Identification of Three Genes Encoding P_{II}-Like Proteins in *Gluconacetobacter diazotrophicus*: Studies of Their Role(s) in the Control of Nitrogen Fixation

Olena Perlova, Alejandro Ureta, Stefan Nordlund, and Dietmar Meletzus

Department of Biochemistry and Biophysics, Stockholm University, SE-106 91 Stockholm, Sweden, and Department of Gene Technology and Microbiology, University of Bielefeld, DE-33615 Bielefeld, Germany

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In our studies on the regulation of nitrogen metabolism in *Gluconacetobacter diazotrophicus*, an endophytic diazotroph of sugarcane, three glnB-like genes were identified and their role(s) in the control of nitrogen fixation was studied. Sequence analysis revealed that one P_{II} protein-encoding gene, glnB, was adjacent to a glnA gene (encoding glutamine synthetase) and that two other P_{II} protein-encoding genes, identified as glnK1 and glnK2, were located upstream of amtR1 and amtR2, respectively, genes which in other organisms encode ammonium (or methylammonium) transporters. Single and double mutants and a triple mutant with respect to the three P_{II} protein-encoding genes were constructed, and the effects of the mutations on nitrogenase expression and activity in the presence of either ammonium starvation or ammonium sufficiency were studied. Based on the results presented here, it is suggested that none of the three P_{II} homologs is required for the expression of nif genes, that the GlnK2 protein acts primarily as an inhibitor of nif gene expression, and that GlnB and GlnK1 control the expression of nif genes in response to ammonium availability, both directly and by relieving the inhibition by GlnK2. This model includes novel regulatory features of P_{II} proteins.

*Gluconacetobacter diazotrophicus* is an endophytic microorganism of the α-proteobacteria that has the capacity to fix molecular nitrogen. It was originally isolated from Brazilian sugarcane (*Saccharum* sp.) varieties and was subsequently isolated from sugarcane cultivars in Mexico, Cuba, and Australia as well as from coffee and pineapple (21, 39). This diazotroph has been suggested to be the primary diazotroph contributing to high levels of biological nitrogen fixation occurring in sugarcane plants (8). Recent studies have shown that *G. diazotrophicus* PAL5 fixes nitrogen inside sugarcane plants; therefore, the transfer of fixed nitrogen to the host plant may be an important factor in plant growth promotion, although the production of phytohormones, such as indole-acetic acid, may play an additional role (37). Several aspects of the diazotrophic physiology of this bacterium have been described (13, 24, 25, 42, 43). However, a detailed understanding of the mechanisms by which nif gene expression is regulated in *G. diazotrophicus* is also of major importance for exploring the benefits of biological nitrogen fixation to the yield of sugarcane and the improvement of this interaction and possibly the interaction of *G. diazotrophicus* with other economically important plants (17, 34, 39), as well as for exploring the potential benefits for both partners involved in this bacterium-plant association.

Biological nitrogen fixation is a highly energy-demanding process catalyzed by nitrogenase. The most common form of nitrogenase is composed of two different enzymes, the Fe protein (dinitrogenase reductase) and the MoFe protein (dinitrogenase) (33). The synthesis and activity of nitrogenase require the expression of several specific nif genes. Recently, the major nif gene cluster of *G. diazotrophicus* was isolated and partially genetically characterized. Among these genes, the nifA gene, encoding the transcriptional activator NiFA, and the nifHDK genes, encoding the nitrogenase Fe and MoFe proteins, were identified (21). Gene expression analysis indicated that the nitrogen-sensing system, i.e., the enzyme GlnD and the regulatory P_{II} protein(s), regulates the expression of the nifA gene in response to nitrogen availability; nifA transcription is repressed in cultures of *G. diazotrophicus* exposed to high levels of ammonium but induced under conditions of nitrogen limitation (40). In some diazotrophs, the activity of NiFA is also controlled by the glnB-encoded P_{II} protein (11, 22). The glnB gene has been cloned from a number of bacterial species and characterized. In addition to glnB, many organisms have multiple genes encoding P_{II} proteins (3). The majority of the α-proteobacteria, including the genera *Sinorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Azospirillum*, and *Rhodobacter*, harbor two P_{II} protein-encoding genes, a glnB-like gene and a glnK-like gene linked to an amtB homolog (except in *Azospirillum brasilense*). The photosynthetic bacterium *Rhodospirillum rubrum* (47) and a member of the β-proteobacteria, *Azorarcus* sp. strain BH72 (26), were found to carry three genes encoding P_{II}-like proteins. Previous reports indicated that in *Klebsiella pneumoniae* and *Azotobacter vinelandii*, GlnK is involved in sensing of the nitrogen status by NiFL (a regulator of NiFA activity), although the mechanisms are not the same in these two organisms (14, 23, 35). The roles of the different P_{II} proteins in the physiology of nitrogen fixation and ammonium assimilation are clearly beginning to be revealed in many diazotrophs (9, 20, 47).

In this study, we describe the identification and mutagenesis of three glnB-homologous genes, glnB, glnK1, and glnK2, as...
part of the ammonium-sensing cascade in *G. diazotrophicus*. In addition, we describe the characterization of the phenotypes of strains with interposon mutations with respect to nitrogenase expression and activity.

**MATERIALS AND METHODS**

**Bacterial strains, phages, and plasmids.** The bacterial strains, phages, and plasmids used in this work are listed in Table 1.

**Media and growth conditions.** *G. diazotrophicus* was grown at 30°C on C2 medium (rich medium) (40) or minimal medium (43) with or without ammonium chloride during studies of the control of expression and activity of nitrogenase genes, wild-type *G. diazotrophicus* was grown at 30°C in C2 medium to a final optical density of 0.8 to 1.0, harvested by centrifugation, washed in 100 mM citrate-phosphate buffer (pH 5.5), and finally resuspended in minimal medium with or without 20 mM NH₄Cl as a sole N source.

To investigate the expression of *nif* genes, wild-type *G. diazotrophicus* and the *pnr* mutants were grown overnight at 30°C in C2 medium to a final optical density at 600 nm of 0.8 to 1.0, harvested by centrifugation, washed in 100 mM citrate-phosphate buffer (pH 5.5), and finally resuspended in minimal medium with or without 20 mM NH₄Cl as a sole N source.

**Construction of a *G. diazotrophicus* genomic library.** Chromosomal DNA from wild-type *G. diazotrophicus* PAL5 was used for the construction of a phage genomic library. For the preparation of chromosomal DNA, cells were grown to stationary phase in C2 medium. Cell lysis and DNA extraction were carried out as described previously (27). Fragments (9 to 23 kb) generated by Sau3A partial digestion were cloned by using a λ EMBL3/BamHI vector kit (Stratagene, Amsterdam, The Netherlands) with *E. coli* MRA(P2) as a host. In vitro packaging kits were also obtained commercially from Stratagene.

**DNA sequencing.** Sequencing of both DNA strands was performed by the dideoxy chain termination method (36) with a DNA sequencing kit (USB Corporation, Cleveland, Ohio) on an ALF automated DNA sequencer (Amersham Biosciences, Freiburg, Germany).

For the nucleotide sequence determination, undirected deletions were generated after thionucleotide end protection followed by the successive actions of exonuclease III of *Escherichia coli* grown in medium containing 10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract per liter (pH 7.5).

To determine the expression of *nif* genes, wild-type *G. diazotrophicus* and the *pnr* mutants were grown overnight at 30°C in C2 medium to a final optical density at 600 nm of 0.8 to 1.0, harvested by centrifugation, washed in 100 mM citrate-phosphate buffer (pH 5.5), and finally resuspended in minimal medium with or without 20 mM NH₄Cl as a sole N source.

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**Southern hybridization.** For Southern hybridization experiments, probes were labeled by the random priming method with a digoxigenin DNA labeling and detection kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s recommendations.

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**Southern hybridization.** For Southern hybridization experiments, probes were labeled by the random priming method with a digoxigenin DNA labeling and detection kit (Boehringer Mannheim, Mannheim, Germany). DNA was transferred to nylon membranes (Schleicher & Schüll, Dassel, Germany) by vacuum blotting for 1 h. After 2 h of prehybridization of 20 ml of prehybridization solution (standard buffer [The DIG System User Guide for Filter Hybridization; Boehringer Mannheim]), the probe (5 to 20 ng/ml in 5 ml of prehybridization solution) was added and hybridization was carried out for 16 h at 68°C. Detection was performed according to Boehringer Mannheim protocols with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium as a substrate.

**PCR amplification and cloning of genes encoding P₄₄-like proteins in *G. diazotrophicus*.** The nucleotide sequences of known *glnB* genes from different bacteria were aligned, and the degenerate primers 5’ ATCATYAAGCCCTT CAARCTG 3’ and 5’ GAARATTTSCGTCRCCRAT 3’ were synthesized (TIBmol, Berlin, Germany) and used in PCRs with wild-type *G. diazotrophicus* PAL5 chromosomal DNA as a template. The PCR products were evaluated by 1.5% agarose gel electrophoresis. Samples with amplified fragments of the ex-
Electrophoresis of G. diazotrophicus. For the introduction of DNA into G. diazotrophicus, cells were grown in C2 medium. At the early- to mid-logarithmic growth phase, cells were collected, washed in ice-cold 10% (vol/vol) glycerol in water, and resuspended in a 1:20 volume of 10% glycerol. Aliquots of 200 μl were used for electrophoresis after the addition of up to 1 μg of the appropriate plasmid DNA. Cells were electrophoresed in a Gene Pulser apparatus (Bio-Rad Laboratories, Munich, Germany) as described previously (40). Cells were then washed from the cuvette with 1 ml of C2 medium, incubated at 28°C for 2 h, collected, and plated on C2 medium containing the corresponding antibiotic.

Western blot analysis and nitrogenase activity. To prepare samples for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), G. diazotrophicus cell suspensions were mixed with a buffer containing 130 mM Tris (pH 6.8), 5% (wt/vol) SDS, 20% (vol/vol) glycerol, 0.003% (wt/vol) bromophenol blue, and 10% (vol/vol) 2-mercaptoethanol and incubated for 5 min at 90°C. Equal amounts of protein were loaded in 12% polyacrylamide–SDS (glutamine synthetase and Fe protein) or 18% polyacrylamide–SDS (Pn) gels, which were run as described previously (43). Gels were electroblotted onto Hybond-P membranes (Amersham Biosciences, Uppsala, Sweden) and probed with antibodies raised against R. rubrum GlnB, glutamine synthetase, or nitrogenase Fe protein. The results were visualized by using an enhanced chemiluminescence detection system (Amersham Biosciences, Uppsala, Sweden). Nitrogenase activity was measured by the acetylene reduction assay (43).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in GenBank under accession numbers AF365037, AF318040, and AF365041.

RESULTS AND DISCUSSION

Cloning and sequence analysis of the glnB and glnK1 genes. To detect Pn protein-encoding genes, PCR was performed with chromosomal DNA isolated from wild-type cells of G. diazotrophicus PALS5 (Table 1). To facilitate the PCR, the degenerate oligonucleotides described in Materials and Methods were used. PCR products of approximately the correct size, 258 bp, were cloned in E. coli by using cloning vector pUC18 (46). To confirm the identities of the cloned PCR products, 10 independent hybrid plasmids were sequenced, followed by a database comparison of the deduced protein sequences. Interestingly, although all deduced protein sequences showed significant similarities to the sequences of known Pn-like proteins, we found that the PCR fragments cloned were not identical but fell into two groups, represented by pOP42 and pOP75, the sequences of which were 70% identical and the gene products of which were 64% identical. Thus, it was evident that G. diazotrophicus harbors at least two glnB-like genes.

In order to obtain the corresponding chromosomal DNA regions carrying the full-length genes as well as the flanking regions, a genomic phage library of G. diazotrophicus was constructed (see Materials and Methods). The resulting phage library was subsequently screened with plasmids pOP42 and pOP75 carrying different glnB-like genes as probes. Southern hybridization experiments with isolated phage library DNA along with chromosomal DNA as a control revealed that plasmid pOP75 hybridized to 2.1-kb SalI and 3.5-kb EcoRI fragments, while pOP42 hybridized to 3.4-kb SalI, 10.0-kb BamHI, and 8.0-kb EcoRI fragments (data not shown). The corresponding restriction fragments generated by the endonucleases EcoRI and BamHI were cloned in E. coli by using vectors pSVB30 and pUC18, resulting in hybrid plasmids pOP7, pOP26, and pOP30 (Table 1).

In order to localize the open reading frames (ORFs) corresponding to the Pn protein-encoding genes, the nucleotide sequences of a 2.5-kb fragment of plasmid pOP26 and a 2.1-kb fragment of plasmid pOP7 were determined by using subclones derived from unidirectional deletions.

An analysis of the nucleotide sequence obtained from pOP26 showed two ORFs of 339 and 1,461 bp separated by 176 bp, with the deduced sequence of ORF1 showing significant similarities to the sequences of known glnB genes of other proteobacteria, with the highest score of 79% identity for the Pn protein of A. brasilense (12). ORF2, located 176 bp downstream, encodes a putative protein of 486 amino acids showing high degrees of identity to the glutamine synthetase proteins of A. brasilense (73%) (12), R. rubrum (71%) (18), and Sinorhizobium meliloti (70%) (2). This linkage resembles the common arrangement of the glnB and glnA genes in the α-proteobacteria. These genes in G. diazotrophicus were therefore designated glnB and glnA.

The nucleotide sequence of a 2.1-kb region of plasmid pOP7 contained two ORFs of 339 and 1,386 bp, with the putative start codon (GTG) of ORF2 overlapping the stop codon of ORF1 (TGCA). An additional putative start codon (ATG) was found to be located 83 bp downstream of ORF1. ORF1 encodes a polypeptide with a molecular mass of 12.4 kDa, the deduced amino acid sequence of which is 67% identical to that of the A. brasilense Pn (GlnZ) protein (11) and 64% identical to those of known glnK genes from E. coli (45) and K. pneumoniae (14), two members of the γ-proteobacteria. ORF2 encodes polypeptides with deduced molecular masses of 47.4 kDa for the product of translation from GTG and 44.4 kDa for the translation product starting at the downstream ATG. The deduced amino acid sequence of the product of this ORF is 49% identical to that of the amtB gene product of A. brasilense (44); therefore, the ORF likely encodes a (methyl)ammonium transporter in this organism. The genes localized on pOP7 were named glnK1 and amtB1.

The physical maps of the corresponding chromosomal DNA regions of G. diazotrophicus were deduced from restriction mapping and hybridization and refined by DNA sequencing data generated from subclones and their deletion derivatives (Table 1 and Fig. 1).

Identification and sequence analysis of a third glnB-like gene. As detailed below, we constructed both single and double mutants affected in glnB and glnK1 in order to investigate whether additional Pn homologs were present in G. diazotrophicus. Western blot analysis of crude protein extracts of the double mutant (UBI 130) with polyclonal anti-R. rubrum Pn antibodies was carried out as described for R. rubrum (19). Surprisingly, we found that a prominent signal of the size observed for Pn-like proteins was still present in the glnB glnK1 double mutant and that the posttranslational modification of a protein of about 13 kDa was still detectable (data not shown). These results raised the question of whether G. diazotrophicus carries a third glnB-like gene.

In order to obtain the corresponding chromosomal DNA regions, the genomic phage library of G. diazotrophicus was screened by plaque hybridization followed by Southern hybridization of isolated phage DNA with pOP8 as a probe. Two hybridizing signals of 2.1 and 3.2 kb were detected by using chromosomal DNA digested with restriction endonuclease SalI (data not shown). One of the hybrid phages isolated carries a SalI fragment of the expected size, 3.2 kb. This fragment
was cloned in *E. coli* by using vector pUC18, with the resulting plasmid being named pOP80, followed by nucleotide sequencing (Fig. 1).

The putative PII-like protein encoded within this DNA fragment has 112 amino acid residues and showed 66% identity with GlnZ of *A. brasilense* as well as high levels of similarity to GlnK and GlnB proteins of other organisms. Directly downstream of this gene, an incomplete ORF was found; the amino acid sequence from this ORF showed a high degree of identity with the amino acid sequences of (methyl)ammonium transporters from different organisms. Based on the proposed nomenclature for *glnB*-like genes (3, 41), the gene described above was named *glnK2*, and the incomplete ORF was named *amtB2*.

**Mutagenesis of glnB-like genes in *G. diazotrophicus***. The arrangement of all three genes directly upstream of other genes encoding proteins involved in nitrogen metabolism suggests that all three are involved in the regulation of assimilation and interconversion of nitrogen compounds in *G. diazotrophicus*, as in other proteobacteria. In order to examine the physiological roles of the GlnB and GlnK proteins, mutants were constructed by using the kanamycin interposon of plasmid pDM6 (32) for *glnB* and the promoterless *gusA*-chloramphenicol resistance gene cassette of plasmid pWM4 (29) for *glnK1*, resulting in hybrid plasmids pOP16 and pOP5 (Table 1). For the mutagenesis of *glnK2*, the tetracycline resistance gene cassette of plasmid p34s-Tc (10) was used. All interposons were cloned as *BamHI* DNA fragments into unique *BglII* restriction sites on plasmids pOP30, pOP7, and pOP80 containing *G. diazotrophicus glnB*, *glnK1*, and *glnK2*, respectively. These constructs, located on suicide plasmids, were then introduced to *G. diazotrophicus* by electroporation and tested for double crossover events by growth on media containing the appropriate antibiotics. Mutants exhibiting an ampicillin-sensitive and kanamycin-, chloramphenicol-, or tetracycline-resistant phenotype were expected to have a chromosomal allele replacement, which was confirmed by Southern hybridization with the chromosomal DNAs of the mutants and the wild-type strain as targets and the entire plasmids carrying *glnK1*, *glnK2*, and *glnB* regions as probes (data not shown).

The construction of the *glnB glnK1* (UBI 130), *glnB glnK2* (UBI 133), and *glnK1 glnK2* (UBI 135) double mutants as well as the *glnB glnK1 glnK2* (UBI 150) triple mutant was carried out by analogous introduction of the corresponding constructs into the verified single (double) mutants followed by selection with the corresponding antibiotic. Allele replacements were

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**FIG. 1.** Physical map of genes encoding PII-like proteins in *G. diazotrophicus*. Relevant restriction sites, interposons used for mutagenesis, and their orientations are indicated. The name of the corresponding mutant is indicated in parentheses. The broken arrow for *amtB2* indicates the part that was not completely sequenced.
Sequence analysis of \textit{G. diazotrophicus} GlnB, GlnK1, and GlnK2. As shown in Fig. 2, glnB, glnK1, and glnK2 all encode 112-amino-acid proteins with a calculated molecular mass of approximately 13 kDa and with the following overall identities: GlnB and GlnK1, 59%; GlnB and GlnK2, 63%; and GlnK1 and GlnK2, 71%. Sequence analysis of the GlnB, GlnK1, and GlnK2 proteins revealed that they share the common distinctive features of PII proteins (3).

As determined for other GlnB proteins, \textit{G. diazotrophicus} GlnB has lysine and glutamate at positions 3 and 5, respectively. The GlnK proteins are distinguished from the GlnB proteins in carrying a hydrophobic residue at position 3 and isoleucine, threonine, or methionine at position 5. \textit{G. diazotrophicus} GlnK1 has leucine and threonine at positions 3 and 5, respectively, while in GlnK2, phenylalanine and isoleucine are located at positions 3 and 5, respectively (3). As for other PII-like proteins, the significance of the presence of these amino acids at these particular positions is still unknown. A common feature of GlnB, GlnK1, and GlnK2 is the tyrosine residue located at position 51, which is likely to be the site for the posttranslational uridylylation of these paralogs (7).

Stretches of highly conserved amino acids corresponding to the T loop, B loop, and C loop in the three-dimensional conformations of these proteins (16, 31) are clearly present in all three \textit{G. diazotrophicus} PII-like proteins (Fig. 2). Interestingly, although the loops are highly conserved in all three PII proteins, there are also distinct differences, e.g., in the T loop, where residue 52 is Ile in GlnB, His in GlnK1, and Gln in GlnK2. Since the loops have been shown to be involved in the interaction of PII proteins with different effector molecules or target proteins (3), these differences could be an indication of the different responses of these paralogs to cellular effectors and/or differences in their abilities to interact with target proteins.

Expression of PII proteins and control of \textit{nif} genes by PII proteins in \textit{G. diazotrophicus}. To verify the expression of all three PII paralogs in \textit{G. diazotrophicus}, Western blot analysis of crude protein extracts was carried out with polyclonal antibodies raised against \textit{R. rubrum} GlnB. As shown in Fig. 3, PII proteins were expressed in all double mutants, indicating that the expression of one PII protein is not dependent on the presence of the others. However, the possibility that the level of expression of one paralog is modulated by those of the other two cannot be excluded, as we have not determined whether the antibodies have different sensitivities to the three PII proteins. Furthermore, no PII-related signal was found when cell extracts from \textit{G. diazotrophicus} glnB glnK1 glnK2 triple-mutant strain UBI 150 were used, probably indicating that no additional PII protein is present in \textit{G. diazotrophicus} (Fig. 3, lanes 5 and 6).

In bacteria, the core of the nitrogen-sensing system is composed of the bifunctional enzyme GlnD and the regulatory PII protein(s). GlnD controls the uridylylation status of PII in response to nitrogen levels, monitored as the glutamine concentration; in turn, PII in different uridylylation states controls the overall cellular response to nitrogen (15). As it has been shown that PII proteins play different roles in the control of nitrogen fixation in diazotrophic bacteria, the effect of the knockout of these proteins on the expression of \textit{nif} genes in \textit{G. diazotrophicus} was studied. The analysis was based on the evaluation of the expression of the Fe protein and nitrogenase activity.

The wild-type and mutant strains were grown in C2 medium (rich medium), and the expression of the \textit{nif} gene (encoding the Fe protein) was studied after transfer of the corresponding strains to nitrogen-free minimal medium. The results of this experiment are shown in Fig. 4. As expected, the induction of the Fe protein and nitrogenase activity occurred in wild-type strains raised against \textit{G. diazotrophicus} glnB glnK1 glnK2, and \textit{G. diazotrophicus} glnB glnK1 glnK2 (Fig. 3, lanes 1 to 5), or nitrogen excess, 20 mM NH$_4^+$ (lane 6).

Experimental conditions were as described in Materials and Methods. Lane 1, wild-type \textit{G. diazotrophicus}; lane 2, strain UBI 130 (glnB glnK2); lane 3, strain UBI 133 (glnB glnK2); lane 4, strain UBI 135 (glnK1 glnK2); lanes 5 and 6, strain UBI 150 (glnB glnK1 glnK2); lane 7, purified His-tagged \textit{R. rubrum} GlnB.

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**FIG. 2.** Sequence alignment of PII-like proteins in \textit{G. diazotrophicus}. The black dots indicate the distinctive presence of conserved amino acids at positions 3 and 5. The horizontal lines represent the putative T loop (T), B loop (B), and C loop (C). The vertical arrow indicates the conserved tyrosine at position 51 in each homolog.

**FIG. 3.** Western blot analysis of PII-like proteins of \textit{G. diazotrophicus} wild-type PAL5, PII-negative double mutants, and a PII-negative triple mutant. Cells were grown under conditions of nitrogen limitation, 1 mM NH$_4^+$ (lanes 1 to 5), or nitrogen excess, 20 mM NH$_4^+$ (lane 6). Experimental conditions were as described in Materials and Methods.
G. diazotrophicus, and similar results were obtained for the glnB, glnK1, and glnK2 single mutants. Interestingly, nifH was also induced in the PII protein triple mutant (Fig. 4, lane 8), indicating that in G. diazotrophicus, none of the PII proteins is required for nif gene expression.

However, in strain UBI 130, where GlnK2 is the only PII paralog present (Fig. 4, lane 5), the expression of the Fe protein was inhibited; no Fe protein was detected after 6 h in nitrogen-free medium. After 20 h, very low levels of the Fe protein were present compared to the levels in the other strains. Since strains UBI 120 (glnB) and UBI 125 (glnK1) showed wild-type induction of nif genes, it can be concluded that GlnK2 has an inhibitory effect on nif gene expression and that GlnB or GlnK1 is required to relieve this effect. In accordance with this observation, strain UBI 133 or UBI 135, expressing only GlnK1 or only GlnB, respectively, showed wild-type induction of nif gene expression.

In many bacteria, the uridylylation state of the PII proteins controls the cellular response to nitrogen levels. Uridylated PII proteins indicate nitrogen limitation, while nonuridylylated forms are related to a cellular state of nitrogen sufficiency (31). The observation that in G. diazotrophicus the expression of nif genes also occurs when all three proteins are absent suggests that the uridylylated forms are not required per se as positive signals. It is therefore reasonable to propose that in G. diazotrophicus, the nonuridylylated forms of the PII proteins inhibit nif gene expression under nitrogen sufficiency, as may be the case in other bacteria (30).

To evaluate this hypothesis, wild-type G. diazotrophicus and all PII mutant strains were grown in C2 medium, and the regulation of nif gene expression was studied after transfer of the cells to minimal medium containing 20 mM NH4+. The results of these studies are shown in Fig. 5. In response to high ammonium levels, nif gene expression was inhibited in the wild type and the G. diazotrophicus PII single mutants (Fig. 5, lanes 1 to 5). However, in strain UBI 150, where no PII proteins are present, nif genes were expressed in spite of high ammonium levels (Fig. 5, lane 8). This result indicates that in G. diazotrophicus, PII Proteins are in fact required for the repression of nif gene expression in response to ammonium. In strain UBI 133 or UBI 135, expressing GlnK1 or GlnB, respectively, as the only PII protein, low levels of the Fe protein were present, a finding which could indicate that GlnK2 is required for complete nif repression.

Figures 4 and 5 show that as a result of the interposon insertion within glnB, strains UBI 120, UBI 130, UBI 133, and UBI 150 have significantly decreased levels of glutamine synthetase compared to the wild type and the other G. diazotrophicus PII mutant strains. This finding probably reflects a polar effect of this mutation on the expression of the glnA gene. However, the lack of normal nif regulation in strains UBI 130 and UBI 150 and, to some extent, in strains UBI 133 and UBI 135 cannot be explained by decreased glutamine synthetase levels per se, since strain UBI 120 showed wild-type control of nif gene expression. Similarly, although the GlnK-AmtB interaction plays a regulatory role in, e.g., A. vinelandii (9), we do not believe that potential polar effects of the glnK1 or glnK2 mutations on the corresponding amtB genes can explain the
lack of nif control, since strains UBI 125 and UBI 127 showed apparent wild-type control of nif gene expression, while in strain UBI 135, ammonium control was not completely abolished.

It is interesting that none of the strains showed Fe protein expression when grown in C2 medium (rich medium) prior to their transfer to LGI medium (minimal medium). This result is somewhat surprising for strain UBI 150, since the inhibition of nif gene expression in response to ammonium was lost (Fig. 5, lane 8). This phenomenon, independent from ammonium-related PII protein signaling, certainly leads to new questions about the regulation of nif gene expression in G. diazotrophicus.

Our results for nifH gene expression (Fig. 4 and 5; summarized in Table 2) suggest that in G. diazotrophicus, the non-uridylated forms of PII proteins are responsible for the inhibition of nif gene expression. However, it was recently reported that a mutation in the 3′ end of the G. diazotrophicus glnD gene allows nif gene expression regardless of the levels of ammonium in the medium (32). This apparent contradiction, i.e., that nif genes are expressed in spite of mutated GlnD, suggests that as a result of this mutation, partially active GlnD with a significantly impaired ability to sense nitrogen (i.e., glutamine) is synthesized, as proposed for the corresponding glnD mutant in E. coli (38).

In conclusion, we report the presence of three PII proteins in G. diazotrophicus, a finding previously shown only for two other bacteria, R. rubrum (47) and Azorarcus sp. strain BH72 (26). In G. diazotrophicus, these proteins are expressed independently of the presence of each other, although we cannot distinguish whether the level of expression of one of the PII proteins is modulated by that of another. This situation is clearly different from that in Azorarcus, where GlnY is expressed only when the other two proteins are deleted (26). The GlnK2 protein clearly has a function different from those of GlnB and GlnK1, as it represses nif gene expression under all conditions tested in the glnB glnK1 mutant. It thus can be proposed that GlnB and GlnK1 are required as positive signals to efficiently relieve repression by GlnK2. When either GlnB or GlnK1 is present alone, the repression of nif gene expression is similar to that of the wild type, but not complete, indicating that GlnK2 has a central role in the regulation of nif gene expression. To our knowledge, this function of GlnK2 is a new feature of PII proteins as central components in the regulation of nitrogen metabolism.

Our studies do not show at what level the three PII proteins exert their action, i.e., which target proteins are affected. It was previously shown that the NtrBC proteins are not required for nitrogen fixation in G. diazotrophicus (28), a finding which could suggest that Nfa is one of the targets for one or more of the PII proteins, as has been shown in A. brasilense (5, 6). An important part of future studies will therefore be to establish the effects on nifA expression and or NifA activity, as well as to determine the capacity of GlnD to catalyze the uridylylation of the three PII proteins present in G. diazotrophicus.

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