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A New Type of Glutamine Synthetase in Cyanobacteria: the Protein Encoded by the *glnN* Gene Supports Nitrogen Assimilation in *Synechocystis* sp. Strain PCC 6803

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A new glutamine synthetase gene, *glnN*, which encodes a polypeptide of 724 amino acid residues (M_r, 79,416), has been identified in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803; this is the second gene that encodes a glutamine synthetase (GS) in this cyanobacterium. The functionality of this gene was evidenced by its ability to complement an *Escherichia coli glnA* mutant and to support *Synechocystis* growth in a strain whose *glnA* gene was inactivated by insertional mutagenesis. In this mutant (strain SJCR3), as well as in the wild-type strain, the second GS activity was subject to regulation by the nitrogen source, being strongly enhanced in nitrogen-free medium. Transcriptional fusion of a chloramphenicol acetyltransferase (*cat*) gene with the 5′-upstream region of *glnN* suggested that synthesis of the second *Synechocystis* GS is regulated at the transcriptional level. Furthermore, the level of *glnN* mRNA, a transcript of about 2,300 bases, was found to be strongly increased in nitrogen-free medium. The *glnN* product is similar to the GS subunits of *Bacteroides fragilis* and *Butyrivibrio fibrisolvens*, two obligate anaerobic bacteria whose GSs are markedly different from other prokaryotic and eukaryotic GSs. However, significant similarity is evident in the five regions which are homologous in all of the GSs so far described. The new GS gene was also found in other cyanobacteria but not in N_2-fixing filamentous species.

Ammonium assimilation takes place in cyanobacteria mainly by the sequential action of glutamine synthetase (GS) and glutamate synthase (25). GS in cyanobacteria is similar to the classical prokaryotic GS type I (GSI) that has been widely studied in enterobacteria and whose structure and regulation are well known (22, 46). Thus, cyanobacterial GS is composed of 12 identical subunits (M_r, about 50,000) arranged in two superimposed hexagonal rings (29, 32). In contrast to the enterobacterial enzyme, cyanobacterial GS is not regulated by adenylylation in response to the nitrogen source (16, 26, 27). However, in *Synechocystis* sp. strain PCC 6803, short-term inactivation of GSI promoted by ammonium has been reported. This inactivation seems to involve a phosphorylated compound (26, 27). In the N_2-fixing, filamentous species *Anabaena* sp. strain PCC 7120 and in the unicellular species *Agmenellum quadruplicatum* (*Synechococcus* sp. strain PCC 7002), control of GS synthesis by the nitrogen source has been shown (31, 44, 45).

The gene that encodes the classical dodecameric GS (*glnA*) has been cloned from several cyanobacteria, such as *Anabaena*, *Synechocystis*, *Calothrix*, and *Agmenellum* spp. (13, 16, 28, 45). The amino acid sequences deduced from the *Anabaena* and *Agmenellum glnA* genes (44, 45) show about 50% identity with the enterobacterial gene, and both are able to complement an *Escherichia coli glnA* mutant (16, 45).

The existence of more than one type of GS in prokaryotes seemed to be restricted to members of the family *Rhizobiaceae*, including the genera *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium*, and to the genus *Streptomyces*, with two different types: one corresponding to the classical prokaryotic structure, GSI (dodecameric), and the other related to eukaryotic GS (GSII) (octameric) (3, 6, 10, 37). Recently, a third GS homologous to prokaryotic GSI has been reported in *Rhizobium* spp. (9, 10, 14, 40).

Besides that, *Bacteroides fragilis* and *Butyrivibrio fibrisolvens*, two members of the family *Bacteroidaceae* which are obligate anaerobic bacteria that live in mammal intestines, contain a GS that differs markedly from all of the GSs previously described in subunit size (M_r, 75,000), structure (hexameric), and amino acid sequence (19, 20, 43).

It has been recently shown that a *glnA* mutant of the cyanobacterium *Agmenellum quadruplicatum* was able to grow in the absence of glutamine, indicating that the *glnA* gene is nonessential for ammonium assimilation and suggesting that another enzyme is responsible for the glutamine synthesis (45).

Here we describe the molecular cloning, sequence, and expression of a novel GS gene (*glnN*) in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. The deduced amino acid sequence revealed 44 and 41% identity with the *B. fragilis* and *B. fibrisolvens* GSs, respectively, but very little homology with other prokaryotic or eukaryotic GSs was detected. Also, we show that both genes *glnA* and *glnN* can be inactivated independently, suggesting that neither of them is essential, in the presence of the other, for nitrogen assimilation in *Synechocystis* sp. strain PCC 6803. Finally, we report the existence of the *glnN* gene in other non-nitrogen-fixing cyanobacteria. The *glnN* gene product and the *B. fragilis* and *B. fibrisolvens* GSs represent a new family of GSs (GS III).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Synechocystis* sp. strain PCC 6803 and its mutants were grown at 30°C with shaking in BG11 medium (36). Alternatively, cultures were bubbled with 1.5% (vol/vol) CO_2 in air. When ammonium was used as the nitrogen source, nitrate was replaced by 10 mM NH_4Cl and the medium was buffered with 20 mM *N*-tris(hy-

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droxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer. For plate cultures, BG11 liquid medium was supplemented with 1% (wt/vol) agar. Kanamycin and chloramphenicol were added to final concentrations of 50 and 20 μg/ml when required. The SFCΩ5, SFC57, and SFF16 strains of *Synechocystis* sp. strain PCC 6803 were used for transcriptional gene fusion experiments (8, 15). Other cyanobacterial strains were grown in liquid BG11 medium. *E. coli* DH5α was used as the host for plasmid preparations. *E. coli* MC 1061 was utilized for gene library construction. Luria broth was supplemented with ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, or chloramphenicol at 20 μg/ml when required. In complementation experiments, glutamine auxotrophic *E. coli* ET6017 [*araD139* Δ(*argF-lac*)*205 flbB5301 pstF25 relA1 rspL150* Δ(*glnG-A* or *glnL-A*)*229 rha-10 deoC1*] (*E. coli* Genetic Stock Center, Yale University, New Haven Conn.) was used. Complementation experiments were performed with glucose minimal medium (33) supplemented with 5 mM glutamine and 40 μg of ampicillin per ml whenever required.

DNA manipulation. All DNA manipulations were performed by following standard procedures (38). DNA fragments were purified from agarose gels with the GeneClean kit (Bio 101, Inc). Total DNA from cyanobacteria was isolated as previously described (5). For Southern hybridizations, DNA was digested and fragments were electrophoresed in 0.7% agarose gels in a Tris-borate-EDTA buffer system (38). Transfer of DNA to nylon Z-Probe membranes (Bio-Rad) and Southern blot hybridizations were performed as previously described (2). For heterologous Southern hybridizations, low-stringency conditions (55°C, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) were used and filters were washed at room temperature. DNA probes were ^{32}P labelled with a nick translation kit (Boehringer Mannhein) using [α-^{32}P]dCTP.

Construction of a *Synechocystis* sp. strain PCC 6803 gene library. Genomic DNA from *Synechocystis* sp. strain PCC 6803 was partially digested with *Sau*3AI and fractionated by centrifugation through a sucrose gradient (38). The fractions containing fragments of 4 to 6 kbp were pooled and ligated to *Bam*HI-digested and dephosphorylated plasmid pBluescript II SK(+). The ligation mixture was used to transform *E. coli* MC 1061. About 6,500 independent colonies containing recombinant clones were obtained.

Insertional mutagenesis of *glnA* and *glnN Synechocystis* genes. A 3-kb *glnA*-containing *Apa*I-*Xba*I fragment from pAM1 (28) was cloned in plasmid pBluescript II SK(+) to generate pJCR3. For mutation of gene *glnA*, a 340-bp internal *Eco*RI fragment of *glnA* was replaced by a 1.3-kb fragment containing a kanamycin resistance gene from Tn*5* (12). The new plasmids were called pJCR5.3(+) and pJCR5.3(−), depending on the antibiotic resistance cassette orientation. Both plasmids were used to transform the *Synechocystis* wild type (WT) as previously described (7). Plasmids for *glnN* mutagenesis were constructed by replacement of a 666-bp internal *Eco*RI fragment of *glnN* by the 1.3-kb kanamycin resistance (Km^r) cassette or by a 1.9-kb chloramphenicol resistance cassette (12), in both orientations, producing pGS2.2(+) and pGS2.2(−) or pGS2.3(+) and pGS2.3(−), respectively. All plasmids were also used for *Synechocystis* transformation.

DNA sequence determination and analysis. Nested deletions of relevant plasmids were performed by using a double-stranded Nested Deletion Kit from Pharmacia. The complete sequence of both strands was determined by the dideoxy-chain termination method (39), with Sequenase 2.0 (USB). Computer analysis was carried out by using the sequence software package of the University of Wisconsin Genetics Computer Group (11). Computer searches for homologies were done by using the FASTA program, and alignments were obtained with Pileup, by using default parameters, and by manual analysis.

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated from mid-exponential-phase cultures of *Synechocystis* sp. strain PCC 6803 as described by Mohamed and Jansson (30), except that before lysis cells were frozen in liquid nitrogen and ground in a mortar while frozen. Separation of RNA on formaldehyde gels, transfer to nylon membranes (Hybond N-plus; Amersham), and prehybridization and hybridization conditions were in accordance with the instruction manuals from Amersham. A 15-μg sample of total RNA was loaded per lane.

Transcriptional gene fusions. Transcriptional gene fusions were constructed in plasmid pFF11, a promoter-probe vector based on the *cat* (chloramphenicol acetyltransferase [CAT]-encoding) gene. A 700-bp *Hin*dIII-*Ava*II fragment upstream of the *glnN* gene was subcloned, in both orientations, in pFF11 (see Fig. 5A). The resulting plasmids, pJCR15(+) and pJCR15(−), were used to transform *Synechocystis* sp. strain SFCΩ5 harboring plasmid pFCΩ5, which is homologous to the incoming vector; this allowed rescue of the incoming vector by homologous recombination with the resident plasmid (15). To check the transformation product, plasmid DNA from *Synechocystis* transformants was isolated as previously described (7) and analyzed by restriction pattern.

CAT activity was assayed in vitro at 37°C by the colorimetric procedure (41). CAT specific activity is reported as the number of micromoles of chloramphenicol acetylated per minute per milligram of protein.

Strains SFF16, which contains a promoterless *cat* gene, and SFC57, which contains the *cat* gene under the control of its own promoter, were used as controls.

GS assay and analytical methods. GS biosynthetic activity was determined in situ from the rate of glutamine formation. In strain SJCR3, GS activity was not detectable by the standard assay previously described for *Synechocystis* sp. strain PCC 6803 GS (26). However, we found appreciable GS-biosynthetic activity when the following reaction mixture was used: 50 mM Tris-HCl (pH 9)–6 mM ATP–5 mM NH_4Cl–50 mM L-glutamate–50 mM $MgCl_2$–0.025% (wt/vol) mixed alkyltrimethylammonium bromide (MTA). To 1 ml of the reaction mixture, 50 μl of a cell suspension in 50 mM Tris-HCl (pH 9) was added. The reaction was started by addition of ATP and continued at 30°C for 15 min. Glutamine was determined by reverse-phase high-performance liquid chromatography as described previously (24). This assay was also used for the WT and SJCR6 strains of *Synechocystis* sp. strain PCC 6803. One unit of GS activity corresponds to the amount of enzyme that catalyzes the synthesis of 1 μmol of glutamine per min. Chlorophyll was measured in methanolic extracts (21). Protein in whole cells was determined by a modified Lowry procedure (23) with ovalbumin as the standard. Protein in cell extracts was determined by the method of Bradford (4) with ovalbumin as the standard.

Nucleotide sequence accession number. The EMBL and GenBank accession number for the sequence described here is X76719.

RESULTS

Insertional mutagenesis of gene *glnA* in *Synechocystis* sp. strain PCC 6803. In a previous work, we cloned the *glnA* gene from *Synechocystis* sp. strain PCC 6803 (28). To generate a *Synechocystis glnA* mutant, we constructed a plasmid containing the *glnA* gene disrupted by a kanamycin resistance cassette

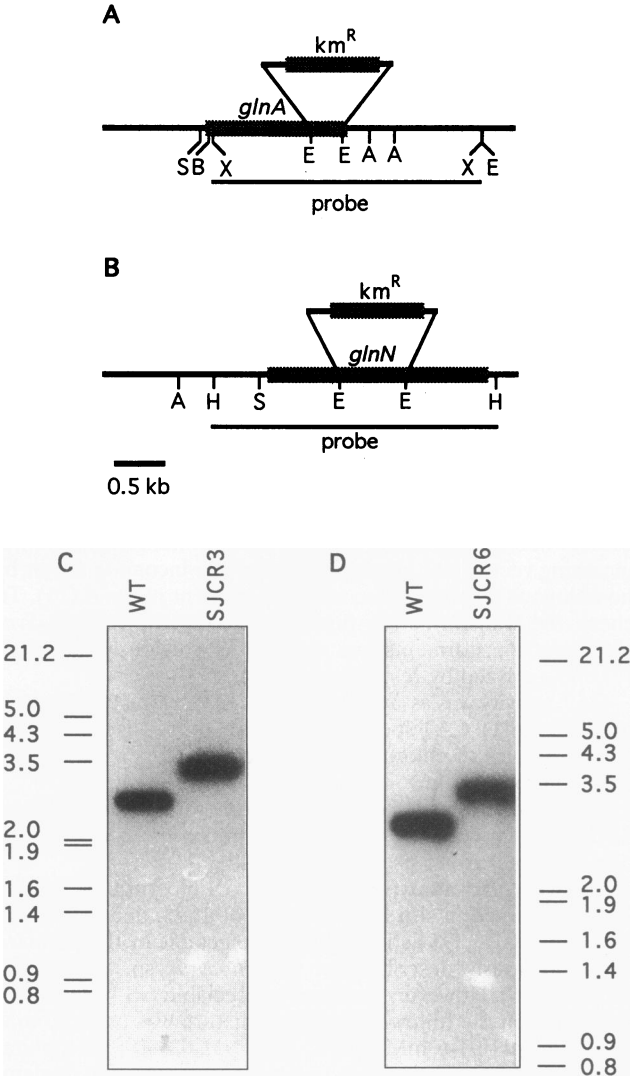

FIG. 1. Southern blot analysis of *Synechocystis* sp. strain PCC 6803 *glnA*::*npt* and *glnN*::*npt* mutants. (A) Structure of the *glnA* region in WT *Synechocystis* sp. strain PCC 6803 and substitution of an internal *Eco*RI fragment of *glnA* by an *npt* (Km^r) gene cassette. (B) Structure of the *glnN* region in WT *Synechocystis* sp. strain 6803 and replacement of an internal *Eco*RI fragment of *glnN* by an *npt* (Km^r) gene cassette. (C) Southern blot of genomic DNAs from the WT and SJCR3 strains. Genomic DNA was digested with *Xmn*I and hybridized by using the 2.66-kb fragment indicated in panel A as a ^{32}P-labelled probe. (D) Southern blot of genomic DNAs from the WT and SJCR6 strains. Genomic DNA was digested with *Hin*dIII and hybridized by using the 2.87-kb fragment indicated in panel B as a ^{32}P-labelled probe. Restriction site abbreviations: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X, *Xmn*I. Fragment sizes are in kilobases.

(Fig. 1A). This plasmid was used for *Synechocystis* WT transformation. Kanamycin-resistant transformants were obtained with similar frequencies in BG11 medium containing nitrate as the nitrogen source and in the same medium supplemented with 0.5 mM glutamine. However, no transformants were obtained when ammonium was used instead of nitrate as the nitrogen source, with or without glutamine. After several segregation rounds, mutants were not glutamine auxotrophic despite being homozygous for the interrupted *glnA* gene. Southern blot analysis was used to confirm the absence of the uninterrupted wild-type copies of *glnA* in genomic DNA (Fig. 1C). Preliminary characterization of SJCR3 indicated that it was able to grow without glutamine in the medium and exhibited very low, but detectable, levels of GS-biosynthetic activity (Table 1). This result strongly suggested the existence of a second gene that encodes a GS in *Synechocystis* sp. strain PCC 6803.

TABLE 1. GS specific activity in WT *Synechocystis* sp. strain PCC 6803 and mutants[a]

Strain	GS sp act[b] with following nitrogen source:		
	Ammonium	Nitrate	Nitrogen starvation[c]
WT	2.3 ± 1.0	27.3 ± 2.5	36.2 ± 2.5
SJCR3 (*glnA* mutant)	ND[d, e]	0.8 ± 0.3	6.6 ± 1.0
SJCR6 (*glnN* mutant)	2.5 ± 1.0	26.7 ± 2.0	28.0 ± 2.0

[a] *Synechocystis* sp. strain 6803 cells were grown in BG11 medium with nitrate or ammonium as the nitrogen source. Cultures were bubbled with 1.5% (vol/vol) CO_2 in air. Cells were collected for a GS assay at the end of the exponential growth phase and contained approximately 10 μg of chlorophyll per ml. The data shown are means of three independent experiments ± the standard errors.

[b] GS activity is expressed as nanomoles of glutamine formed per milligram of protein in the biosynthetic assay carried out as described in Materials and Methods.

[c] For nitrogen starvation, cells grown in BG11 medium with nitrate as the nitrogen source were harvested at the end of the exponential growth phase, washed, and transferred to BG11 medium lacking a nitrogen source for 20 h.

[d] Because of the absence of growth of SJCR3 in the presence of ammonium, GS activity was measured 20 h after the cells were transferred from nitrate-containing medium to ammonium-containing medium.

[e] ND, not detected.

Cloning and sequence of the *glnN* gene. To identify and clone the gene that codes for this second GS, we tried to complement an *E. coli glnA* mutant (ET 6017; unable to grow on minimal medium in the absence of glutamine) with a gene library from *Synechocystis* sp. strain PCC 6803. After transformation of *E. coli* ET 6017, one colony able to grow in minimal medium and harboring a plasmid (pBE2) which again complemented the *E. coli* mutant was isolated. The *E. coli* clone showed appreciable GS activity. pBE2 was analyzed with restriction enzymes and showed a 4.5-kb *Synechocystis* DNA insert with a restriction map different from that of the known *glnA* region of *Synechocystis* strains (Fig. 1A and B). A 2.85-kb fragment able to complement the *E. coli glnA* mutant was subcloned from pBE2 into plasmid pBluescript II SK(+), and both strands were sequenced. Only a 2,172-bp open reading frame that encodes a 724-amino-acid protein was found (Fig. 2). The new GS gene was designed *glnN*.

The fact that in the original plasmid, pBE2, gene *glnN* was placed in the opposite direction with respect to the *lacZ* promoter from the pBluescript II SK(+) vector suggested that the *glnN* gene is transcribed from its own promoter in *E. coli* ET 6017.

Sequence analysis of the *glnN* gene. Comparison of the deduced amino acid sequence of the *glnN* gene with the available data bases by using the FASTA program revealed that *glnN* is homologous to the *glnA* genes of *B. fragilis* (44% identity) and *B. fibrisolvens* (41% identity) (Fig. 3) but has no significant homology (less than 20% identity) with any other GS sequence from prokaryotic or eukaryotic organisms. The amino acid sequence homology between prokaryotic and eukaryotic enzyme subunits GSI and GSII, respectively, is approximately 15% (35); however, it is possible to identify five regions conserved in both GS types (34). Figure 4 shows an alignment of the five corresponding regions of the *glnN* protein product from *Synechocystis* sp. strain PCC 6803, GSs from *B.*

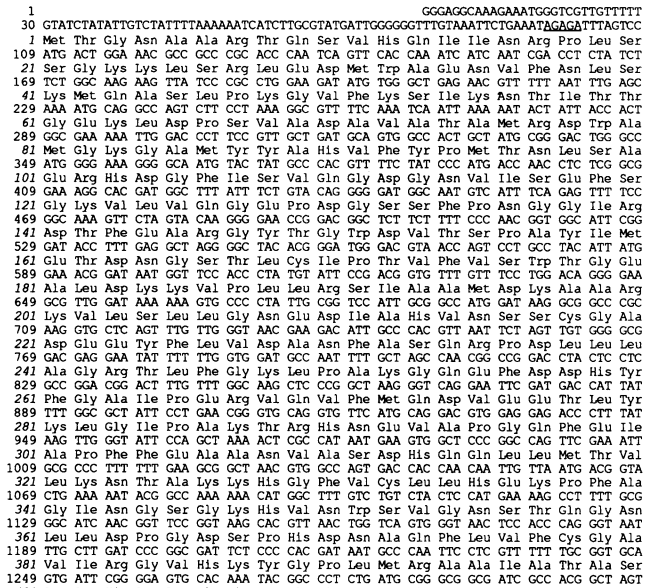

FIG. 2. Nucleotide sequence of the *Synechocystis* sp. strain PCC 6803 *glnN* gene and deduced amino acid sequence. A putative ribosome-binding site is underlined.

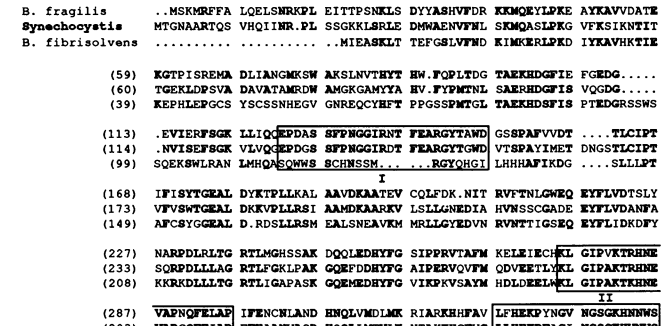

FIG. 3. Comparison of the predicted amino acid sequences of the *Synechocystis* sp. strain PCC 6803 *glnN* product, *B. fragilis* GS, and *B. fibrisolvens* GS. The five regions conserved in GSI and GSII are boxed. Identical residues are in boldface type.

fragilis and *B. fibrisolvens*, and other representative prokaryotic and eukaryotic GSs. Critical amino acids involved in catalytic function are conserved among the three different types of GS (see Discussion). The size of both the *Synechocystis* and *B. fragilis* GS enzyme subunits (about 725 amino acids) is approximately 270 and 350 amino acids longer than the GSI and GSII subunits, respectively. However, a higher level of similarity was found in the central fragment of the protein, where the five homologous regions, shown in Fig. 4, are located, decreasing significantly in the amino- and carboxyl-terminal regions.

Insertional mutagenesis of the *glnN* gene. To examine the role of *glnN* in *Synechocystis* sp. strain PCC 6803, an internal *Eco*RI fragment of *glnN* was replaced by a kanamycin resistance cassette (Fig. 1B). Kanamycin-resistant colonies were obtained in BG11 medium with nitrate or ammonium as the nitrogen source at the same frequency and without any further requirement. Complete segregation of *glnN* mutant strain named SJCR6 was confirmed by Southern hybridization of genomic DNA (Fig. 1D). Strain SJCR6 grew normally under all of the conditions tested, indicating that *glnN* is not required for nitrogen assimilation in the presence of the WT *glnA* gene. We tried to obtain a *glnA glnN* double mutant by transforming strain SJCR3 with plasmid pGS2.3, which contains the *glnN* gene interrupted by a chloramphenicol resistance (Cm^r) cassette. Cm^r Km^r colonies were obtained in BG11 medium and cultured for several rounds of segregation in the same nitrate-containing medium supplemented with glutamine (0.5 mM). Analysis by Southern hybridization showed only partial chromosomal segregation (data not shown). An increase of the chloramphenicol concentration in the medium provoked absence of growth of this strain, indicating that the *glnA glnN* double mutant was not viable.

Effect of nitrogen feeding in *glnA* and *glnN* mutants. We characterized the mutants affected in each of the GS genes with respect to response to the nitrogen source. The duplication time of *glnA* mutant strain SJCR3 in medium containing nitrate as the nitrogen source was affected only slightly compared with that of the WT (12 h versus 9.5 h, respectively). As mentioned above, the *glnA* mutant was unable to grow (duplication time, higher than 100 h) in the presence of ammonium (in medium with or without nitrate). When SJCR3 cells grown in medium containing nitrate as the nitrogen source were transferred to ammonium containing medium, growth was halted and after 20 h in these conditions, GS-biosynthetic activity was not detected (Table 1). In contrast, in medium containing nitrate as the nitrogen source, and especially in the absence of combined nitrogen, GS levels increased dramatically, indicating that the expression or activity of the *glnN* product is regulated by the nitrogen status of the cell. The *glnN* mutant grew at the same rate as the WT and exhibited the

FIG. 4. Alignment of the five regions conserved in the three GSIII sequences and other GSs from the following organisms: *Zea mays*, *Pisum sativum*, *Homo sapiens*, *Bradyrhizobium japonicum* (GSII), *B. cereus*, *Clostridium acetobutyricum*, *M. voltae*, *Anabaena* sp. strain PCC 7120, *Streptomyces coelicolor*, *E. coli*, and *S. solfataricus*. Conservative amino acid changes in the three GS types are in boldface type. Amino acids residues identical in all of the sequences are marked with asterisks.

ammonium-mediated regulation of GSI activity previously described (Table 1) (27); however, the increase of GS activity in nitrogen-starved cells was significantly smaller in the *glnN* mutant strain than in the WT (Table 1), supporting the view that under nitrogen stress, about 20% of the total GS activity of the *Synechocystis* WT corresponds to the GS encoded by *glnN*.

Regulation of *glnN* gene expression. To determine the level at which *glnN* product regulation occurs, we subcloned the 5′-upstream region of *glnN* in pFF11, a promoter-probe plasmid based on the *cat* reporter gene and constructed for testing of promoters in *Synechocystis* sp. strain PCC 6803 (15). A 700-bp *Hin*dIII-*Ava*II fragment upstream of the *glnN* gene (Fig. 5A) was cloned in pFF11 in both orientations. The resulting plasmids [pJCR15(+) and pJCR15(−)] were used to transform the SFCΩ5 strain, generating strains SJCR15(+) and SJCR15(−), which were chloramphenicol sensitive (Cm^s) in medium with nitrate or ammonium as the nitrogen source. The CAT activity levels of both strains grown either in nitrate or in ammonium-containing medium or after 20 h of nitrogen starvation were determined. As shown in Fig. 5B, significant levels of CAT activity were detected only after 20 h of nitrogen starvation in strain SJCR15(+). The CAT activity levels of strains SFC57 (Cm^r) and SFF16 (Cm^s) were not affected by the nitrogen conditions. These data suggest that transcription of the *glnN* gene is strongly activated under nitrogen starvation, even in the WT strain.

Levels of *glnN* mRNA were determined by Northern blot analysis. Total RNA was extracted from mid-exponential-phase WT *Synechocystis* cells grown on nitrate or ammonium

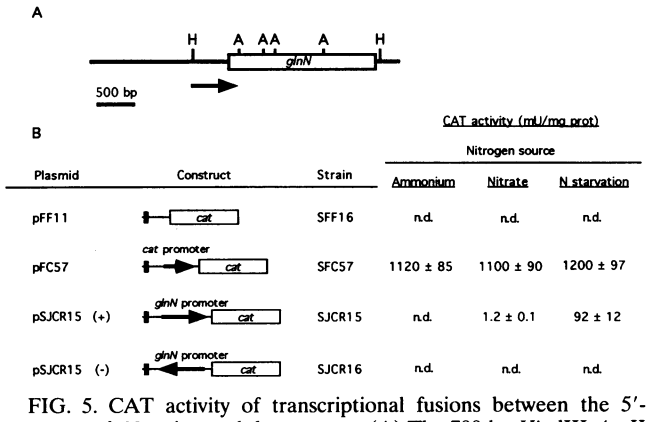

FIG. 5. CAT activity of transcriptional fusions between the 5′-upstream *glnN* region and the *cat* gene. (A) The 700-bp *Hin*dIII-*Ava*II upstream *glnN* fragment was subcloned in promoter-probe plasmid pFF11. (B) Levels of CAT activity were determined either in nitrate or in ammonium-containing medium or 20 h after transferring the cells from nitrate to nitrogen-free medium. Strains SFF16, which contain a promoterless *cat* gene, and SFC57, which contain the *cat* gene under the control of its own promoter, were used as controls. Means and standard errors resulting from two independent experiments are shown. n.d., not detected. Restriction site abbreviations: A, *Ava*II; H, *Hin*dIII. The coliphage T4 gene 32 transcription terminator is represented by the heavy vertical bar.

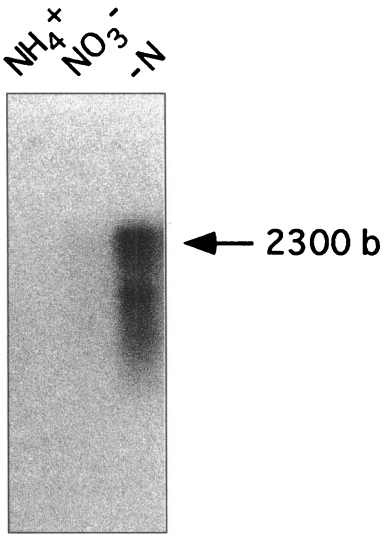

FIG. 6. Northern blot of total RNA isolated from *Synechocystis* WT cells grown in ammonium (NH_4^+) or nitrate (NO_3^-) or from cells grown in nitrate and transferred for 20 h to nitrogen-free medium (−N). Total RNA was denatured, electrophoresed in a 1% agarose gel, blotted, and hybridized with a 666-bp *Eco*RI-*Eco*RI *glnN* probe. A 15-μg sample of total RNA was loaded per lane. Transcript size was estimated by comparison with 23S, 16S, and 5S rRNAs (30). b, bases.

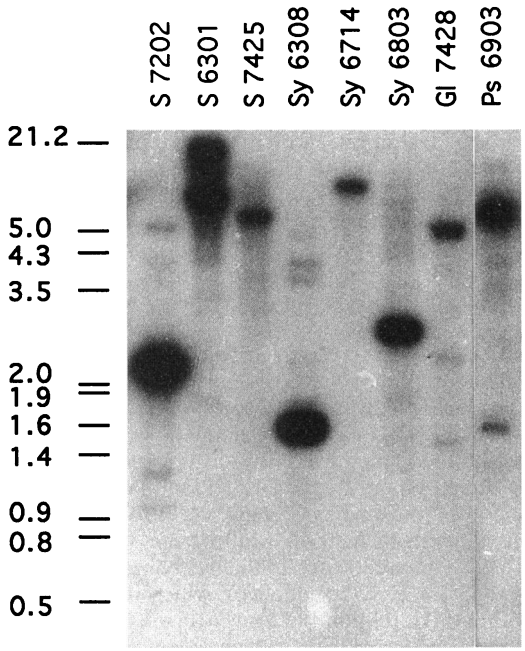

FIG. 7. Southern hybridization of genomic DNAs from different cyanobacterial strains. Genera are abbreviated as follows: S, *Synechococcus*; Sy, *Synechocystis*; Gl, *Gloeocapsa*; Ps, *Pseudanabaena*. Two micrograms of genomic DNA was digested with *Hin*dIII, except for the DNAs from *Synechococcus* sp. strain PCC 7202 and *Synechocystis* sp. strain PCC 6308, which were digested with *Dra*I. For *Synechocystis* sp. strain PCC 6803 and *Synechocystis* sp. strain PCC 6714, 0.4 μg of DNA was used. DNA was electrophoresed in an agarose gel and blotted onto a nylon membrane. The blot was probed with a 666-bp *Eco*RI-*Eco*RI fragment of *glnN*. Fragment sizes are in kilobases.

or after 20 h of nitrogen starvation. After denaturation, separation on agarose, and blotting onto a nylon membrane, the RNA was probed with the 666-bp *Eco*RI-*Eco*RI fragment of *glnN*. A single transcript of about 2,300 bases was detected in the lane containing RNA from nitrogen-starved cells, suggesting that *glnN* is transcribed as a monocistronic message (Fig. 6). Total RNA extracted from nitrate-grown cells gave a much weaker hybridization signal (Fig. 6), and no signal was obtained with RNA from ammonium-grown cells.

Presence of the *glnN* gene in cyanobacteria. To establish whether the *glnN* gene found in *Synechocystis* sp. strain PCC 6803 is a peculiarity of this organism or is present in other cyanobacteria, heterologous Southern hybridization analysis of genomic DNAs from a variety of phylogenetically diverse cyanobacteria was done. A 666-bp *Eco*RI fragment corresponding to the central region of *glnN*, which is more conserved with respect to the *B. fragilis glnA* gene, was used as the hybridization probe. Strong hybridization signals were observed with the genomic DNAs from the unicellular cyanobacteria *Synechococcus* sp. strains PCC 7202, PCC 6301, and PCC 7425, *Synechocystis* sp. strains PCC 6308 and PCC 6714, and *Gloeocapsa* sp. strain PCC 7428 (Fig. 7). A strong signal was also observed with DNA from the section III (36) filamentous cyanobacterium *Pseudoanabaena* sp. strain PCC 6903 (Fig. 7). However, no significant signals were observed with DNAs from the filamentous cyanobacteria *Anabaena* sp. strains ATCC 29413 and PCC 7120; *Nostoc* sp. strains PCC 6720, PCC 7413, and PCC 6705; and *Calothrix* sp. strain PCC 7601, which all correspond to section IV, or *Fischerella* sp. strain UTEX 1829, which belongs to section V (data not shown).

DISCUSSION

Construction of a *Synechocystis* sp. strain 6803 *glnA* mutant. In cyanobacteria, GS plays a central role in nitrogen assimilation, especially because it is the first enzymatic step linking nitrogen and carbon metabolism (25). Many studies have been devoted to determine the effect of nutritional conditions on the regulation of expression and activity of GS (16, 26, 31, 44, 45). In a previous work, we have reported the inactivation of the *glnA* gene in *Synechocystis* sp. strain PCC 6803 which contained, integrated into the chromosome, the *Anabaena* sp. strain PCC 7120 *glnA* gene (28). However, attempts to obtain a *glnA* mutant from the *Synechocystis* WT were unsuccessful. Since *Synechocystis* sp. strain PCC 6803 exhibits glutamate dehydrogenase activity (17), which could support ammonium assimilation in a potential *glnA* mutant, and glutamine can be taken up by the cells (18), we tried again to generate a *Synechocystis glnA* mutant. Herein, we report data that support the obtaining of an insertional inactivated *glnA* mutant (SJCR3) of *Synechocystis* sp. strain PCC 6803 that, unexpectedly, does not require glutamine for growth. *Synechocystis glnA* mutant SJCR3 was able to grow at a rate similar to that of the WT strain in medium containing nitrate as the nitrogen source but was unable to grow in ammonium-containing medium. SJCR3 GS-biosynthetic activity was very low in medium with nitrate and not detectable after 20 h in ammonium-containing medium (Table 1). The absence of detectable GS activity in the presence of ammonium may be the reason for the inability of strain SJCR3 to grow under these conditions. Recently, another *glnA* mutant of the cyanobacterium *A. quadruplicatum* (*Synechococcus* sp. strain PCC 7002) has been reported (45). This mutated strain did not require glutamine either and, unlike the SJCR3 mutant, grew at the WT rate with either nitrate or ammonium as the sole nitrogen source. Both results suggest the existence of a second GS that would be able to

support nitrogen assimilation in the absence of the *glnA* product.

Sequence analysis of the *glnN* gene. The second GS gene, here designed *glnN*, has been cloned by complementation of an *E. coli glnA* mutant. The *glnN* gene encodes a new type of GS that differs widely in size, structure, and amino acid sequence from the previously known GSI and GSII of prokaryotes. This alternative *Synechocystis* GS is only homologous (about 40 to 45% identity) to the *B. fragilis* and *B. fibrisolvens* GSs and can be considered a third type of GS in prokaryotes (19, 43). In fact, it is now possible to define a third family of GSs in bacteria (GSIII) which is not restricted to a single taxonomic group.

The structure of the GS from *Salmonella typhimurium* has been extensively characterized (1, 46), and five β-sheets involved in the GS active site and conserved between eukaryotic and prokaryotic GSs have been defined. All of these regions are found in the second *Synechocystis* GS and in *B. fragilis* GS, but only three of them (regions II, III, and IV) could be clearly identified in *B. fibrisolvens* GS (Fig. 4). In total, 17 amino acid positions remain unchanged in all of the 14 sequences analyzed; 16 of them are located in the five conserved regions, and 1 is Asp-233 (of *E. coli* sequence), which is located between regions II and III. Region I of *Synechocystis* GSIII contains the amino acid sequence DGSS, which is conserved in all of the sequences except that of the *B. fragilis* GS, where it is replaced by DASS, and in that of *B. fibrisolvens* (WWSS). Regions II and V are related, with two Mn^{2+} cations associated at each active site. Glutamic acid residues present in these two regions of all of the GSs have been recognized as ligands to Mn^{2+} ions. In region III, the putative ATP-binding site, seven residues are identical in GSI and GSII and are also identical in GSIII. Region IV, considered the glutamate-binding site, is characterized by the sequence NR---(I/V)R(I/V). In GSIII, the second Arg residue is absent and Ile or Val is replaced by Phe.

The molecular mass of *Synechocystis* GSIII, as deduced from the predicted amino acid sequence, is similar to that of the *B. fragilis* GS, about 75 kDa (43), and very different from those of GSI and GSII, about 50 and 40 kDa, respectively. Assuming that the data reported for the purified *B. fragilis* GS are correct, native *Synechocystis* GSIII may be composed of six identical subunits arranged in an hexameric structure. In this regard, work is now in progress to purify GSIII overexpressed in *E. coli* ET 6017 to determine the physicochemical and kinetic properties of this novel GS.

Function of the *glnN* gene. Data shown in Table 1 indicate that *Synechocystis* GSIII is not essential for nitrogen assimilation in the WT strain. In fact, the growth rates of *glnN* mutants were not affected. However, attempts to obtain a double mutant affected in both GS genes have been unsuccessful.

GSIII activity was drastically affected by nitrogen availability; thus, the GS activity of strain SJCR3 increased dramatically in nitrogen-free medium, with ammonium acting as a repressor. In this regard, data obtained with the WT strain by *cat* transcriptional fusions or Northern blot analysis (Fig. 5 and 6) suggest that regulation occurs mainly at the transcriptional level. In addition, comparison of the GS activities from the *Synechocystis* WT and the *glnN* mutant indicates that, in the absence of nitrogen, the increase in GS activity is partially (about 20%), due to GSIII, suggesting that under nitrogen stress all possible ways to capture ammonium are triggered. The fact that sequences homologous to *glnN* have been identified in different groups of cyanobacteria which are unable to fix dinitrogen but not in nitrogen fixers, together with the pattern of activity regulation, suggests that possession of GSIII gives a selective advantage, when a combined nitrogen source is not present, to cyanobacteria that are unable to fix dinitrogen.

Horizontal transference from plants has been proposed to explain the presence of GSII in bacteria (6, 42). In the same way, horizontal transference has been suggested to explain why in a GS phylogenetic tree *Sulfolobus solfataricus* GS is placed with the gram-negative bacteria while GS from another archaebacterium, *Methanococcus voltae*, is found with the gram-positive bacteria (42). The *Synechocystis* sp. strain PCC 6803 *glnN* gene shows typical *Synechocystis* codon usage. Besides, the *Synechocystis* GSIII sequence has approximately the same degree of homology to *B. fragilis* or *B. fibrisolvens* GS as that which exists between *Synechocystis* sp. strain PCC 6803 GSI and *Bacillus cereus* GSI (35a). These data, together with the very different ecological niches of the family *Bacteroidaceae* and the genus *Synechocystis*, seem to discard the idea of lateral gene transfer in this case.

The presence of this type of GS in two taxonomic groups as different as cyanobacteria and the family *Bacteroidaceae* suggests the possibility that a homologous protein exists in other bacterial groups. In conclusion, GSIII represents a novel type of GS in cyanobacteria that, together with the *B. fragilis* and *B. fibrisolvens* GSs, constitutes a new family of GSs in prokaryotes.

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REFERENCES

1. **Almasy, R. J., C. A. Janson, R. Hamlin, N.-H. Xuong, and D. Eisenberg.** 1986. Novel subunit-subunit interaction in the structure of glutamine synthetase. Nature (London) **323**:304–309.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1992. Current protocols in molecular biology. Greene Publishing and Wiley-Interscience, New York.
3. **Behrmann, I., D. Hillemann, A. Pühler, E. Strauch, and W. Wohlleben.** 1990. Overexpression of a *Streptomyces viridochromogenes* gene (*glnII*) encoding a glutamine synthetase similar to those of eukaryotes confers resistance against the antibiotic phosphinothricyl-alanyl-alanine. J. Bacteriol. **172**:5326–5334.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Cai, Y., and C. P. Wolk.** 1990. Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. J. Bacteriol. **172**:3138–3145.
6. **Carlson, T. A., and B. K. Chelm.** 1986. Apparent eukaryotic origin of glutamine synthetase II from the bacterium *Bradyrhizobium japonicum*. Nature (London) **322**:568–570.
7. **Chauvat, F., L. De Vries, A. Van der Ende, and G. A. Van Arkel.** 1986. A host-vector system for gene cloning in the cyanobacterium Synechocystis PCC 6803. Mol. Gen. Genet. **204**:165–191.
8. **Chauvat, F., J. Labarre, and F. Ferino.** 1988. Gene transfer system for the cyanobacterium Synechocystis PCC 6803. Plant Physiol. Biochem. **26**:629–637.
9. **Chiurazzi, M., R. Meza, M. Lara, A. Lahm, R. Defez, M. Iaccario, and G. Espin.** 1992. The *Rhizobium leguminosarum* biovar *Phaseoli glnT* gene, encoding glutamine synthetase III. Gene **119**:1–8.
10. **de Bruijn, F. J., S. Rossbach, M. Schneider, P. Ratet, S. Messmer, W. W. Szeto, F. M. Ausubel, and J. Schell.** 1989. *Rhizobium meliloti* 1021 has three differentially regulated loci involved in glutamine

biosynthesis, none of which is essential for symbiotic nitrogen fixation. J. Bacteriol. **171**:1673–1682.

11. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
12. **Elhai, J., and C. P. Wolk.** 1988. A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. Gene **68**:119–138.
13. **Elmorjani, K., S. Liotenberg, J. Houmard, and N. Tandeau de Marsac.** 1992. Molecular characterization of the gene encoding glutamine synthetase in the cyanobacterium *Calothrix* sp. PCC 7601. Biochem. Biophys. Res. Commun. **189**:1296–1302.
14. **Espin, G., S. Moreno, M. Wild, R. Meza, and M. Iaccarino.** 1990. A previously unrecognized glutamine synthetase expressed in *Klebsiella pneumoniae* from the *glnT* locus of *Rhizobioum leguminosarum*. Mol. Gen. Genet. **223**:513–516.
15. **Ferino, F., and F. Chauvat.** 1989. A promoter-probe vector-host system for the cyanobacterium *Synechocystis* PCC 6803. Gene **84**:257–266.
16. **Fisher, R., R. Tuli, and R. Haselkorn.** 1981. A cloned cyanobacterial gene for glutamine synthetase function in *Escherichia coli*, but the enzyme is not adenylylated. Proc. Natl. Acad. Sci. USA **78**:3393–3397.
17. **Florencio, F. J., S. Marqués, and P. Candau.** 1987. Identification and characterization of glutamate dehydrogenase in the unicellular cyanobacterium *Synechocystis* PCC 6803. FEBS Lett. **223**:37–41.
18. **Flores, E., and A. M. Muro-Pastor.** 1990. Mutation and kinetic analysis of basic amino acid transport in the cyanobacterium *Synechocystis* sp. PCC 6803. Arch. Microbiol. **154**:521–527.
19. **Goodman, H. J. K., and D. R. Woods.** 1993. Cloning and nucleotide sequence of the *Butyrivibrio fibrisolvens* gene encoding a type III glutamine synthetase. J. Gen. Microbiol. **139**:1487–1493.
20. **Hill, R. T., J. R. Parker, H. J. K. Goodman, D. T. Jones, and D. R. Woods.** 1989. Molecular analysis of a novel glutamine synthetase of the anaerobe *Bacteroides fragilis*. J. Gen. Microbiol. **135**:3271–3279.
21. **MacKinney, G.** 1941. Absorption of light by chlorophyll solution. J. Biol. Chem. **140**:315–322.
22. **Magasanik, B., and F. C. Neidhardt.** 1987. Regulation of carbon and nitrogen utilization, p. 1318–1325. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger, (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
23. **Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membranes and lipoprotein samples. Anal. Biochem. **87**:206–210.
24. **Marqués, S., F. J. Florencio, and P. Candau.** 1989. Ammonia assimilating enzymes from cyanobacteria: *in situ* and *in vitro* assay using high-performance liquid chromatography. Anal. Biochem. **180**:152–157.
25. **Meeks, J. C., C. P. Wolk, W. Lockau, N. Schilling, P. W. Shaffer, and W.-S. Chien.** 1978. Pathways of assimilation of $[^{13}N]N_2$ and $^{13}NH_4^+$ by cyanobacteria with and without heterocysts. J. Bacteriol. **134**:125–130.
26. **Mérida, A., P. Candau, and F. J. Florencio.** 1991. *In vitro* reactivation of *in vivo* ammonium-inactivated glutamine synthetase from *Synechocystis* sp. PCC 6803. Biochem. Biophys. Res. Commun. **181**:780–786.
27. **Mérida, A., P. Candau, and F. J. Florencio.** 1991. Regulation of glutamine synthetase activity in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 by the nitrogen source: effect of ammonium. J. Bacteriol. **173**:4095–4100.
28. **Mérida, A., E. Flores, and F. J. Florencio.** 1992. Regulation of *Anabaena* sp. strain PCC 7120 glutamine synthetase activity in a *Synechocystis* sp. strain PCC 6803 derivative strain bearing the *Anabaena glnA* gene and a mutated host *glnA* gene. J. Bacteriol. **174**:650–654.
29. **Mérida, A., L. Leurentop, P. Candau, and F. J. Florencio.** 1990. Purification and properties of glutamine synthetase from the cyanobacteria *Synechocystis* sp. strain PCC 6803 and *Calothrix* sp. strain PCC 7601. J. Bacteriol. **172**:4732–4735.
30. **Mohamed, A., and C. Jansson.** 1989. Influence of light on accumulation of photosynthesis-specific transcripts in the cyanobacterium *Synechocystis* 6803. Plant Mol. Biol. **13**:693–700.
31. **Orr, J., and R. Haselkorn.** 1982. Regulation of glutamine synthetase activity and synthesis in free-living and symbiotic *Anabaena* spp. J. Bacteriol. **152**:626–635.
32. **Orr, J., L. M. Keefer, P. Keim, T. D. Nguyen, T. Wellems, R. L. Heinrikson, and R. Haselkorn.** 1981. Purification, physical characterization and NH_2-terminal sequence of glutamine synthetase from the cyanobacterium *Anabaena* 7120. J. Biol. Chem. **256**: 13091–13098.
33. **Pahel, G., D. M. Rothstein, and B. Magasanik.** 1982. Complex *glnA-glnL-glnG* operon of *Escherichia coli*. J. Bacteriol. **268**:469–475.
34. **Pesole, G., M. P. Bozzetti, C. Lanave, G. Preparata, and C. Saccone.** 1991. Glutamine synthetase gene evolution: a good molecular clock. Proc. Natl. Acad. Sci. USA **88**:522–526.
35. **Rawlings, D. E., W. A. Jones, E. G. O'Neil, and D. R. Woods.** 1987. Nucleotide sequence of the glutamine synthetase gene and its controlling region from the acidophilic autotroph *Thiobacillus ferrooxidans*. Gene **53**:211–217.

35a. **Reyes, J. C., and F. J. Florencio.** Unpublished data.

36. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herman, and R. Y. Stanier.** 1979. Genetics assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111**:1–61.
37. **Rossbach, S., J. Schell, and F. J. de Bruijn.** 1988. Cloning and analysis of *Agrobacterium tumefaciens* C58 loci involved in glutamine biosynthesis: neither the *glnA* (GSI) nor the *glnII* (GSII) gene plays a special role in virulence. Mol. Gen. Genet. **212**:38–47.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
40. **Shatters, R. G., Y. Liu, and M. L. Kahn.** 1993. Isolation and characterization of a novel glutamine synthetase from *Rhizobium meliloti*. J. Biol. Chem. **256**:13091–13098.
41. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. **43**:737–755.
42. **Smith, M. W., D.-F. Feng, and R. F. Doolittle.** 1992. Evolution by acquisition: the case for horizontal gene transfers. Trends Biochem. Sci. **17**:489–493.
43. **Southern, J. A., J. R. Parker, and D. R. Woods.** 1987. Novel structure properties and inactivation of glutamine synthetase cloned from *Bacteroides fragilis*. J. Gen. Microbiol. **133**:2437–2446.
44. **Tumer, N. E., S. J. Robinson, and R. Haselkorn.** 1983. Different promoters for the *Anabaena* glutamine synthetase gene during growth using molecular or fixed nitrogen. Nature (London) **306**: 337–342.
45. **Wagner, S. J., S. P. Thomas, R. I. Kaufman, B. T. Nixon, and S. E. Stevens, Jr.** 1993. The *glnA* gene of the cyanobacterium *Agmenellum quadruplicatum* PR-6 is nonessential for ammonium assimilation. J. Bacteriol. **175**:604–612.
46. **Yamashita, M. M., R. J. Almassy, C. A. Janson, D. Cascio, and D. Eisenberg.** 1990. Refined atomic model of glutamine synthetase at 3.5 Å resolution. J. Biol. Chem. **264**:17681–17690.