JOURNAL OF BACTERIOLOGY, Feb. 1993, p. 604–612
0021-9193/93/030604-09$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 175, No. 3

The *glnA* Gene of the Cyanobacterium *Agmenellum quadruplicatum* PR-6 Is Nonessential for Ammonium Assimilation

STEPHEN J. WAGNER,[1]† SELWIN P. THOMAS,[2] R. ILENE KAUFMAN,[1] B. TRACY NIXON,[1] AND S. EDWARD STEVENS, JR.[2]*

Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802,[1] and Department of Biology, Memphis State University, Memphis, Tennessee 38152[2]

Received 8 July 1992/Accepted 18 November 1992

The *glnA* gene of the cyanobacterium *Agmenellum quadruplicatum* PR-6 (*Synechococcus* sp. strain PCC 7002) was isolated by complementing an *Escherichia coli* strain auxotrophic for glutamine (YMC11) with a PR-6 cosmid library. PR-6 *glnA* is a single-copy gene that encodes a deduced amino acid sequence that is highly homologous to the deduced *glnA* amino acid sequences reported for other bacteria. No homology was found between the PR-6 *glnA* flanking sequences and the *ntrB*, *ntrC*, or *glnB* genes of other bacteria. Northern (RNA) and primer extension analyses of PR-6 RNA revealed one predominant and several minor *glnA* transcripts of about 1.5 to 1.7 kb. The steady-state amounts of these transcripts increased three- to fivefold when the cells were starved for nitrogen. However, we found that mutant PR-6 cells lacking *glnA* were still able to use nitrate or ammonium as a sole nitrogen source. Although no RNA homologous to an internal fragment of the *glnA* gene could be detected in the mutant cells, they retained about 60% of wild-type glutamine biosynthetic activity. The mutant cells were more sensitive than the wild-type cells to methionine sulfoximine, a transition state analog of glutamate, a result that might indicate the presence of an additional glutamine synthetase; however, cell extracts of wild-type PR-6 cells and those lacking *glnA* were both able to use carbamyl phosphate instead of ammonium as a nitrogen donor for the synthesis of glutamine, a result that indicates the use of carbamyl phosphate synthetase to assimilate ammonium and produce glutamine.

The cyclic conversion of glutamate to glutamine, catalyzed by glutamate synthase (glutamine-oxoglutarate aminotransferase; EC 2.6.1.53) and glutamine synthetase (GS; L-glutamate-ammonia ligase [ADP forming]; EC 6.3.1.2), plays an essential role in bacterial nitrogen assimilation (38). In addition to the well-known role that ammonium assimilation plays in the intermediate metabolism of heterotrophs, ammonium assimilation in cyanobacteria and other photoautotrophs can also be intimately related to the formation of bilins found in the light-harvesting proteins of the phycobilisomes, to the synthesis of chlorophyll found in the photosystem I and II reaction centers, and to the production of hemes found in the cytochromes involved in electron transport. This is because photoautotrophs, unlike heterotrophs and mammalian cells, frequently use glutamate rather than glycine and succinate as the precursor of δ-aminolevulinic acid, the metabolite from which chlorophylls, sirohemes, vitamin B_{12}, hemes, and bilins are derived (13, 14).

In enteric bacteria, the *glnA* gene product, GS, constitutes all GS activity. The two *glnA* promoters are controlled by a nitrogen regulatory system (*ntr*) that also regulates numerous other genes involved in nitrogen metabolism (38). One of the two *glnA* promoters resembles the general bacterial consensus promoter and is activated by the catabolite-activating protein and repressed by the *ntrC* gene product (46). The other *glnA* promoter utilizes the alternate sigma factor σ^{54} (*rpoN*; also called *ntrA* and *glnF*). RNA polymerase containing σ^{54} binds to and promotes transcription from a sequence (*ntr* promoter) −12 to −24 bp upstream from the transcription start site (22, 30, 32, 46). Activation of this promoter also requires activated *ntrC* gene product (32). The transcriptional regulation of *glnA* by the *ntr* system works in conjunction with the posttranslational regulation of GS by reversible adenylylation (38).

The regulation of GS in enteric bacteria and in members of the family *Rhizobiaceae* is similar in that each includes a dual-promoter system that utilizes a general consensus promoter and an *ntr*-regulated promoter. However, in *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* species, this dual transcriptional regulation is partitioned between two separate *gln* genes: (i) a constitutively expressed *glnA* gene that encodes an enzyme similar in structure to the GS of enteric bacteria (11, 12, 19, 24, 50, 55), and (ii) an *ntr*-regulated *glnII* gene that encodes an enzyme, GS2, similar in structure to the GSs of higher organisms, such as plants (10, 11, 19, 24, 41, 49, 50). The two isoforms of GS in rhizobia differ in their molecular mass, subunit interactions, regulation, and heat lability (16, 26, 27, 37, 51). Recently, a third locus, *glnT*, was identified. *glnT* encodes an *ntr*-regulated operon in *Rhizobium meliloti* that is capable of complementing an *Escherichia coli* glutamine auxotroph. None of the three *gln* loci, by themselves, are essential for growth in *R. meliloti* (19).

Glutamate dehydrogenase (GDH) is another potential ammonium-assimilating enzyme that is ordinarily active at high external ammonium concentrations (47). The enzyme catalyzes the formation of glutamate by NADPH-dependent reductive amination of 2-oxoglutarate (47). The gene for GDH (47), which has been designated *gdh* (or *gdhA*), *gdhD*, and *gdhA* in *E. coli*, *Klebsiella aerogenes*, and *Salmonella*

* Corresponding author.
† Present address: The Jerome H. Holland Laboratory, American Red Cross, Rockville, MD 20855.

TABLE 1. Strains, bacteriophages, and plasmids used in this study

Strain, bacteriophage, or plasmid	Relevant characteristics or genotype	Source or reference
Strains		
Escherichia coli		
HB101	*recA hsdR hsdM pro leu lacZ supE*	6
YMC11	*thi-1 endA hsdR* Δ(*glnA-ntrC*)	3
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15*	69
C600	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44*	1
Agmenellum quadruplicatum		
PR-6	Wild type	60
PR-6102	Kan^r *Bam*HI-*Bgl*II *glnA*	This work
Bacteriophages		
λ467	*b221 cI857 Oam Ppm rex*::Tn*5*	4
M13mp18		69
M13mp19		69
Plasmids		
pHC79	Amp^r Tet^r *cos*	31
pSWA3	Amp^r Tet^r *cos glnA*$^+$ cosmid	This work
pSW177	Amp^r Tet^r *cos glnA*::Tn*5* B20	This work
pSW210	pSWA3 *Eco*RI subclone; *gln*$^+$	This work
pUC4K	Amp^r Kan^r	62
pFB682	Amp^r *R. meliloti glnA* region in pBR322	19
pFB691	Tet^r *R. meliloti glnII* region in pBR322	19
pFB6162	Cam^r *R. meliloti glnT* region in pACYC184	19
pBJ196A	Tet^r *B. japonicum glnII* region in pBR322	12
M13 RF1.2	1.2-kb *Eco*RI fragment (*glnA* upstream region) cloned into mp19	This work
M13 RF2.1	2.1-kb *Eco*RI fragment (*glnA* downstream region) cloned into mp19	This work
pIK1	Amp^r Kan^r Tn*903-kan* replacing the *Bam*HI-*Bgl*II fragment of the 3.9-kb *glnA* region of PR-6 cloned into pUC19 (see Fig. 3)	This work

typhimurium, respectively, has been characterized in these enteric bacteria but, to our knowledge, not in any cyanobacterium.

We recently isolated the *glnA* gene from the cyanobacterium *Agmenellum quadruplicatum* PR-6 by complementation of *E. coli* YMC11 with a PR-6 cosmid library. We report that PR-6 *glnA* is a single-copy gene whose transcripts are up-regulated during nitrogen starvation; however, the *glnA* gene is not essential for growth on either nitrate or ammonium as a sole nitrogen source.

(Preliminary aspects of some of this work have been described by Wagner et al. [64]).

MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, media, and antibiotics. The bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in LB medium (39), 2× medium TY (tryptone and yeast extract) buffered with 100 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) (pH 6.5), or minimal M63 medium (5). YMC11 was grown in LB medium supplemented with 0.2% glutamine. PR-6 cultures were grown in A$^-$ medium (56) supplemented with 0.1% sodium nitrate or 0.03% ammonium chloride in the presence of 10 to 100 microeinsteins of light emitted from Sylvania F96T12 cool-white fluorescent bulbs. Liquid cultures were incubated at 39°C and bubbled with a 0.5% carbon dioxide-in-air mixture. For nitrogen starvation assays, cultures grown to an optical density at 600 nm of 0.8 were collected by centrifugation, resuspended in A$^-$ medium, and incubated with CO_2 and light for various times. Plate cultures were incubated at 30 to 32°C in air. Antibiotics were added at the following final concentrations: ampicillin, 50 μg/ml for *E. coli* and 2 μg/ml for PR-6; tetracycline, 10 μg/ml for *E. coli*; kanamycin, 50 μg/ml for *E. coli* and 200 μg/ml for PR-6; and streptomycin and spectinomycin, 100 μg/ml for *E. coli* and 25 μg/ml for PR-6.

Plasmid DNA isolation. Most plasmids were isolated by alkaline lysis. Cosmids were amplified with chloramphenicol prior to extraction by boiling (39).

Isolation of genomic DNA and cellular RNA. Large preparations of *A. quadruplicatum* PR-6 genomic DNA were isolated as described by de Lorimier et al. (21). Small preparations of PR-6 and *E. coli* genomic DNAs were isolated as described by Buzby (8). RNA was isolated as described by Golden et al. (28).

PR-6 genomic library construction. PR-6 genomic DNA fragments of 30 to 40 kb were generated by partial *Eco*RI digestion, purified, and ligated to vector pHC79 as described by Meyerowitz et al. (42). Bacteriophage lambda packaging extracts were purchased from Stratagene (La Jolla, Calif.) and used in accordance with the specifications suggested by the manufacturer.

Tn*5* mutagenesis. Tn*5*-mediated plasmid mutagenesis was carried out as described by de Bruijn and Lupski (18).

DNA sequencing and primer extension analysis. The preparation of nested DNA deletions, the cloning of deletion fragments into M13, and DNA sequencing have been de-

scribed elsewhere (65). The cloned fragments collectively spanned the region of interest and provided both strands for analysis. Ambiguities in the sequence arising from any clone were resolved by sequencing clones that contained overlapping templates. Primers were purchased from Pharmacia (Uppsala, Sweden) and Midland Certified Reagent Co. (Midland, Tex.). The initial codon was inferred by examining the usage of preferred codons, homology to the appropriate genes of other organisms, and the presence of a probable ribosome binding site. Reverse transcriptase and T4 polynucleotide kinase were used to conduct primer extension analysis as described previously (2) with the oligonucleotide 5'-CTTGGATCAGCCGTAAAACATCGGTTGCTG-3', which anneals to bases 11 to 40 of the *glnA* coding region.

Hybridization and preparation of hybridization probes. Hybridization probes were prepared by the random priming method with agarose-purified DNA fragments and reagents purchased from Boehringer (Mannheim, Germany) and New England Nuclear (Wilmington, Del.). Southern blotting and stringent or nonstringent DNA-DNA hybridizations were carried out as previously described (33). Northern (RNA) blotting (35 μg of RNA per lane) and stringent DNA-RNA hybridizations were performed with Hybond-N membranes (Amersham) in accordance with the instructions provided by the manufacturer. Radioactivity present in specific bands was measured with a Betagen Betascope 603 blot analyzer. A probe from the *psaK* gene of PR-6 (kindly provided by Wendy Schluchter and Don Bryant), which encodes a component of photosystem I, was used to provide an internal control.

Construction of a *glnA* deletion mutant. A deletion of the entire *glnA* coding region was constructed via a four-fragment ligation: the region upstream of *glnA* (700-bp fragment, *Pst*I [from the M13 RF1.2 sequencing vector] to *Bam*HI [upstream of *glnA*]), a 1.3-kb *Bam*HI kanamycin resistance cassette (from pUC4K), the region downstream of *glnA* (400-bp fragment, *Bgl*II [downstream of *glnA*] to *Eco*RI [from the M13mp19 2.2 sequencing vector]), and a 2.7-kb *Eco*RI-to-*Pst*I pUC13 vector. The result of this four-fragment ligation was pIK1, which was used to introduce the deletion into PR-6 via homologous recombination. Transformation in PR-6 was performed as previously described (8, 9, 56). Transformants resistant to kanamycin were purified twice to avoid the isolation of heterozygotes and to aid in the segregation of mutant from wild-type alleles. Deletion mutants (double crossover events) were distinguished from heterozygotes (single crossover events) and illegitimate recombinants by hybridization.

Biosynthetic assay. Permeabilized cell glutamine biosynthetic activity was determined by the procedure of Paone and Stevens (43, 44), except that high-pressure liquid chromatography (HPLC) was used to quantitate glutamine rather than radioactivity. Activity was determined at 39°C in a reaction mixture typically containing 0.1 ml of 500 mM Tris-HCl (pH 7.8), 0.1 ml of 100 mM ATP (pH 7.5), 0.1 ml of 200 mM NH_4Cl, 0.1 ml of 500 mM glutamate, 0.1 ml of 200 mM $MgCl_2$, 0.5 ml of cell suspension in 50 mM Tris-HCl (pH 7.8), and 0.070 ml of the detergent Nonidet P-40 (0.5% [wt/vol]) as a permeabilizing agent. Assays with carbamyl phosphate were done by use of 0.1 ml of 200 mM dilithium salt of carbamyl phosphate (pH 7.8) in place of NH_4Cl. After 30 min of incubation, found in preliminary assays to be within a linear response time, the reaction was stopped by the addition of 0.1 ml of 1 N HCl and neutralized by the addition of 0.1 ml of 1 M Tris. Following centrifugation, 20 μl of the supernatant was treated with 100 μl of a derivitization solution containing 54 mg of *o*-phthaldialdehyde, 1 ml of methanol, 9 ml of borate buffer (pH 7.5), and 0.2 ml of β-mercaptoethanol. After 90 s of incubation at room temperature, 20 μl was injected into a Waters HPLC via a Waters Ubk manual injector. The glutamine formed was quantified as its *o*-phthaldialdehyde derivative by use of a Waters 600E system controller and a Waters 990 photodiode array detector interfaced to an NEC (Milford, Mass.) APC IV Power Mate 2 advanced personal computer and a C_{18} Bondapak column with 20 mM phosphate buffer (pH 6.8)–methanol (64:36) as described by Martin et al. (40).

GDH aminating activity was determined at 30°C in a reaction mixture containing 85 mM Tris-HCl buffer (pH 8.0), 10 mM 2-oxoglutarate, 50 mM NH_4Cl, and 0.2 mM NADPH (25). The reaction was started by the addition of NH_4Cl and monitored spectrophotometrically at 340 nm with an SLM-Aminco (Urbana, Ill.) DW-2000 spectrophotometer. Control experiments were done to correct for ammonium-independent endogenous oxidation of NADPH.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to EMBL and assigned the accession number Z13965.

RESULTS

Isolation of PR-6 *glnA*. Packaged cosmids were used to infect *E. coli* YMC11, and transformants were selected for the ability to grow on ammonium as a sole nitrogen source. All Gln^+ transformants grew very slowly, producing visible colonies after 3 to 4 days of growth. Of five independently isolated Gln^+ clones, all contained cosmids with nearly identical *Eco*RI banding patterns on agarose gels (data not shown). One of the complementing cosmids, pSWA3, was mutagenized with Tn*5*. Mutant cosmids were transformed into YMC11, and transformants were screened for growth on medium in which the sole source of nitrogen was ammonium. One Gln^- mutant was isolated, and the Tn*5* mutation was mapped to a 2.1-kb *Eco*RI fragment. Subcloning experiments with pSWA3 and pSW183 demonstrated that complementation of the Gln^- phenotype of YMC11 required the presence of 2.1-, 1.2-, and 0.6-kb *Eco*RI fragments.

One subclone, pSW210, contained only the 2.1-, 1.4-, and 0.6-kb *Eco*RI fragments, and YMC11 bearing pSW210 grew more quickly on ammonium than did YMC11 transformed with the other subclones. Whole-cell extracts of YMC11 cells containing pSW210 produced 70 U of GS activity (1 U equals 1 nmol of glutamine produced per min per mg of protein), while no activity was detected in YMC11 cells lacking this plasmid. Plasmid pSW210 was used in subsequent experiments.

DNA sequence and deduced amino acid sequence. Nested deletions of the 2.1-, 1.4-, and 0.6-kb *Eco*RI fragments were constructed, cloned, and sequenced. The DNA sequence at the two *Eco*RI fragment junctions was confirmed by sequencing across them with oligonucleotide primers and a double-stranded pSW210 template. The nucleotide and deduced amino acid sequences of PR-6 *glnA* and flanking regions are shown in Fig. 1. The 1,422-bp gene encodes a deduced protein with an apparent molecular mass of 53,027 daltons and whose amino acid sequence is highly homologous to GS I amino acid sequences from a variety of bacteria (data not shown). Ten to 13 bp upstream of the coding sequence is a probable ribosome binding site, GAGG.

Modulation of *glnA* transcript levels in response to nitrogen starvation. Using the 0.6-kb *Eco*RI fragment internal to the coding region of *glnA* (Fig. 1) to probe Northern blots

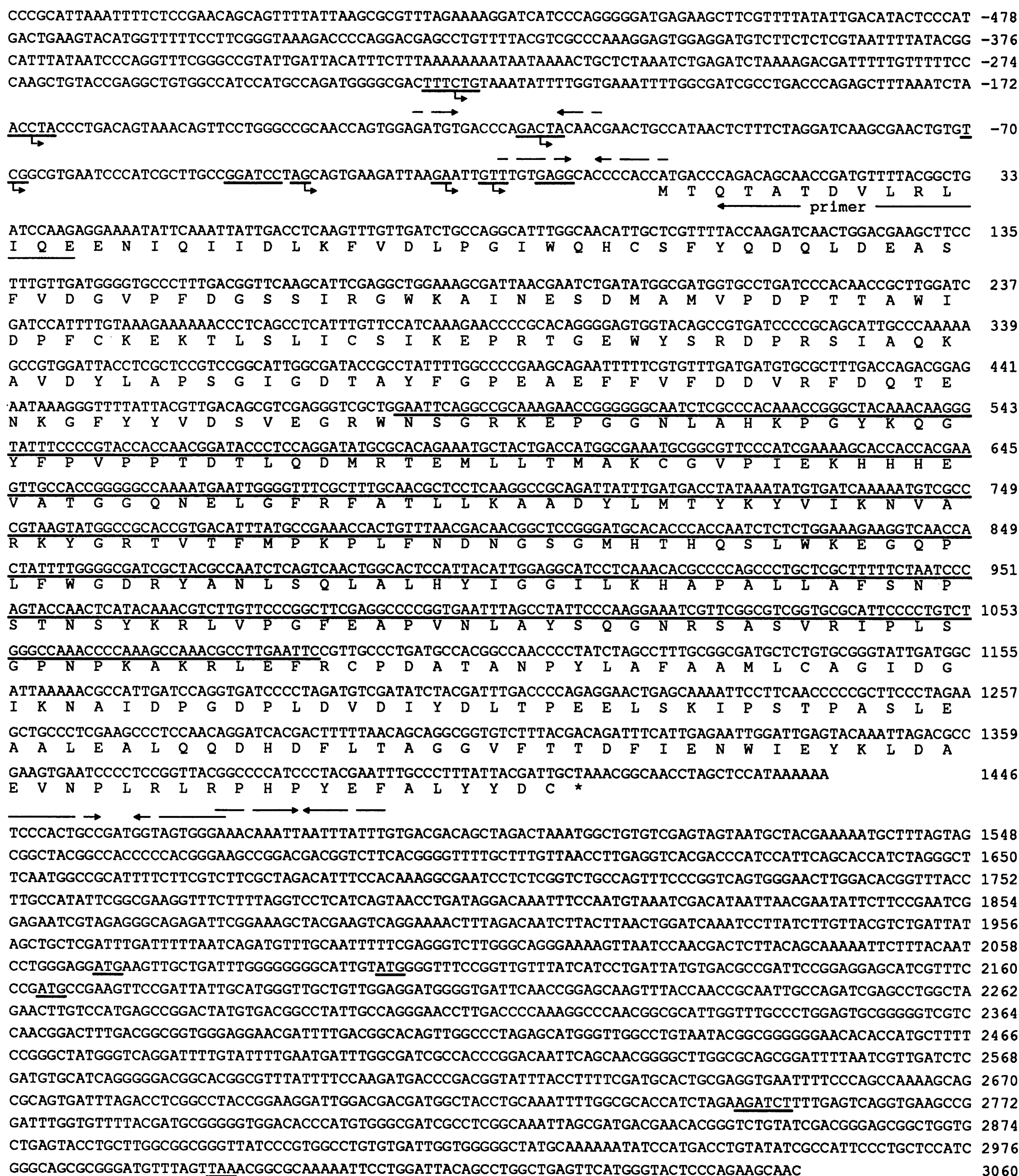

FIG. 1. Nucleotide and deduced amino acid sequences of *A. quadruplicatum* PR-6 *glnA* and flanking regions. Base pairs are numbered relative to the coding region of *glnA*. Underlined elements include sites for restriction endonucleases *Bam*HI (bp −46 to −41) and *Bgl*II (bp 2748 to 2753), used to construct deletion or insertion mutants (see also Fig. 4); a putative Shine-Dalgarno sequence for *glnA* (bp −13 to −10); a 0.6-kb *Eco*RI restriction fragment internal to the *glnA* coding region (bp 483 to 1086), used in Southern and Northern assays (see Fig. 2, 4, and 6); and potential initiation and termination codons of a downstream open reading frame (bp 2068 to 3000). Opposing arrows above the sequence identify inverted repeats. Underlined bases with subscripted arrows mark potential transcription start sites identified by extending the indicated primer (see Fig. 3).

containing RNA that had been isolated from PR-6 cells grown in A medium supplemented with 0.1% sodium nitrate, we observed *glnA* transcripts ranging from 0.8 to 1.7 kb (Fig. 2A). The presence of excess 16S RNA was probably responsible for depleting the signal in the 1.5-kb region. The broad nature and downward smearing of the hybridization signal suggest RNA degradation; however, only a limited amount of rRNA was seen to be degraded in the agarose gel. The

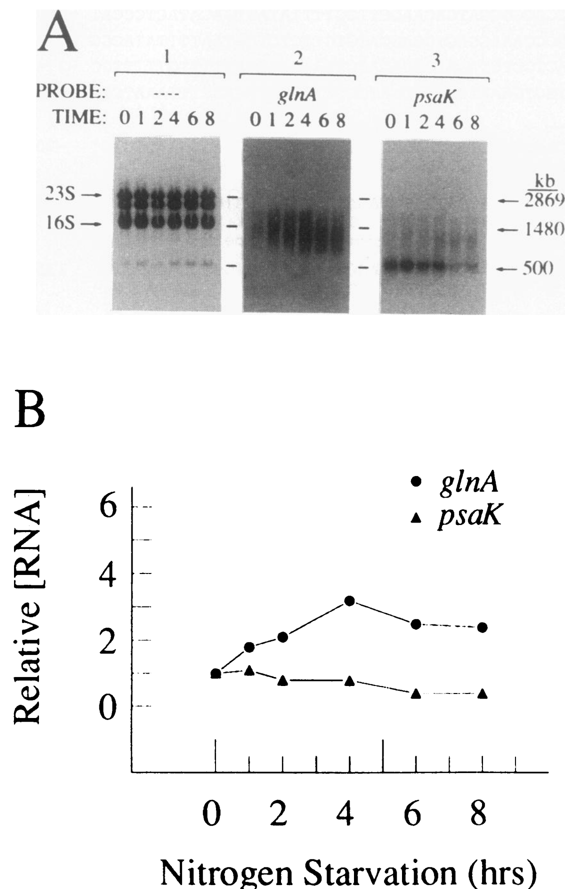

FIG. 2. Northern analysis of *glnA* transcripts in nitrogen-starved PR-6 cells. (A) RNA (35 μg) isolated from PR-6 cells incubated for 0 to 8 h in A medium lacking a nitrogen source was electrophoresed in agarose (panel 1) and blotted onto a nylon membrane. The blot was probed first with *glnA* (0.6-kb *Eco*RI fragment, panel 2) and then with *psaK* as a control (panel 3). Transcript sizes were estimated by comparison with 23S (35) and 16S (58) rRNAs and their breakdown products (23). (B) The amount of *glnA* or *psaK* hybridization signal is plotted against the time of nitrogen starvation. For three separate experiments, the average increase for *glnA* transcripts was 5.3 ± 2.0.

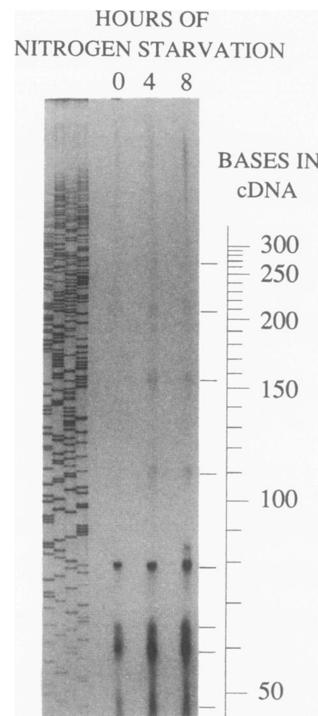

FIG. 3. Primer extension analysis of *glnA* transcripts in nitrogen-starved PR-6 cells. An end-labeled oligonucleotide complementary to codons 3 to 14 of the *glnA* coding region (Fig. 1) was annealed to 50 μg of RNA isolated from PR-6 cells starved for nitrogen for 0, 4, or 8 h and extended with avian reverse transcriptase. Extended products were electrophoresed in sequencing gels next to an M13 dideoxy sequencing ladder. The potential transcript start sites are summarized in Fig. 1.

amount of RNA hybridizing to *glnA* increased three- to fivefold when PR-6 cells were transferred from A medium containing nitrate to A medium lacking nitrate and incubated for 0 to 8 h (Fig. 2). In contrast, hybridization of the same blots with a probe specific for the *psaK* gene showed that the levels of its transcripts were diminished during the nitrogen starvation period (Fig. 2B). Primer extension reactions with RNA from the 0-, 4-, and 8-h nitrogen-starved cultures confirmed the increase in RNA levels during nitrogen starvation (Fig. 3). Several 5′ endpoints were detected; the levels of all of them were elevated in the samples from nitrogen-starved cells. The most prominant distinct band corresponded to a position about 80 bases upstream of the translation initiation codon of *glnA* (Fig. 1).

Construction and characterization of *glnA* mutants. The cloned DNA containing the *glnA* homolog was used to construct loss-of-function mutations to further assess the role of *glnA* in the nitrogen metabolism of PR-6. Initially, Ω fragment insertions (45) in the *glnA* coding region were used to replace the wild-type gene. Although Southern analysis of genomic DNA confirmed that the mutants lacked wild-type *glnA* DNA, the mutations did not prevent the cells from growing at wild-type rates on nitrate or ammonium as the sole nitrogen source (data not shown). To eliminate the possibility that a mutant but nonetheless functional *glnA* gene had been constructed, we deleted the entire *glnA* coding region and replaced it with a *Bam*HI kanamycin resistance cassette to create strain PR-6102 (Fig. 4A). Hybridization of four independently derived deletion mutants with a mixed probe containing the three *Eco*RI fragments of pSW210 or separately prepared fragments (Fig. 4B) confirmed that the expected 2.4-kb *Eco*RI fragment had replaced the 2.1-, 1.2-, and 0.6-kb wild-type fragments. Strong hybridization to the 2.4-kb *Eco*RI fragment was also seen when identical blots were probed with the *Bam*HI kanamycin resistance cassette, although no corresponding band hybridizing to genomic DNA was observed. No hybridization to genomic DNA of PR-6 or PR-6102 was seen when the pHC79 vector of pSW210 was used in control experiments (data not shown).

As was seen for the insertion mutants, no growth defect was observed for deletion mutant PR-6102 when it was cultured in A medium containing 0.1% sodium nitrate or 0.03% ammonium chloride as the sole nitrogen source (Fig. 5). When RNA was isolated from PR-6102 cultures grown on A medium with nitrate and hybridized to the 0.6-kb *Eco*RI fragment internal to the *glnA* coding region, the 1.5-kb transcript seen in wild-type cells was not detected (Fig. 6).

Glutamine biosynthetic activities of PR-6 and PR-6102. We

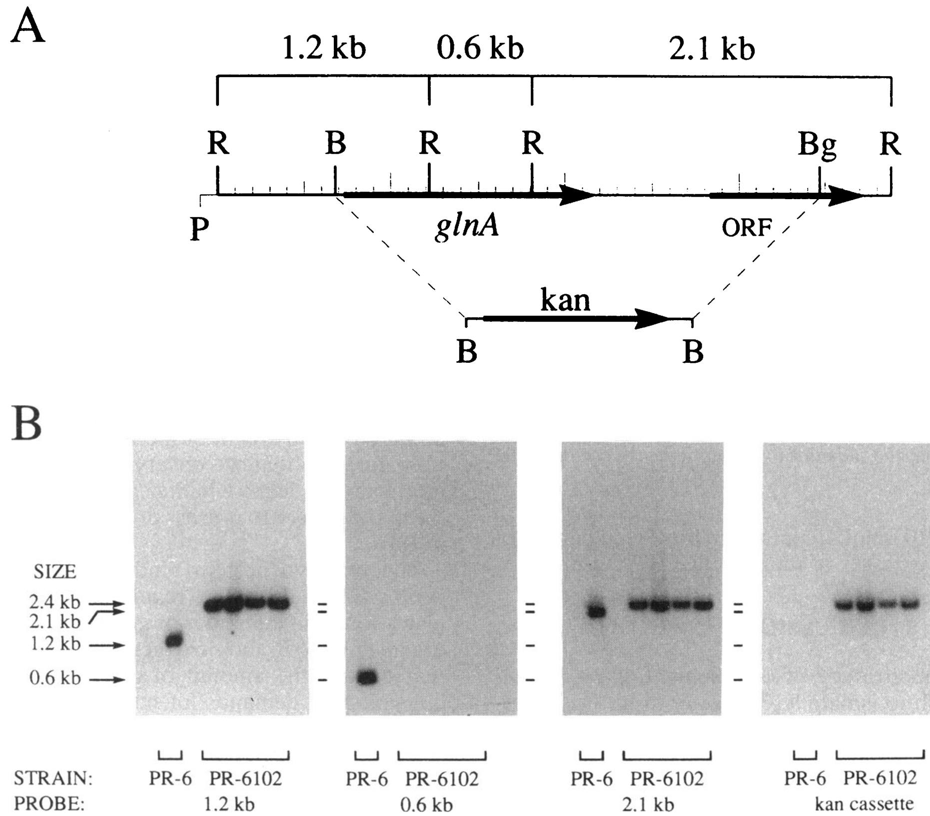

FIG. 4. Construction of a *glnA* deletion strain of PR-6. (A) A restriction map of the region of PR-6 genomic DNA encoding all of *glnA* and a downstream open reading frame (ORF) is shown above the region replaced with the Tn*903-kan* gene (R, *Eco*RI; B, *Bam*HI; P, *Pst*I; Bg, *Bgl*II). (B) Southern analysis of DNA from wild-type PR-6 cells and cells of four independently derived mutants (strain PR-6102). Identically prepared blots bearing DNA digested with *Eco*RI were hybridized under stringent conditions with a 1.2-, 0.6-, or 2.1-kb *Eco*RI restriction fragment of the wild-type *glnA* coding region, isolated from clone pSW210, or the Tn*903-kan* gene, isolated from pUC4K.

measured whole-cell glutamine biosynthetic activity in exponential-phase cells of PR-6 and PR-6102 by determining the rate of production of glutamine from glutamate (Fig. 7). The synthesis of glutamine depended on the concentrations of ammonium (Fig. 7A) and glutamate (Fig. 7B) substrates for extracts of both mutant PR-6102 and wild-type PR-6 cells. However, maximal rates of glutamine synthesis in extracts of PR-6102 cells were only about 50 to 75% those in extracts of wild-type cells. When ammonium was omitted from these mixtures, neither PR-6 nor PR-6102 produced glutamine from glutamate, although both could use carbamyl phosphate as an amino donor (125 nmol of glutamine produced per mg of protein per min by extracts of wild-type

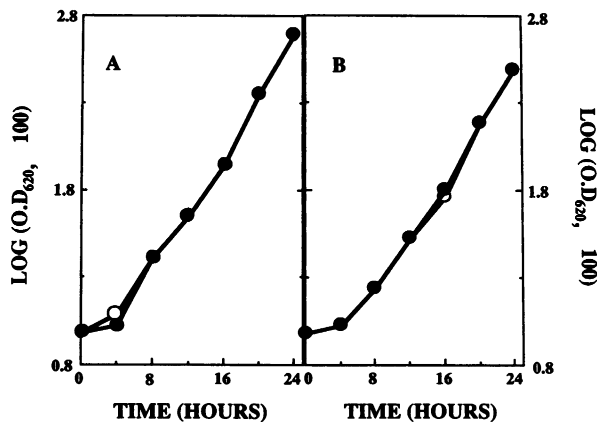

FIG. 5. Growth curves for wild-type strain PR-6 (○) and *glnA* deletion mutant PR-6102 (●) in A medium supplemented with NH_4^+ (A) and NO_3^- (B). OD_{620}, optical density at 620 nm.

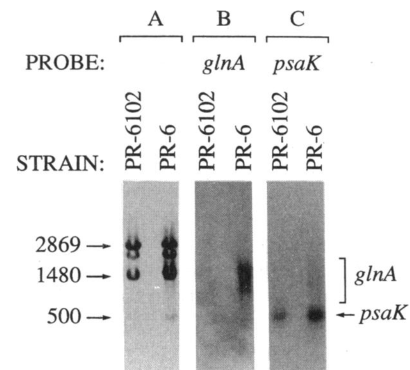

FIG. 6. Northern analysis of *glnA* transcripts in deletion mutant PR-6102. RNA (35 μg) isolated from PR-6 or PR-6102 cells grown to an optical density at 600 nm of 0.7 in A medium supplemented with nitrate was electrophoresed in agarose (A) and blotted onto a nylon membrane. The blot was then hybridized successively to *glnA* (B) and *psaK* (C) probes as described in the legend to Fig. 2.

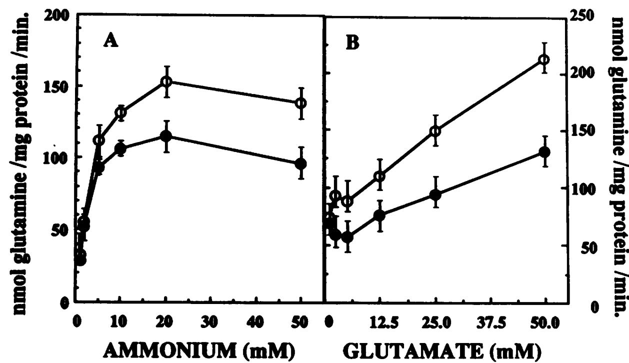

FIG. 7. Glutamine synthesis by permeabilized whole cells of PR-6 (○) and PR-6102 (●) in the presence of various concentrations of ammonium (A) and glutamate (B). Error bars represent one standard deviation from the mean of three replicate determinations.

PR-6 cultures and 210 nmol of glutamine produced per mg of protein per min by extracts of mutant PR-6102 cultures).

DISCUSSION

We have used the strategy of complementing an *E. coli* glutamine auxotroph to isolate a *glnA* gene from the cyanobacterium *A. quadruplicatum* PR-6. This approach also has been used successfully to isolate *glnA* from other organisms (24, 50, 53–55). The deduced amino acid sequence of GS encoded by *glnA* of PR-6 is highly homologous (75% identity) to the deduced amino acid sequences of GSs from *Anabaena* sp. strain 7120 (59) and from other bacteria.

In PR-6, *glnA* appears to be a single-copy gene. Using Northern analysis and primer extension reactions, we observed multiple transcripts that ranged in size from 0.8 to 1.7 kb and that contained the *glnA* coding region. On the basis of the DNA sequence of *glnA*, functional transcripts would need to have at least 1,450 bases. These data are consistent with *glnA* being expressed as a monocistronic RNA, with most transcripts beginning about 80 bases upstream of the *glnA* coding region and terminating just beyond the coding region. The additional 5' endpoints could represent additional *glnA* promoters, as has been seen for the cyanobacterium *Anabaena* sp. strain 7120 (59), or any of the mapped endpoints could arise from the processing of a larger RNA transcript. Direct demonstration of promoter function upstream of *glnA* will be needed to distinguish between these possibilities. We did not find any good matches between the putative promoter regions and known consensus promoter elements. Twenty-five bp downstream of the coding region is a GC-rich region that could form a hairpin loop with a predicted stability of −14.4 kcal (ca −60.2 kJ) (Fig. 1). This GC-rich sequence, which overlaps by 1 nucleotide a downstream AT-rich inverted repeat, could potentially serve as a transcriptional terminator.

The amount of glutamine biosynthetic activity in extracts of PR-6 cells was previously shown to vary about fivefold in response to growth conditions (43, 44). The three- to fivefold increase in the *glnA* transcript levels that was detected upon starvation of PR-6 cells for nitrogen could account for such a variation. How PR-6 cells regulate *glnA* transcription remains to be determined. The coding region downstream of PR-6 *glnA* displayed no significant homology to any genes in current data bases, and we observed no phenotypic change in PR-6 when this putative gene was interrupted with a fragment containing a gene conferring resistance to streptomycin or spectinomycin (data not shown) or when it was partially deleted, as in the PR-6 *glnA* deletion mutant described here.

The *ntcA* gene of *Synechococcus* sp. strain PCC 7942 (*Anacystis nidulans* R2), identified as a gene needed for the optimal expression of ammonium-repressible activities, which include GS activity, was recently shown to encode a protein similar to the known transcriptional activators FnR, FixK, Crp, and CysR (61). Perhaps a similar gene will be found to be involved in the regulation of *glnA* in PR-6. Two potential binding sites for a protein with the DNA binding motif typical of this family of regulators, $TGTN_{10}ACA$ (20), are located upstream of PR-6 *glnA*. One site overlaps the probable Shine-Dalgarno sequence, and one is centered 118 bp upstream of the initiation codon (Fig. 1). This latter site is about 80 bp upstream of the most likely transcription initiation start site that we observed for *glnA* (Fig. 1). It will be interesting to learn whether an *ntcA* homolog and either of these sites serve to repress or activate the expression of *glnA* in PR-6.

The putative protein binding sites located upstream of PR-6 *glnA* could reflect regulation that is similar to mechanisms found in a number of gram-negative bacteria. In these organisms, *ntrB* and *ntrC* genes encode regulatory proteins that control the amount of *glnA* transcription to adjust to physiological demands for nitrogen given available levels of environmental ammonium (see references 38 and 57 for a review). NtrC belongs to the σ^{54}-dependent family of transcriptional activators that exist in both gram-negative (36) and gram-positive (17) eubacteria. In gram-negative bacteria, these activators also possess helix-turn-helix-type DNA binding motifs similar to that of CrP (and presumably NtcA). For example, the consensus binding sequence of the σ^{54}-dependent activator NifA is also $TGTN_{10}ACA$ (7); thus, the putative binding sites upstream of *glnA* in PR-6 could bind a conserved NtrC-like protein. In enteric bacteria, protein P_{II} controls a regulatory cascade by affecting an interaction between NtrB and NtrC. A possible homolog of the *glnB* gene encoding P_{II} has been identified in PR-6 (34).

However, to our knowledge, no one has described a σ^{54}-dependent promoter in PR-6, and no obvious candidates are present in the region upstream of *glnA*. We have thus far failed to detect the presence of the *ntrB* or *ntrC* gene in PR-6, even though hybridization experiments with a probe from the highly conserved central portion of the *ntrC* gene did identify several DNA fragments (63). The PR-6 DNA fragment that is most homologous only matches about 20 bp of the *ntrC* probe. This region encodes a DNA binding motif that is common to a number of otherwise unrelated proteins (48), and the flanking sequence of the PR-6 DNA encodes proteins very similar to the *S. typhimurium* oligopeptide permease components OppD and OppF. This result is in sharp contrast to what we have seen for *R. meliloti* and a *Bradyrhizobium* sp. (*Parasponia* sp.) (33), which contain several σ^{54}-dependent transcriptional activators homologous to NtrC. DNA fragments encoding the central regions of those activators hybridized strongly to several regions of the *ntrC* probe.

Perhaps, as is the case for a number of gram-negative bacteria, the signalling capability of P_{II} is used in PR-6 to regulate nitrogen metabolism. However, the protein may communicate with elements of a transcription control system that differs from the one used in gram-negative bacteria. It is clear that P_{II} is part of a transduction mechanism that signals to proteins, other than NtrB and NtrC, involved in

transcriptional regulation, since it also regulates the activity of adenylyltransferase, the enzyme that governs the adenylylation state of GS in enteric bacteria.

PR-6 must have at least one gene other than *glnA* whose product can be used in the assimilation of ammonium, since we observed no change in the ability of PR-6 cells to utilize ammonium as the sole nitrogen source when *glnA* was deleted and in the absence of detectable GDH activity. Whole-cell extracts of both the deletion strain and its wild-type parent could use carbamyl phosphate as an amino donor to synthesize glutamine from glutamate, a result that might indicate the use of a carbamyl phosphate synthetase as an alternative to GS for catalyzing ammonium assimilation (15). It is also possible that PR-6 possesses glutamine biosynthetic activity related to the aminotransferase of bacilli that can convert Glu-tRNA^{Gln} to Gln-tRNA^{Gln} given a suitable amide donor. This pathway, known to exist in gram-positive eubacteria (67, 68) and *Halobacterium* species (29, 66), was implicated to exist in the cyanobacterium *Synechocystis* sp. as well as in plant and animal organelles (52). However, the *glnA* deletion strain of PR-6 was significantly more sensitive to methionine sulfoximine, a transition state analog of glutamate, than was the wild-type strain (data not shown). Unless the inhibition was indirect, this result might indicate the presence of an alternate GS in PR-6. Additional GSs are encoded by the *glnII* and *glnT* genes of bacteria from the *Rhizobiaceae* family (11, 12, 19, 24, 50, 55). Thus far we have been unable to detect in the PR-6 genome DNA that hybridizes to probes for these genes from *Bradyrhizobium japonicum* and *R. meliloti*. It is clear that additional work is needed to clarify how PR-6 cells achieve the regulation of *glnA* and to explain why *glnA* is nonessential for ammonium assimilation.

ACKNOWLEDGMENTS

We thank Wendy Schluchter, Laurie Caslake, and Don Bryant for providing DNA encoding *psaK* from PR-6, help preparing RNA blots, and comments on the manuscript; J. Somerville and F. J. de Bruijn for the kind gift of several *gln* gene probes; and Mary Ann Billingsley for typing the manuscript.

We acknowledge a generous unrestricted gift from Johnson & Johnson (to S.E.S.) and NIH grant GM40404 (to B.T.N.) for support of this research. Salary for R.I.K. was paid in part from PHS grant GM31626 to D. A. Bryant. Publication of the manuscript was supported by the W. Harry Feinstone Chair of Excellence in Molecular Biology (held by S.E.S.).

REFERENCES

1. **Appleyard, R. K.** 1954. Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. Genetics **39:**440–452.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York.
3. **Backmann, K., Y. M. Chen, and B. Magasanik.** 1981. Physical characterization of the *glnA-glnG* region of the *E. coli* chromosome. Proc. Natl. Acad. Sci. USA **78:**3743–3747.
4. **Berg, D. E.** 1977. Insertion and excision of the transposable kanamycin resistance determinant Tn5, p. 205–212. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Berman, M. L., L. W. Enquist, and T. J. Silhavy.** 1981. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *E. coli*. J. Mol. Biol. **41:**459–472.
7. **Buck, M., S. M. Miller, M. Drummond, and R. Dixon.** 1986. Upstream activator sequences are present in the promoters of nitrogen fixation genes. Nature (London) **320:**374–378.
8. **Buzby, J. S.** 1984. Gene expression and recombination in a cyanobacterium: development of plasmid cloning vectors. Ph.D. thesis. Pennsylvania State University, University Park.
9. **Buzby, J. S., R. D. Porter, and S. E. Stevens, Jr.** 1985. Expression of an *Escherichia coli lacZ* gene on a plasmid vector in a cyanobacterium. Science **230:**805–807.
10. **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.
11. **Carlson, T. A., M. L. Guerinot, and B. K. Chelm.** 1985. Characterization of the gene encoding glutamine synthetase I (*glnA*) from *Bradyrhizobium japonicum*. J. Bacteriol. **162:**698–703.
12. **Carlson, T. A., G. B. Martin, and B. K. Chelm.** 1987. Differential transcription of the two glutamine synthetase genes of *Bradyrhizobium japonicum*. J. Bacteriol. **169:**5861–5866.
13. **Castelfranco, P. A., and S. I. Beale.** 1981. Chlorophyll biosynthesis, p. 375–421. *In* P. K. Stumpf and E. E. Conn (ed.), The biochemistry of plants, vol. 8. Academic Press, Inc., New York.
14. **Castelfranco, P. A., and S. I. Beale.** 1983. Chlorophyll biosynthesis: recent advances and areas of current interest. Annu. Rev. Plant Physiol. **34:**241–278.
15. **Chen, C. H., C. Van Baalen, and F. R. Tabita.** 1987. DL-7 azatryptophan and citrulline metabolism in the cyanobacterium *Anabaena* sp. strain 1F. J. Bacteriol. **169:**1114–1119.
16. **Darrow, R. A., and R. R. Knotts.** 1977. Two forms of glutamine synthetase in free-living root-nodule bacteria. Biochem. Biophys. Res. Commun. **78:**554–559.
17. **Debarbouille, M., I. Martin-Verstraete, A. Klier, and G. Rapoport.** 1991. The transcriptional regulator LevR of *Bacillus subtilis* has domains homologous to both sigma-54- and phosphotransferase system-dependent regulators. Proc. Natl. Acad. Sci. USA **88:**2212–2216.
18. **de Bruijn, F. J., and F. R. Lupski.** 1984. The use of transposon Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids—a review. Gene **27:**131–149.
19. **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.
20. **de Crombrugghe, B., S. Busby, and H. Buc.** 1984. Cyclic AMP receptor protein: role in transcription activation. Science **224:**831–838.
21. **de Lorimier, R., D. A. Bryant, R. D. Porter, W. L. Liu, E. Jay, and S. E. Stevens, Jr.** 1984. Genes for the α and β subunits of phycocyanin. Proc. Natl. Acad. Sci. USA **81:**7946–7950.
22. **Dixon, R. A.** 1984. The genetic complexity of nitrogen fixation, the ninth Fleming lecture. J. Gen. Microbiol. **130:**2745–2755.
23. **Doolittle, W. R.** 1972. Ribosomal ribonucleic acid synthesis and maturation in the blue-green alga *Anacystis nidulans*. J. Bacteriol. **111:**316–324.
24. **Filser, M. M. K., C. Moscatelli, A. Lamberti, E. Vincze, M. Guida, G. Salzano, and M. Iaccarino.** 1986. Characterization and cloning of two *Rhizobium leguminosarum* genes coding for glutamine synthetase activities. J. Gen. Microbiol. **132:**2561–2569.
25. **Florencia, F. J., S. Marques, and P. Candau.** 1987. Identification and characterization of a glutamate dehydrogenase in the unicellular cyanobacterium *Synechocystis* PCC 6803. FEBS Lett. **223:**37–41.
26. **Fuchs, R. L., and D. L. Keister.** 1980. Identification of two glutamine synthetases in *Agrobacterium*. J. Bacteriol. **141:**996–998.
27. **Fuchs, R. L., and D. L. Keister.** 1980. Comparative properties of glutamine synthetases I and II in *Rhizobium* and *Agrobacterium* spp. J. Bacteriol. **144:**641–648.

28. **Golden, S., J. Brusslan, and R. Haselkorn.** 1987. Genetic engineering of the cyanobacterial chromosome. Methods Enzymol. **153:**215–231.
29. **Gupta, R.** 1984. *Halobacterium volcanii* tRNAs: identification of 41 tRNAs covering all amino acids and sequences of 33 class I tRNAs. J. Biol. Chem. **259:**9461–9471.
30. **Hirschmann, J., P. K. Wong, K. Sei, J. Kenner, and S. Kustu.** 1985. Products of nitrogen regulatory genes *ntrA* and *ntrC* of enteric bacteria activate *glnA* transcription in vitro: evidence that *ntrA* product is a sigma factor. Proc. Natl. Acad. Sci. USA **82:**7525–7529.
31. **Hohn, B., and J. Collins.** 1980. A small cosmid for efficient cloning of large DNA fragments. Gene **11:**291–298.
32. **Hunt, T. P., and B. Magasanik.** 1985. Transcription of *glnA* by purified *Escherichia coli* components: core RNA polymerase and the products of *glnF*, *glnG*, and *glnL*. Proc. Natl. Acad. Sci. USA **82:**8453–8457.
33. **Jiang, J., B. Gu, L. M. Albright, and B. T. Nixon.** 1989. Conservation between coding and regulatory elements of *Rhizobium meliloti* and *Rhizobium leguminosarum dct* genes. J. Bacteriol. **171:**5244–5253.
34. **Kaufmann, R. I., B. T. Nixon, and D. A. Bryant.** Unpublished data.
35. **Kumano, M., N. Tomioka, and M. Sugiura.** 1983. The complete nucleotide sequence of a 23S rRNA gene from a blue-green alga, *Anacystis nidulans*. Gene **24:**219–225.
36. **Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss.** 1989. Expression of sigma-54 (*ntrA*)-dependent genes is probably united by a common mechanism. Microbiol. Rev. **53:**367–376.
37. **Ludwig, R. A.** 1980. Physiological roles of glutamine synthetases I and II in ammonium assimilation in *Rhizobium* sp. 32H1. J. Bacteriol. **141:**1209–1216.
38. **Magasanik, B.** 1982. Genetic control of nitrogen assimilation in bacteria. Annu. Rev. Genet. **16:**135–168.
39. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Martin, F., A. Suzuki, and B. Hirel.** 1982. A new high-performance liquid chromatography assay for glutamine synthetase and glutamate synthase in plant tissues. Anal. Biochem. **125:**24–29.
41. **Martin, G. B., K. A. Chapman, and B. K. Chelm.** 1988. Role of the *Bradyrhizobium japonicum ntrC* gene product in differential regulation of the glutamine synthetase II gene (*glnII*). J. Bacteriol. **170:**5452–5459.
42. **Meyerowitz, E. M., G. M. Guild, L. S. Prestidge, and D. S. Hogness.** 1980. A new cosmid vector and its use. Gene **11:**271–282.
43. **Paone, D. A.** 1981. Purification and initial studies on the regulation of glutamine synthetase in *Agmenellum quadruplicatum*. Ph.D. thesis. Pennsylvania State University, University Park.
44. **Paone, D. A., and S. E. Stevens, Jr.** 1981. Nitrogen starvation and the regulation of glutamine synthetase in *Agmenellum quadruplicatum*. Plant Physiol. **67:**1097–1100.
45. **Prentki, P., and H. M. Krisch.** 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
46. **Reitzer, L., and B. Magasanik.** 1985. Expression of *glnA* in *E. coli* is regulated at tandem promoters. Proc. Natl. Acad. Sci. USA **82:**1979–1983.
47. **Reitzer, L. J., and B. Magasanik.** 1987. Ammonia assimilation and the biosynthesis of glutamine, glutamate, aspartate, asparagine, L-alanine, and D-alanine, p. 302–320. *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.
48. **Ronson, C. W., P. M. Astwood, B. T. Nixon, and F. M. Ausubel.** 1987. Deduced products of C4-dicarboxylate transport regulatory genes of *Rhizobium leguminosarum* are homologous to nitrogen regulatory gene products. Nucleic Acids Res. **15:**7921–7934.
49. **Rossbach, S., J. Schell, and F. J. de Bruijn.** 1987. The *ntrC* gene of *Agrobacterium tumefaciens* C58 controls glutamine synthesis (GSII) activity, growth on nitrate and chromosomal but not Ti-encoded arginine catabolism pathways. Mol. Gen. Genet. **209:**419–426.
50. **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.
51. **Rossi, M., R. Defez, M. Chiurazzi, A. Lamberti, A. Fuggi, and M. Iaccarino.** 1989. Regulation of glutamine synthetase isoenzymes in *Rhizobium leguminosarum* biovar viceae. J. Gen. Microbiol. **135:**629–637.
52. **Schon, A., C. G. Kannangara, S. Gough, and D. Soll.** 1988. Protein synthesis in organelles requires misaminoacylation of tRNA. Nature (London) **331:**187–190.
53. **Scolnik, P. A., J. Virosco, and R. Haselkorn.** 1983. The wild-type gene for glutamine synthetase restores ammonia control of nitrogen fixation to *gln* (*glnA*) mutants of *Rhodopseudomonas capsulata*. J. Bacteriol. **155:**180–185.
54. **Snustad, D. P., J. P. Hunsperger, B. M. Chereskin, and J. Messing.** 1988. Maize glutamine synthetase cDNAs: isolation by direct genetic selection in *Escherichia coli*. Genetics **120:**1111–1124.
55. **Somerville, J. E., and M. L. Kahn.** 1983. Cloning of the glutamine synthetase I gene from *Rhizobium meliloti*. J. Bacteriol. **156:**168–176.
56. **Stevens, S. E., Jr., and R. D. Porter.** 1980. Transformation in *Agmenellum quadruplicatum*. Proc. Natl. Acad. Sci. USA **77:**6052–6056.
57. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:**450–490.
58. **Tomioka, N., and M. Sugiura.** 1983. The complete nucleotide sequence of a 16S ribosomal RNA gene from a blue-green alga, *Anacystis nidulans*. Mol. Gen. Genet. **191:**46–50.
59. **Tumer, N. E., S. E. Robinson, and R. Hazelkorn.** 1983. Different promoters for the *Anabaena* glutamine synthetase gene during growth using molecular or fixed nitrogen. Nature (London) **306:**337–342.
60. **Van Baalen, C.** 1962. Studies on marine blue-green algae. Bot. Mar. **4:**129–139.
61. **Vega-Palas, M. A., E. Flores, and A. Herrero.** 1992. NtcA, a global nitrogen regulator from the cyanobacterium *Synechococcus* that belongs to the Crp family of bacterial regulators. Mol. Microbiol. **6:**1853–1859.
62. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
63. **Wagner, S. J., M. W. McKinstry, and B. T. Nixon.** Unpublished data.
64. **Wagner, S. J., S. E. Stevens, Jr., and B. T. Nixon.** 1988. Isolation of *glnA* from the cyanobacterium PR-6, p. 351–353. *In* S. E. Stevens, Jr., and D. A. Bryant (ed.), Light-energy transduction in photosynthesis: higher plant and bacterial models. The American Society of Plant Physiologists, Rockville, Md.
65. **Wagner, S. J., S. E. Stevens, Jr., B. T. Nixon, D. H. Lambert, R. G. Quivey, Jr., and F. R. Tabita.** 1988. Nucleotide and deduced amino acid sequence of the *Rhodobacter sphaeroides* gene encoding form II ribulose-1,5-bisphosphate carboxylase/oxygenase and comparison with other deduced form I and II sequences. FEMS Microbiol. Lett. **55:**217–222.
66. **White, B. N., and S. T. Bayley.** 1972. Further codon assignments in an extremely halophilic bacterium using a cell-free protein-synthesizing system and ribosomal binding assay. Can. J. Biochem. **50:**601–609.
67. **Wilcox, M.** 1969. γ-Glutamyl phosphate attached to glutamine-specific tRNA: a precursor of glutaminyl-tRNA in *Bacillus subtilis*. Eur. J. Biochem. **11:**405–412.
68. **Wilcox, M., and M. Nirenberg.** 1968. Transfer RNA as a cofactor coupling amino acid synthesis with that of protein. Proc. Natl. Acad. Sci. USA **61:**229–236.
69. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.