylation site in

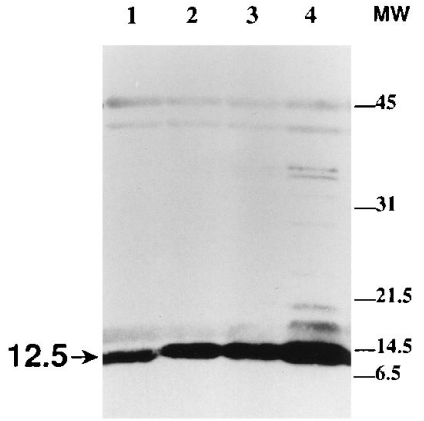

FIG. 2. Immunoblot analysis by anti-P_{II} antiserum 1 of proteins transferred onto nitrocellulose after separation by SDS-PAGE. Lanes 1 and 2, wild-type strain; lanes 3 and 4, *glnB* null mutant strain 7606; lanes 1 and 3, conditions of nitrogen excess; lanes 2 and 4, conditions of nitrogen limitation. MW, protein molecular weight standards (numbers are in thousands).

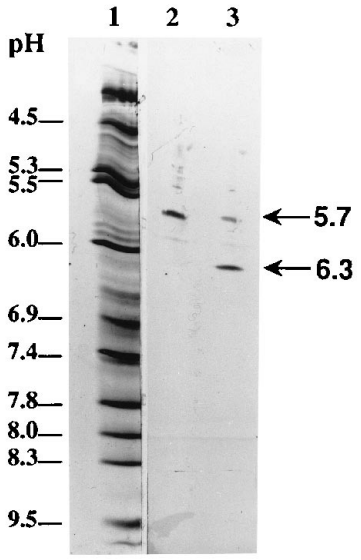

FIG. 3. Identification by immunodetection of P_{II} and P_Z separated by IEF gel electrophoresis under nondenaturing conditions. Samples were prepared from cells grown under nitrogen limitation. P_{II} and P_Z isoforms were revealed by anti-P_{II} antiserum 1. Lane 1, pH gradient; lane 2, *glnB* null mutant strain 7628; lane 3, wild-type strain.

the *E. coli* protein, is conserved in P_Z, in agreement with its cell N-status-dependent uridylylation. Some differences between P_{II} and P_Z are located at the C and N termini in addition to two other small internal regions. The residues between positions 64 and 74 are more hydrophilic in P_Z (data not shown). This region is part of the central β5-αB-β6 structure of the *E. coli* P_{II}, as defined in reference 7, and thus may correspond to a difference in secondary structure. The region of dissimilarity between positions 94 and 99 is predicted to have no effect on the hydropathy profile and belongs to a variable region among P_{II} proteins. However, these results and those obtained by immunodetection demonstrate that P_{II} and P_Z are structurally very similar.

Testing for phenotypes associated with *glnB*. The existence of *glnZ*, whose product was present under all physiological conditions tested, led us to complete the phenotypic characterization of the previously described P_{II}. It was found that the 7606 *glnB* null mutant strain was Nif^- and that nitrogen fixation was fully restored after introduction of pAB914 (12). The same data were obtained with the *glnB* null mutant strain 7628, constructed for this work. It was also shown, by using *glnB*- and *glnA-lacZ* fusions, that in *A. brasilense* the absence of P_{II} led to a higher expression of the *glnBA* cotranscript and to a decrease of *glnA* transcript under conditions of nitrogen excess, but no difference was observed under conditions of nitrogen limitations (12). This indicated a regulatory role of P_{II} in both *glnB* and *glnA* expression and showed that the *glnA* transcription, in the *glnB* mutant strains, was driven mainly through the *glnB* promoter under nitrogen excess, in contrast to transcription in the wild-type strain (12).

Since the two *glnB* null mutant strains are prototrophs, we wondered whether the GS adenylylation process was also dependent on P_{II} in *A. brasilense.*

(i) Modulation of GS activity by ammonia. GS activity was assayed in both the wild-type and *glnB* null mutant strains (Table 1). Deadenylylation was maximal under conditions of nitrogen fixation and was minimal in cultures grown in the presence of ammonia and/or glutamine. The proportion of active GS (the nonadenylylated form) was similar in the wild-type and mutant strains under all physiological conditions tested. The adenylylation-deadenylylation ratio in the *glnB*::*kan* mutant strains was unchanged by the presence of a functional *glnB* gene on plasmid pAB914. This indicates that P_{II} is not essential in the nitrogen control of the modulation of GS activity. However, for every experimental condition tested, the level of GS synthesis was lower in the *glnB* mutant strains than in the wild type. This was partly due to the absence of *glnB* product, although the complementation by P_{II} does not restore the wild-type GS synthesis level. Consequently, the low level of GS in the *glnB* null mutant strains is most probably due to an artifactual effect of the *kan* insertion. Thus, the presence of the *kan* cartridge in the *glnB* gene could impair the stability of mRNA initiated from the *glnB* promoters in the mutants. As a consequence, the expression of *glnA* in this mutant could be essentially from the *glnA* promoter.

(ii) Swarming motility on semisolid medium. *A. brasilense* synthesizes two types of flagella. The polar flagellum is responsible for swimming motility, and the lateral flagella, synthesized only on semisolid or solid media, are required for swarming and play a major role in chemotaxis (20, 28, 33). It was reported that an *A. brasilense ntrA* (*rpoN*) mutant was entirely devoid of both polar and lateral flagella (34). Since the *ntr* system seems to be involved in flagellum synthesis, motility tests were performed with *ntr* and *glnB* mutant strains available in our collection. Cells of *glnB* null mutant strains 7606 and 7628 were motile in liquid media (swimming) and grew at rates

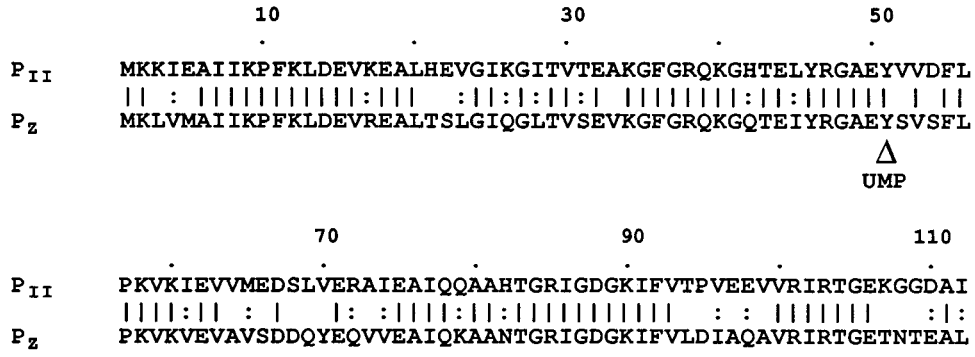

FIG. 4. Comparison of the amino acid sequence of P_{II} with that of the predicted *glnZ* product (P_Z) of *A. brasilense*. Identical amino acids are indicated by vertical bars. Dots indicate residues with similar properties. Numbering corresponds to the P_{II} amino acid sequence. The conserved Tyr-51 residue, involved in the uridylylation process in enteric bacteria, is indicated as UMP.

TABLE 1. GS specific activity in *A. brasilense* Sp7 and *glnB* null mutant strains grown under various physiological conditions[a]

Strain[b]	$-NH_4^+$, +0.5% O_2			$+NH_4^+$, +air			$+NH_4^+$, +Gln, +air		
	GS sp act (U)[c]		% GS[d]	GS sp act (U)		% GS	GS sp act (U)		% GS
	$-Mg^{2+}$	$+Mg^{2+}$		$-Mg^{2+}$	$+Mg^{2+}$		$-Mg^{2+}$	$+Mg^{2+}$	
Sp7	1.18	1.11	94	1.41	0.11	8	1.48	0.09	6
7606 (*glnB*::*kan*)	0.21	0.17	81	0.37	0.08	22	0.42	0.05	12
7628 (*glnB*::*kan*)	0.25	0.21	84	0.32	0.06	19	0.44	0.05	11
7606(pAB914)	0.20	0.16	80	NT	NT	NT	0.35	0.04	11
7628(pAB914)	0.28	0.24	85	NT	NT	NT	0.40	0.04	10

[a] Culture conditions are indicated as follows: $+NH_4^+$, 20 mM ammonia; $-NH_4^+$, the absence of any nitrogen source in minimal medium; Gln, 200 μg of L-glutamine per ml. Data are the means of at least four independent experiments; the standard deviation was about 15%. NT, not tested.

[b] Strains 7606 (12) and 7628 (see Materials and Methods) carry the *kan* cartridge at a different location in the coding sequence of *glnB*. pAB914 carries a functional copy of *glnB* (12).

[c] One unit of GS specific activity (transferase) corresponds to 1 μmol of L-glutamyl hydroxamate produced per min per mg of protein. $-Mg^{2+}$, total amount of GS present (both the adenylylated and nonadenylylated forms of the enzyme are active); $+Mg^{2+}$, amount of nonadenylylated GS present (the activity of adenylylated subunits is completely inhibited).

[d] Percent nonadenylylated GS.

similar to that of the wild-type cells. They displayed the wild-type mixed pattern of flagellation, with a single polar flagellum and numerous lateral flagella, as observed by electron microscopy (data not shown). However, despite the presence of lateral flagella, *glnB* null mutants were impaired in their swarming properties. In semisolid medium, the swarming distance was half that of the wild-type strain Sp7 (Fig. 5, sectors A and B). Complementation with a functional *glnB* (pAB914) in strain 7606 (or 7628; data not shown) completely restored the swarming range (Fig. 5C). The impaired swarming properties of *glnB* mutants are not dependent on the nitrogen source concentration of the minimal medium, as observed when 1 to 20 mM NH_4Cl is used. Thus, P_{II} is not involved in the synthesis of lateral flagella but contributes to the swarming motility.

DISCUSSION

We report evidence that *A. brasilense* contains two different but structurally similar P_{II} proteins. The second one, named P_Z, is present in *glnB*-disrupted strains and displays a pI of 5.7, compared with a pI of 6.3 for the P_{II} protein. P_Z is encoded by a previously unidentified gene, named *glnZ*, which was cloned and sequenced. Hybridization data with total DNA showed that this gene is not located in the vicinity of *glnA*, in contrast to the first identified *glnB* gene. The amino acid sequences and sizes (12.5 kDa) of the P_{II} and P_Z polypeptides are very similar.

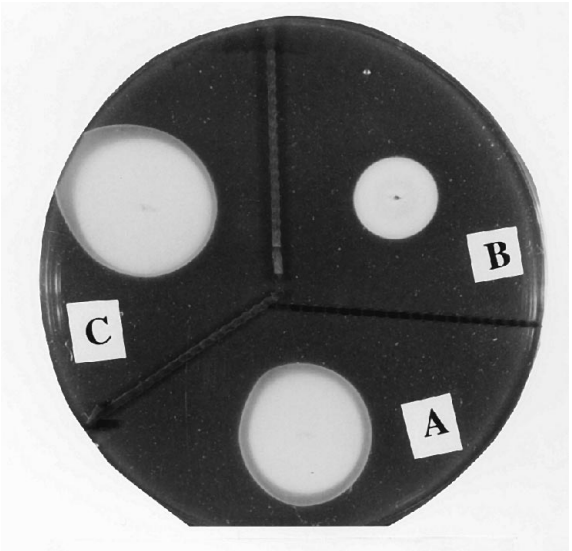

FIG. 5. Swarming spreads of *A. brasilense* on semisolid minimal medium. (A) Wild-type strain; (B) *glnB* null mutant strain 7606; (C) strain 7606 bearing plasmid pAB914.

P_{II} has been described as the central signal transmitter of the cell's N status in studies with the enteric bacteria *E. coli* and *K. pneumoniae*. Under conditions of nitrogen limitation, the uridylyltransferase covalently modifies P_{II} by uridylylation (1, 6, 31). Under conditions of nitrogen excess, the uridylyl-removing activity of uridylyltransferase is stimulated. The modification state of P_{II} is involved in the control of biosynthetic pathways mediated by the NtrB-NtrC system (in particular, GS synthesis) and of GS activity by reversible adenylylation (reviewed in references 27 and 32). The *glnB* product in *Rhodobacter capsulatus* and *Rhizobium leguminosarum* may play similar roles (3, 17).

The fact that *A. brasilense* contains two proteins structurally similar to classical P_{II} raises several questions. In particular, what are the functions of each protein, what regulates their production and activity, and are they posttranslationally modified in response to the cell's metabolic status?

Previous studies (11, 12) and data reported here indicate that in *A. brasilense* the protein we called P_{II} (i) is synthesized at a high level under conditions of nitrogen limitation and at a lower level under conditions of nitrogen excess, (ii) is required for nitrogen fixation by activating NifA, (iii) is involved in the swarming motility of the bacteria, and (iv) is not involved in GS activity by adenylylation. In addition, pAB914/10, which carries the *A. brasilense glnB* gene, does not complement an *E. coli glnB* deletion in strain RB9060 (26a). Thus, the *A. brasilense* P_{II} differs in function from the classical P_{II} of enterobacteria. The involvement of P_{II} in the regulation of the swarming motility is interesting. Since polar and lateral flagella are intact in *glnB* null mutant strains, the impaired swarming is probably due to a defect in the control of the general energy metabolism. It has been suggested that P_{II} participates in the coordination of both nitrogen and carbon assimilation (22). Possibly the regulation of the proton flux towards flagella motor rotation (reviewed in reference 26) could be impaired in the mutant strains.

In the absence of *glnZ* mutations, nothing is known about the role of P_Z, except that P_Z cannot compensate for a defect in P_{II}. However, P_Z might be involved in some functions performed by P_{II} in other species (see above). *A. brasilense* possesses a two-component NtrB-NtrC system and a GS subjected

to modification of activity by adenylylation. In addition, the immunological analysis reported here shows that P_Z is synthesized under conditions of both nitrogen excess and nitrogen limitation. This resembles the regulation of *glnB* expression in *E. coli* (25, 35). Thus, it is possible that P_Z is functionally closer to the classical P_{II} than the protein called P_{II} in *A. brasilense*, because it was first identified. Moreover, *A. brasilense* P_Z appears to share higher degrees of similarity with *E. coli* P_{II} and P_{II}-like proteins than with *A. brasilense* P_{II}. These observations open a new area for research in *A. brasilense.*

[^{3}H]uridine radiolabeling experiments showed that P_Z is uridylylated under conditions of nitrogen limitation, most probably at the conserved Tyr-51 residue by analogy with *E. coli*, *K. pneumoniae*, and *R. leguminosarum* (9, 21, 31). However, [^{32}P]phosphate radiolabeling experiments revealed a significant signal under condition of both nitrogen limitation and nitrogen excess. It is possible that this was due to a gradually decreasing uridylylation of P_{II} monomers in the presence of ammonia, revealed by the higher sensitivity of the ^{32}P labeling. Alternatively, the possibility that P_Z might, in addition to its uridylylation, be modified by phosphorylation has to be kept in mind. No conclusion concerning the modification of P_{II} activity can be drawn from these experiments. However, the Tyr-51 is also conserved in P_{II}, and this suggests the possibility of uridylylation of this protein as well. The absence of a serine residue at position 49, the target for P_{II} phosphorylation in *Synechococcus* sp. (16), indicates that there is no such phosphorylation of P_{II} in *A. brasilense*. However, another type of modification could also be considered.

The coexistence of two different P_{II} proteins, encoded by two different structural genes, in *A. brasilense* is a feature likely widespread in prokaryotes. Two open reading frames located between *nifH* and *nifD* and exhibiting a similarity with P_{II} were reported in some methanogens (30). A *glnB*-like gene just downstream of the *mdl* (multidrug resistance-like) gene in *E. coli*, which might encode a P_{II}-like protein, has been also found (2). Then, the presence of a P_{II}-like protein in *E. coli*, possibly with functions similar to those of P_{II}, was recently reported (36). This P_{II}-like protein of *E. coli* is likely to be synthesized only under nitrogen limitation conditions, in contrast to the *A. brasilense* P_Z. Indeed, we have observed a 12.5-kDa polypeptide, cross-reacting with the *A. brasilense* anti-P_{II} antiserum 1, in *E. coli* and *K. pneumoniae glnB* mutant strains (unpublished data), confirming and suggesting, respectively, the coexistence of P_{II} and P_{II}-like proteins in these species. However, in *K. pneumoniae* a strong uridylylated band was found in the wild-type strain, but there is no evidence for an uridylylated protein in a *glnB*::*Tn5* mutant strain (13).

Immunodetection revealed only one P_{II} protein in *Synechococcus* PCC7942 (15) and in *R. leguminosarum* (9). Note that a *glnB* gene is found in various species, including the gram-negative bacterium *Haemophilus influenzae*, whose only natural host is humans (14), and the gram-positive bacterium *Bacillus subtilis* (38), but no *glnB* gene was identified by screening the complete nucleotide sequence of *Mycoplasma genitalium* (18).

If *A. brasilense* P_{II} and P_Z have the classical trimeric structure (37), the substantial structural conservation between the two may allow the assembly of heteromers. This could lead to functionally different P_{II} proteins. There might be a particular role for P_Z in *A. brasilense* for fine-tuning the signal transmission pathway of the cellular N status.

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