# 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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Received 13 October 1993/Accepted 29 December 1993

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<sub>2</sub>-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

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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<sub>2</sub> in air. When ammonium was used as the nitrogen source, nitrate was replaced by 10 mM NH<sub>4</sub>Cl and the medium was buffered with 20 mM *N*-tris(hy-

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 Synechocvstis sp. strain PCC 6803 were used for transcriptional gene fusion experiments (8, 15). Other cyanobacterial strains were grown in liquid BG11 medium. E. coli DH5a 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  $\Delta(argF-lac)205$  flbB5301 pstF25 relA1 rspL150  $\Delta(glnG-A \text{ or }$ 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  $\mu$ g 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, lowstringency conditions (55°C,  $5 \times SSC [1 \times SSC is 0.15 M NaCl$ plus 0.015 M sodium citrate]) were used and filters were washed at room temperature. DNA probes were <sup>32</sup>P labelled with a nick translation kit (Boehringer Mannhein) using [ $\alpha$ -<sup>32</sup>P]dCTP.

Construction of a Synechocystis sp. strain PCC 6803 gene library. Genomic DNA from Synechocystis sp. strain PCC 6803 was partially digested with Sau3AI and fractionated by centrifugation through a sucrose gradient (38). The fractions containing fragments of 4 to 6 kbp were pooled and ligated to BamHI-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 ApaI-XbaI fragment from pAM1 (28) was cloned in plasmid pBluescript II SK(+) to generate pJCR3. For mutation of gene glnA, a 340-bp internal EcoRI fragment of glnA was replaced by a 1.3-kb fragment containing a kanamycin resistance gene from Tn5 (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 EcoRI fragment of glnN by the 1.3-kb kanamycin resistance (Km<sup>r</sup>) 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 doublestranded 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- $\mu$ g 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 transformatis 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<sub>4</sub>Cl-50 mM L-glutamate-50 mM MgCl<sub>2</sub>-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 EcoRI fragment of glnA by an npt (Km<sup>T</sup>) gene cassette. (B) Structure of the glnN region in WT Synechocystis sp. strain 6803 and replacement of an internal EcoRI fragment of glnN by an npt (Km<sup>T</sup>) gene cassette. (C) Southern blot of genomic DNAs from the WT and SJCR3 strains. Genomic DNA was digested with XmnI and hybridized by using the 2.66-kb fragment indicated in panel A as a <sup>32</sup>P-labelled probe. (D) Southern blot of genomic DNAs from the WT and SJCR6 strains. Genomic DNA was digested with HindIII and hybridized by using the 2.87-kb fragment indicated in panel B as a <sup>32</sup>P-labelled probe. Restriction site abbreviations: A, ApaI; B, BamHI; E, EcoRI; H, HindIII; S, SaII; X, XmnI. 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

TABLE 1. GS specific activity in WT *Synechocystis* sp. strain PCC 6803 and mutants<sup>a</sup>

	GS sp act <sup>b</sup> with following nitrogen source:							
Strain	Ammonium	Nitrate	Nitrogen starvation <sup>c</sup>					
WT SJCR3 (glnA mutant) SJCR6 (glnN mutant)	$2.3 \pm 1.0 \\ \text{ND}^{d, e} \\ 2.5 \pm 1.0$	$27.3 \pm 2.5 \\ 0.8 \pm 0.3 \\ 26.7 \pm 2.0$	$\begin{array}{r} 36.2 \pm 2.5 \\ 6.6 \pm 1.0 \\ 28.0 \pm 2.0 \end{array}$					

<sup>a</sup> 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<sub>2</sub> in air. Cells were collected for a GS assay at the end of the exponential growth phase and contained approximately 10  $\mu$ g of chlorophyll per ml. The data shown are means of three independent experiments  $\pm$  the standard errors.

<sup>b</sup> 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.

<sup>c</sup> 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.

<sup>d</sup> 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 nitratecontaining medium to ammonium-containing medium.

e ND, not detected.

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.

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.

30	GTATCTATATTGTCTATTTTAAAAAATCATCTTGCGTATGATTGGGGGGGTTTGTAAATTCTGAAATAGAGATTTAGTCC									TCC										
1	Met	Thr	Gly	Asn	Ala	Ala	Arg	Thr	Gln	Ser	Val	His	Gln	Ile	Ile	Asn	Arg	Pro	Leu	Ser
109	ATG	ACT	GGA	AAC	GCC	GCC	CGC	ACC	CAA	TCA	GTT	CAC	CAA	ATC	ATC	AAT	CGA	CCT	CTA	TCT
169	TCT	GGC	AAG	AAG	TTA	TCC	CGC	CTG	GAA	GAT	ATG	TGG	GCT	GAG	ASD	GTT	TTT	ASD	TTG	AGC
41	Lys	Met	Gln	Ala	Ser	Leu	Pro	Lys	Gly	Val	Phe	Lys	Ser	Ile	Lys	Asn	Thr	Ile	Thr	Thr
229	AĀA	ATG	CAG	GCC	AGT	CTT	CCT	AÃA	GGC	GTT	TTC	AĀA	TCA	ATT	AÃA	AAT	ACT	ATT	ACC	ACT
61	Gly	Glu	Lys	Leu	Asp	Pro	Ser	Val	Ala	Asp	Ala	Val	Ala	Thr	Ala	Met	Arg	Asp	Trp	Ala
289	Met	GIV	LVS	Glv	Ala	Met	Tvr	TVr	Ala	His	Ual Val	Pho	TVr	Pro	Mot	Thr	Asn	LOU	Sor	GCC Ala
349	ATG	GGG	AAA	GGG	GCA	ATG	TAC	TAT	GCC	CAC	GTT	TTC	TAT	CCC	ATG	ACC	AAC	CTC	TCG	GCG
101	Glu	Arg	His	Asp	Gly	Phe	Ile	Ser	Val	Gln	Gly	Asp	Gly	Asn	Val	Ile	Ser	Glu	Phe	Ser
409	GAA	AGG	CAC	GAT	GGC	TTT	ATT	TCT	GTA	CAG	GGG	GAT	GGC	AAT	GTC	ATT	TCA	GAG	TTT	TCC
469	GCC	LYS	GTT	CTA	GTA	CAA	GLA	GIU	Pro	GAC	GLA	Ser	Ser	TTT	Pro	ASD	GLY	GLY	11e	Arg
141	Asp	Thr	Phe	Glu	Ala	Arg	Gly	Tyr	Thr	Gly	Trp	Asp	Val	Thr	Ser	Pro	Ala	Tyr	Ile	Met
529	GAT	ACC	TTT	GAG	GCT	AGĞ	GGC	TÂC	ACG	GGÂ	TGG	GAC	GTA	ACC	AGT	CCT	GCC	TÁC	ATT	ATG
161	Glu	Thr	Asp	Asn	Gly	Ser	Thr	Leu	Cys	Ile	Pro	Thr	Val	Phe	Val	Ser	Trp	Thr	Gly	Glu
191	GAA Ala	ACG	GAT Aco	LVC	GGT	Val	ACC	LON	TGT	ATT	Ser	ACG	GIG Ala	Ala	Mot	Aco	TGG	ACA	GGG	GAA
649	GCG	TTG	GAT	AAA	AAA	GTG	ccc	CTA	TTG	CGG	TCC	ATT	GCG	GCC	ATG	GAT	AAG	GCG	GCC	CGC
201	Lys	Val	Leu	Ser	Leu	Leu	Gly	Asn	Glu	Asp	Ile	Ala	His	Val	Asn	Ser	Ser	Cys	Gly	Ala
709	AAG	GTG	CTC	AGT	TTG	TTG	GGT	AAC	GAA	GAC	ATT	GCC	CAC	GTT	AAT	TCT	AGT	TGT	GGG	GCG
769	ASP	GIU	GIU	TYT	TTT	TTC	CTC	ASP	ALA	ASD	Phe	ALA	Ser	GIN	Arg	Pro	Asp	Leu	Leu	Leu
241	Ala	Glv	Arg	Thr	Leu	Phe	Glv	Lvs	Leu	Pro	Ala	Lvs	Glv	Gln	Glu	Phe	Asp	Asp	His	Tvr
829	GCC	GGA	CGG	ACT	TTG	TTT	GGC	AAG	CTC	CCG	GCT	AAG	GGT	CAG	GAA	TTC	GAT	GAC	CAT	TAT
261	Phe	Gly	Ala	Ile	Pro	Glu	Arg	Val	Gln	Val	Phe	Met	Gln	Asp	Val	Glu	Glu	Thr	Leu	Tyr
889	TTT	GGC	GCT	ATT	CCT	GAA	CGG	GTG	CAG	GTG	TTC	ATG	CAG	GAC	GTG	GAG	GAG	ACC	CTT	TAT
949	AAG	TTG	GGT	ATT	CCA	GCT	AAA	ACT	CGC	CAT	AAT	GAA	GTG	GCT	CCC	GGC	CAG	TTC	GAA	ATT
301	Ala	Pro	Phe	Phe	Glu	Ala	Ala	Asn	Val	Ala	Ser	Asp	His	Gln	Gln	Leu	Leu	Met	Thr	Val
1009	GCG	ccc	TTT	TTT	GAA	GCG	GCT	AAC	GTG	GCC	AGT	GAC	CAC	CAA	CAA	TTG	TTA	ATG	ACG	GTA
321	Leu	Lys	Asn	Thr	Ala	Lys	Lys	His	Gly	Phe	Val	Cys	Leu	Leu	His	Glu	Lys	Pro	Phe	Ala
341	Glv	Ile	Asn	Glv	Ser	Glv	Lvs	His	Val	Asn	Tro	Ser	Val	Glv	Asn	Ser	Thr	Gln	Glv	Asn
1129	GGC	ATC	AAC	GGT	TCC	GGT	AAG	CAC	GTT	AAC	TGG	TCA	GTG	GGT	AAC	TCC	ACC	CAG	GGT	AAT
361	Leu	Leu	Asp	Pro	Gly	Asp	Ser	Pro	His	Asp	Asn	Ala	Gln	Phe	Leu	Val	Phe	Cys	Gly	Ala
1189	TTG	CTT	GAT	CCC	GGC	GAT	TCT	CCC	CAC	GAT	AAT	GCC	CAA	TTC	CTC	GTT	TTT	TGC	GGT	GCA
1249	GTG	ATT	CGG	GGA	GTG	CAC	AAA	TAC	GGC	CCT	CTG	ATG	CGG	GCG	GCG	ATC	GCC	ACG	GCT	AGT
401	Asn	Asp	His	Arg	Leu	Gly	Ala	Asn	Glu	Ala	Pro	Pro	Ala	Ile	Met	Ser	Val	Tyr	Leu	Gly
1309	AAT	GAC	CAC	CGT	TTG	GGG	GCC	AAC	GAA	GCT	CCC	CCG	GCC	ATT	ATG	TCC	GTT	TĂT	CTG	GGT
421	Thr	GIN	Leu	GIU	GIU	Val	Phe	GIU	GIN	11e	Lys	Thr	GLY	Ser	Val	Lys	Asp	Ser	Lys	Lys
441	Lvs	Glv	Val	Met	Asp	Leu	Glv	Val	Asp	Val	Leu	Pro	Asp	Leu	Thr	LVS	Aso	Ala	Glv	AAA Asp
1429	AAA	GGC	GTG	ATG	GAT	CTG	GGÂ	GTT	GAT	GTA	CTT	CCA	GAT	TTA	ACC	AAG	GAC	GCT	GGC	GAT
461	Arg	Asn	Arg	Thr	Ser	Pro	Phe	Ala	Phe	Thr	Gly	Asn	Arg	Phe	Glu	Phe	Arg	Ala	Val	Gly
1489	CGA	AAC	CGT	ACT	TCT Vol	200	TTT	GCC	TTC	ACT	GGT	AAC	CGT	TTT	GAA	TTC	CGA	GCA	GTG	GGC
1549	TCC	AGT	CAG	TCG	GTT	TCT	GGT	CCG	CTA	ATT	GTG	CTG	AAC	ACC	ATG	CTG	GCT	GAC	TCC	CTT
501	Asn	Trp	Ile	Gly	Asp	Arg	Leu	Glu	Ala	Glu	Leu	Ala	Lys	Gly	Leu	Asp	Leu	Asp	Thr	Ala
1609	AAC	TGG	ATT	GGC	GAT	CGT	CTG	GAG	GCG	GAG	TTG	GCC	AAG	GGA	TTG	GAC	TTG	GAC	ACT	GCC
1669	11e	Leu	ACC	CTC	Leu	Lys	GIU	11e	Met	GIU	ASD	HIS	GIY	GIN	Val	11e	Phe	GIY	GLY	Asn
541	Glv	Tvr	Ser	Glu	Glu	Trp	His	Lvs	Met	Ala	Val	Glu	Glu	Arg	Glv	Leu	Ala	Asn	Leu	Ara
1729	GGC	TAT	TCC	GAA	GAA	TGG	CAT	AAA	ATG	GCA	GTG	GAA	GAA	CGG	GGT	TTG	GCC	AAC	TTG	CGT
561	Thr	Thr	Ala	Asp	Ala	Leu	Pro	Val	Leu	Lys	Glu	Lys	Tyr	Ile	Glu	Asp	Leu	Phe	Glu	Lys
501	ACT	ACT	GCC	GAT	GCT	TTG	CCG	GIG	CIG	AAG	GAG	AAG	TAT	ATC	GAA	GAC	CTA	TTT	GAA	AAA
1849	ACT	GGT	GTA	TTG	ACT	CCA	GTG	GAG	TTG	GAA	AGC	CGC	TTT	GAA	GTC	TAC	GCT	GAG	CAG	TAC
601	Ile	Leu	Ser	Ile	Glu	Val	Glu	Ala	Lys	Leu	Val	Val	Ser	Met	Ala	Lys	Thr	Val	Ile	Tyr
1909	ATC	CTC	TCC	ATT	GAA	GTG	GAA	GCC	AĀA	CTG	GTG	GTC	AGC	ATG	GCC	AAA	ACG	GTC	ATT	TAT
621 1969	Pro	ALA	ALA	val	GIU	Tyr	Leu	Ser	LYS	Leu	Ser	Ser	Thr	Ile	Ala	Ser	Leu	Ser	Gly	Leu
641	Glv	Ile	Asp	Phe	Glu	Lvs	Glu	Ser	AnA	Lvs	Lvs	Ile	ALC	ALL	Len	Thr	Asp	Glp	Met	Val
2029	GGC	ATT	GAT	TTT	GAA	ÂÂG	GAA	AGT	GCC	AAA	AAG	ATC	GCT	GAT	TTG	ACC	AAC	CAA	ATG	GTC
661	Gly	Arg	Val	Ala	Lys	Leu	Ser	Glu	Ala	Met	Ala	Lys	His	Asp	Phe	Ala	Asn	Thr	Glu	Glu
2089	GGT	CGT	GTC	GCC	AAA	CTA	AGT	GAA	GCT	ATG	GCT	AAA	CAC	GAC	TTT	GCC	AAC	ACC	GAA	GAG
2149	AAG	TTG	CAG	TAT	TGC	GCC	CAA	ACT	CTC	CGT	CCC	CTG	ATC	GAT	GAA	GTA	CGG	ACT	TTT	GCC
701	Asp	Ala	Leu	Glu	Gly	Glu	Ile	Ala	Asp	Ser	Phe	Trp	Pro	Leu	Pro	Thr	Tyr	Gln	Glu	Met
2209	GAT	GCC	CTG	GAA	GGG	GAA	ATT	GCC	GAT	AGT	TTC	TGG	ccc	CTG	CCT	ACC	TAC	CAG	GAA	ATG
721	Leu	Phe	Ile	Lys	STO	TCC		~~~~				~~~ ~	ACC	а т.с. т.		amc.cr	man			000
2209	CAAC	GCTT	TTGG/	AAGT	TTGGG	GGGG	-1410	ACCI	CAG	20004	-1996	JINA	-ACCI	-ich	LUCA	NIGC.	1999(	JIAA	4GTC0	LULT

GGGAGGCAAAGAAATGGGTCGTTGTTTT

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.

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 EcoRI 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

в.	fragilis	MSKMRFFA	LQELSNRKPL	EITTPSNKLS	DYYASHVFDR	KKNOEYLPKE	AYKAVVDATE
8yı	echocystis	MTGNAARTOS	VHQIINR.PL	SSGKKLSRLE	DMWAENVFNL	SKMQASLPKG	VFKSIKNTIT
в.	fibrisolvens			MIEASKLT	TEFGSLVFND	KIMKERLPKD	I YKAVHKTIE
	(59)	KGTPISREMA	DITANGMESH	AKSLNUTHYT	HW. FORLTDG	TARKHDGFIR	FGEDG
	(60)	TGEKLOPSVA	DAVATAMEDW	AMGKGAMYYA	HV. FYPMTNL	SAERHDGFIS	VOGDG
	(39)	KEPHLEPGCS	YSCSSNHEGV	GNREOCYBET	PPGSSPMTGL	TAEKHDSFIS	PTEDGRSSWS
	(/						
	(113)	. EVIERFSGK	LLIQUEPDAS	SFPNGGIRNT	FEARGYTAWD	GSSPAFVVDT	TLCIPT
	(114)	. NVISEFSGK	VLVQCEPDGS	SFPNGGIRDT	FEARGYTGWD	VTSPAYIMET	DNGSTLCIPT
	(99)	SQEKSWLRAN	LMHQASOWWS	SCHNSSM	RGYQHGI	LHHH <b>AFIKD</b> G	SLLLPT
				I			
	(168)	IFISYTCEAL	DYKTPLLKAL	AAVDRAATEV	COLFDK.NIT	RVFTNLGWEQ	EYFLVDTSLY
	(173)	VFVSWTGEAL	DKKVPLLRSI	AAMDKAARKV	LSLLGNEDIA	HVNSSCGADE	EYFLVDANFA
	(149)	AFCSYGGEAL	D. RDSLLRSM	EALSNEAVEM	MRLLGYEDVN	RVNTTIGSEQ	EYFLIDKDFY
	(227)	NARPDLRLTG	RTLMGHSSAK	DOOLEDHYFG	SIPPRVTAFM	KELEIECHKL	GIPVKTRHNE
	(233)	SQRPDLLLAG	RTLFCKLPAK	<b>GOR</b> FD <b>DHYFG</b>	AIPERVQVFM	<b>ODVEETLYKL</b>	GIPAKTRHNE
	(208)	KKRKDLLLTG	RTLIGAPASK	GOEMEDHYFG	VIRPKVSAYM	HOLDEELWKL	GIPAKTKHNE
							II
	(287)	VAPNOFELAP	IFENCHLAND	HNOLVMDLMK	RIARKHHFAV	LFHERPYNGV	NGSGKHNNWS
	(293)	VAPGOFEIAP	FFEAANVASD	HOOLIMIVLK	NTAKKHGFVC	LLHERPFAGI	NGSGKHVNWS
	(268)	VAPSQHELAP	VFETANIAVD	HNQLTMEVMK	KVADKHNYAC	LLHERPFEGV	NGSGKHNNWS
		II				II	I
	(347)	LCTDTGINLF	APGKNPKGNM	LFLTFLVNVL	MMVHKNQDLL	<b>RASIMSAGN</b> S	ERLGANEAPP
	(353)	VGNSTQGNLL	DPGDSPHDNA	<b>OFLVF</b> CGAVI	RGVHKYGPLM	RAAIATASND	HRLGANEAPP
	(328)	ICTDIGINLL	DPGKNPGENI	PFLVFLMSVI	AAVDEYAPIL	RLSVASAGND	HRLGGNEAPP
	(407)	AILSIFLGSO	LSATLDEIVR	<b>OVTNSKMTPE</b>	EKTTLKLGIG	RIPEILLDTT	DRNRTSPFAF
	(413)	AIMSVYLGTQ	LEEVFEQI	.KTGSVKDSK	KKGVMDLGVD	VLPDLTKDAG	DRNRTSPFAF
	(388)	AIISIFVGDE	LAEVLKAV	. EAGEAYKAA	GKSQMTWEQQ	YFT.FTKDNT	DRNRTSPFAF
							IV
	(467)	TGNRFEFRAA	GSSANCAAAM	IAIMAAMANQ	LNEFKASVDK	LMEEGIGKDE	AIFRILKENI
	(470)	TGNRFEFRAV	GSSQSVSGPL	IVLNTMLADS	LNWIGDRLEA	ELAKGLDLDT	AILTVLKEIM
	(444)	TGNKFESDGG	HSSVA.NGRY	GPOHMOLOKE	VATLNAKLSA	YSGDELKE	KVKEVLKETL
	150.51	V NORT TOP	DOVORTH TO				
	(526)	TASELIRFEG	DGISEEW.KQ	EAARRGLINI	CHVPEALMHY	MDNQSRAVLI	GERIFNETEL
	(530)	ENHGQVIFGG	NGYSEEWHKM	AVEERGLANL	RTTADALPVL	KEKYTEDLITE	KTGVLTPVEL
	(501)	LAHKRVLPNG	NGYTDEWVE.	EAAKRGLPNL	KALPDCMPYW	ISDESIDLET	RHGIFTKEEI
	(500)						
	(586)	ACREEVELER	TIMEVQUESE	VLGDLAINHL	VPIAVSIONR	LLENLCRMRE	IFSELETEVM
	(590)	ESRPEVIALO	TILSIEVEAK	LVVSBARTVI	TPAAVETLSK	LSSTIASLSG	L. GIDPERE
	(560)	ISRIELLEN	ISKSIHIESL	TMOEMIRKDL	TEGLVAIERD	LSKEIVQKKS	LDGDC
	1646)		TOURNON TWO				BYL BOLDDUL
	(040)	CAUKALLIKE	TTNONUCEUR	LVRUMIEARE	VANNALNFKE	NAPAILLIVK	DIMORUDITES
	(048)	CALELOUITO	TOKSCARMON	A DELAMAKHD	FANILERLQ.	TACYVECTUR	F DEDEVRIPA
	(010)	CHILLIGVIKS	DESSALMGE	ABOADTEDIK	NACONICALE	TROTIESTVL	ADRUGLANIA
	(706)			T.PTE (720)			
	(701)	DALEGEIADE	FNDI.DTV/PM	TETE (724)			
	(676)	DEABALTOEV	VISVOTVCEN	TESTO (700)			
	(0/0)	our and IF ER	17019110004				

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.

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<sup>r</sup>) cassette. Cm<sup>r</sup> Km<sup>r</sup> colonies were obtained in BG11 medium and cultured for several rounds of segregation in the same nitratecontaining 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

Dimays	(100)	ID CODING AND INTERIAR II NOT INCOMMENT CONTINUE (147)	
P.sativum	(55)	Y <b>DGSS</b> TNQAPG.KDS <b>E</b> VILYPQAIFKD <b>P</b> FRRGNNIL <b>VIC</b> DVYTPA (98)	
H.sapiens	(62)	F <b>DGSS</b> TLQSEG.SNSDMYLVPAAMFRDPFRKDPNKL <b>VLC</b> EVFKYN (105)	
B.japonicum GSII	(43)	FDGSSTQQAEG.HSSDCVLKPVAVFPDAAR.TNGVLVMCEVMMPD (85)	
B.fragilis	(128)	P <b>DASS</b> FPNGGIRNTF <b>BA</b> RGYTAWDGSS <b>P</b> AFVVDTTLCIPTIFI (170)	
Synechocystis GSII	I(129)	PDGSSFPNGGIRDTFEARGYTGWDVTSPAYIMETDNGSTLCIPTVFV (175)	
B.fibrisolvens	(115)	OWWSSCHNSSMBGYOHG(LHHHAFIKDGSLLLPTAFC (151)	
B.cereus	(52)	FDGSSIEGFVRIEESDMYLYPDLDTWVIFPWTAEKGKVARLICDIYNAD (100)	
C.acetobutvricum	(52)	FDGSSIDGEVRIEESDMNLRPNLDSEVIEPWRPOOGKVARLICDVYKPD (100)	
M.voltae	(58)	FDGSSTEGEVETEDSDMVLKPDLSTLSVLPWEPSEKSVARTICDVYRKN (106)	
Anabaena 7120	(50)	FDGSSIEGWKAINESDMTMVLDPNTAWIDPFME. VPTLSTVCSIKEPB (96)	
S.coelicolor	(49)	FDGSSIRGFOATHESDMSLRPDLSTARVDPFRR. DKTLNINFFTHDPT (95)	
F coli	(49)	FDGSSIGGWKGINESDMVIMEDASTAVIDEFFA DSTLITECDILEEG (95)	
S solfataricus	(53)	IDGSSIRGETSIYESDMULLPVPFTMTLIPWSP GVA RVLCKVFWGG (98)	
5.5011ucui icus	(00)		
7 mays	(233)	ACTNICCTNCRUM PCOWRYOUCPSUCTFACTHINISRYTIPRIT FOACUUTIDERIOG DWAGACHTNYST	(305)
D estivum	(192)	ACTIVISCINCE WILL COMPENSION CONTRACT AND A CONTRAC	(254)
H capiene	(186)	ACTAINCE THE THE THE THE ACTAIN AND A THE ACTAIN AND A THE ACTAINST AN	(258)
B japonioum CCII	(160)	A CALLECTARE CALL CALLECTER CONCERNMENT AND THE DESCRIPTION AT THE CALLECTARE AND CALLECTARE AND A CALLECTAR	(235)
B. japonicum GSII	(102)	AGINGEGINASVA. KEYASIYI GKOSKKAAD DAMMAKIDIKLI. DKIGIDERKKI DODINA OSAMAANISI	(233)
Superboundie CETT	1 ( 2 9 2 )	LOTPARTING VA. FRYE BLAF IF ENCHLAND MULTUD MARTAK. RHEFAVLF HERF INGV NGS GRANNES L	(357)
P fibricalyana	(252)	Le TEANING VA. FEYER THE FEARWASD RYCHEMEVAN VER VAR VAL THE AND A CONSTRUCTION OF A	(328)
P. corous	(170)	LOIPARINGNA, FSYNSLAF YFEIANIAYDANULIMEYMRAVAD, RAWIACLEBERFFEGY, AGGAMANNSI MCFEFRYUNDWA BCAUFINEWYNNATSCONTATEVIWWRTAD KUGIUATEMBWBIYGY WGCAMAGI	(250)
C. sectobuturiour	(179)	MORELEASHIBVA.FGQHBIDERIANAIKSCDDIQIERLVVIIAA.KHGLHAIFHERELIGVRGSGHBCHLSL	(230)
C.acecobucyricum	(100)	MOTE LEASTING VALEGYND I DER IDER IDER IDER INDER INTER VALE VALE VALE VALE VALE VALE VALE VALE	(29)
M.VOILae	(182)	LGUN TRAUMENA.PGQHEVDERIUNAVRIADSVIIERIIIRILAR.QGGVLAIEMEREFEGN.RGSGMECNQSI	(233)
Anabaena /120	(205)	LEVPIERNHHEVAIGEQUELEGEREGELIEADWLHIINIVIRNVAR.RIGHIVIEMEREIGEDRESEMECHUSI	(277)
S.COULCOID	(200)	SG LVURAUMANAGA AG AN AMANANANA KUKANA AN	(272)
E.COII	(202)	MGLVVEAHHEVAIAGQNEVAIRINIMIKADEIQIIRIVVNNVKRIGKIAIFMIKEMIGD	(273)
5.Sollataricus	(199)	ELITEATHHAVATUUVETUUREVUTUREVUTUREVATURAANUEUVVIEME <u>KETUU</u> . <u>BULUMA</u> INLAL * * * * * * * * * *	(2/1)
7	(350)	VANDCCSTDWCD DTEAKC KCVTEDBDDASNMD DYTWTCLL (398)	
P estivum	(308)	VANDOSSTRVCP DEFEND KGYPENDERASNMD DYVVTSDI (347)	
H saniens	(316)	VANDSASTRTDP TVCOPK KCYPEDBRSANCD DESUTEI (355)	
B isponioum CETT	(200)	VARAGAGENTEL FOUNDE VERVENTER BERNEGOD BYGTERE (330)	
B fragilie	(457)		
Supechocyctic CSIT	T (460)	DENERSE AFTER NOT NOT THE DESCRIPTION $(495)$	
B fibricolvene	(435)	DONDERSPRETC N KERSCHESSVAN CKYCOHMA (458)	
B corolle	(313)	ANNE OF UPTA SECTOR RUBURGADAN PYLUMATI (350)	
C acetobutyricum	(312)	CENTRAL TRUDA ARGACE RUE RUE CONSENS DE LA CONSENSE (349)	
M voltae	(316)	New FRITE TODA ADCKCT DURGEDE SON E LETERS (377)	
Anabaana 7120	(330)	ACING AST DT SCHNOKAL RIEP CONTEN DY IFAM (378)	
S coelicolor	(335)	OPWORANDELT CONDENS REAL REAL REAL REAL REAL REAL (374)	
E coli	(333)	VANASAAMAALI.ISSALKAK.KTBEKALUASSAEIDAISAD (377)	
S colfatarious	(337)	ARANGASARATAT STATE ARAN ARA BARATATA ARANGASARATA ARANGASARATA (377)	
5.SUIId(dIICUS	(337)	NO <u>HA</u> SY <u>A</u> A <u>T</u> EATINOMBAAK, K <u>I</u> IGEEDESIK <u>E</u> IDAEAAD (SII) *	

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.

Α

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 HindIII-AvaII 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 SFC $\Omega$ 5 strain, generating strains SJCR15(+) and SJCR15(-), which were chloramphenicol sensitive (Cm<sup>s</sup>) 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<sup>r</sup>) and SFF16 (Cm<sup>s</sup>) 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-exponentialphase WT *Synechocystis* cells grown on nitrate or ammonium

			A	н И		
	500 bp	→				
в				CAT	activity (mU/r	ma prot)
-					Nitrogen sourc	e
Plasmid		Construct	Strain	Ammonium	Nitrate	N starvation
pFF11		t cat	SFF16	nd	n.d.	nd.
pFC57		cat promoter	SFC57	1120 ± 85	1100 ± 90	1200 ± 97
pSJCR15	(+)	gInN promoter	SJCR15	n.d.	1.2 ± 0.1	92 ± 12
pSJCR15	(-)	gInN promoter	SJCR16	nd.	n.d.	n.d.

FIG. 5. CAT activity of transcriptional fusions between the 5'upstream glnN region and the cat gene. (A) The 700-bp HindIII-AvaII 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, AvaII; H, HindIII. 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.

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 EcoRI-EcoRI 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 EcoRI 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



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 *Hind*III, except for the DNAs from *Synechococcus* sp. strain PCC 7202 and *Synechocystis* sp. strain PCC 6308, which were digested with *DraI*. For *Synechocystis* sp. strain PCC 6803 and *Synechocystis* sp. strain PCC 6714,  $0.4 \mu g$  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.

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  $\beta$ -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<sup>2+</sup> 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 grampositive 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.

#### ACKNOWLEDGMENTS

We thank A. Vioque, P. Candau, S. Chávez, J. Casadessús, J. M. Lora, and M. García Guerrero for advice and helpful discussions and M. I. Muro-Pastor for construction of the *Synechocystis* gene library. The contribution to the earlier results of the students of the 1990–1991 Advanced Biochemistry course is also acknowledged.

This work was supported by grants from DGICYT (PB91-0127) (Spain) and by the Junta de Andalucía.

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