Regulation of Expression of Glutamine Synthetase in a Symbiotic *Nostoc* Strain Associated with *Anthoceros punctatus*

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A characteristic of N_2 -fixing cyanobacteria in symbiotic associations appears to be release of N_2 -derived NH_4^+ . The specific activity of the primary ammonium-assimilating enzyme, glutamine synthetase (GS), was found to be three- to fourfold lower in *Nostoc* sp. strain 7801 grown in symbiotic association with the bryophyte *Anthoceros punctatus* than in free-living *Nostoc* sp. strain 7801. Quantitative immunological assays with antisera against GS purified from *Nostoc* sp. strain 7801 and from *Escherichia coli* indicated that similar amounts of the GS protein were present in symbiotic ($50 \mu g \text{ mg}^{-1}$) and free-living ($68 \mu g \text{ mg}^{-1}$) cultures. The conclusion from these experiments is that GS is regulated by a posttranslational mechanism in *Anthoceros*-associated *Nostoc* sp. strain 7801. However, the results of comparative catalytic and immunological experiments between N_2 - and NH_4^+ -grown free-living *Nostoc* sp. strain 7801 implied control of GS synthesis. A correlation was not observed between the level of GS expression and the extent of symbiotic heterocyst differentiation in *Nostoc* sp. strain 7801 associated with *A. punctatus*.

Dinitrogen-fixing cyanobacteria assimilate N_2 -derived or exogenously supplied NH_4^+ almost exclusively by the sequential activities of glutamine synthetase (GS) (L-glutamate:ammonia ligase [ADP-forming], EC 6.3.1.2) and glutamate synthase (L-glutamate:ferredoxin oxidoreductase [transaminating], EC 1.4.7.1) (the glutamate synthase cycle [29]) (18). Isotopic labeling experiments and in vitro enzyme assays have established that the glutamate synthase cycle also operates in dinitrogen-fixing cyanobacteria in symbiotic associations with species of the water fern *Azolla* (20, 28), the bryophyte *Anthoceros* (19), and certain lichens (26, 27). However, a characteristic of these symbiotically associated cyanobacteria also appears to be a decreased capacity to assimilate ammonium; consequently, a substantial fraction of the N_2 -derived NH_4^+ is made available for growth of the associated eucaryotic tissue.

A lower in vivo ammonium assimilation activity of associated cyanobacteria could result from repressing synthesis of GS, the initial enzyme of the cycle, inhibiting its activity, or depriving it of substrate. In the *Anabaena* sp. symbiont of *Azolla*, GS activity is reportedly regulated by repression of synthesis (22, 23, 36). The enzyme in *Nostoc* spp. from lichen associations has variously been reported as being inhibited catalytically (31) or transcriptionally repressed (36).

Procedures have been established for pure culture of the bryophyte *Anthoceros punctatus* L. and reconstitution of its symbiotic association with various isolates and mutant strains of *Nostoc* spp. (7). In situ, symbiotic *Nostoc* sp. strain 7801 assimilates approximately 10% of its fixed nitrogen, and in vivo, it metabolizes exogenous NH_4^+ at a fivefold lower rate than free-living *Nostoc* sp. strain 7801 (19).

In this paper, we present evidence that the depressed nitrogen assimilation activities of symbiotic *Nostoc* sp.

strain 7801 are paralleled by lower in vitro GS catalytic activity and that the low activity was a consequence of an undefined posttranslational mechanism rather than of its synthesis. We further show that the expression of GS activity in symbiotically associated *Nostoc* sp. strain 7801 does not correlate with the extent of its heterocyst differentiation.

MATERIALS AND METHODS

Cultures. Pure cultures of *A. punctatus* L. gametophyte tissue were obtained, the symbiotic association was reconstituted with pure cultures of the original isolate, *Nostoc* sp. strain 7801, and the associated tissues were grown as described previously (7) and used not less than 3 months or more than 12 months after reconstitution. Prior to experimentation, symbiotically associated cultures were treated with penicillin G for 2 days to remove any epiphytic growth of *Nostoc* sp. strain 7801 and then allowed to recover for 14 days (7). Ammonium-treated cultures were supplemented with 2.5 mM NH_4Cl immediately after penicillin treatment and were transferred every 3 to 5 days to keep the NH_4Cl concentration above 1 mM. The concentration of ammonium in the medium was monitored by direct assay (34).

Nostoc sp. strain 7801 was grown in free-living culture in the combined nitrogen-free medium of Allen and Arnon (1), diluted fourfold. The liquid cultures were incubated under continuous light at 7 W m^{-2} from cool-white fluorescent lamps on orbital shakers (175 rpm) at room temperature. When noted, cultures were supplemented with 2.5 mM NH_4Cl plus, as buffer, 5.0 mM 3-(N-morpholino)propanesulfonic acid (MOPS; Sigma Chemical Co., St. Louis, Mo.).

Salmonella typhimurium TA831 (2) and its GS deletion mutant SK35 (11) were gifts from S. Kustu. *S. typhimurium* was grown at 37°C with aeration on the minimal medium E of Vogel and Bonner (38) supplemented with 0.4% glucose and 0.3 mM histidine. The medium for strain SK35 was supplemented with 3 mM glutamine.

Isolation of symbiotic *Nostoc*. Symbiotic *Nostoc* sp. strain 7801 was isolated from *A. punctatus* tissue by the basic

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TABLE 1. Purification of GS from *Nostoc* sp. strain 7801

Step	Protein (mg)	Total activity (U) ^a	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Crude extract	653.03	447	0.69	100	1
(NH ₄) ₂ SO ₄ precipitate	79.05	234	2.96	52	4.3
Blue-Sepharose	2.70	141	52.22	32	76
HPLC	1.80	124	68.99	28	100

^a One unit equals 1 μ mol of gamma-glutamylhydroxamate formed per min.

procedure of Meeks et al. (19), with the following modifications. Two days prior to isolation, the *Anthoceros-Nostoc* cultures were transferred to medium without glucose to reduce the amount of mucilage in the symbiotic cavities within the *A. punctatus* tissue. After Ficoll-Ludox gradient centrifugation, the pellet fraction was washed and suspended in 5 mM MOPS, pH 7.8. This fraction was layered onto a Ludox discontinuous gradient of densities 0.15, 0.20, 0.25, and 0.30 g ml⁻¹ and centrifuged at $5,500 \times g$ for 10 min in a horizontal rotor (Sorvall model HB-4; DuPont Instruments, Newton, Conn.) at 4°C. The layer at 0.30 g ml⁻¹ was removed and washed twice in buffer, and any contaminating *A. punctatus* tissue was removed with the aid of a stereomicroscope. Ludox AM (colloidal silica) was a gift from E. I. Du Pont de Nemours & Co., Wilmington, Del.

GS activity assay. The transferase assay of Shapiro and Stadtman (33), with modifications described previously (15), was used to monitor GS activity.

Purification of GS from *Nostoc* sp. strain 7801. Antibodies to *Nostoc* sp. strain 7801 GS were prepared by using enzyme purified from free-living cultures. Cultures were grown in a 10-liter illuminated fermentor (Microferm; New Brunswick Scientific Co., Inc., Edison, N.J.) for 7 days at 30°C, under cool-white fluorescent lamps at 10 W m⁻², with the aeration modifications as noted (5). Cells were harvested with a Sorvall continuous-flow apparatus and a fixed-angle rotor (Sorvall model SS-34) at $8,000 \times g$ and 4°C. The yield was generally about 10 g (wet weight) of cells. The fermentor contents were checked for contamination by heterotrophic bacteria before harvest by plating a sample on nutrient agar and the full-strength medium of Allen and Arnon (1) supplemented with glucose and ammonium. Cells were broken in a French pressure cell at 16,000 lb in⁻² in the buffer of Orr et al. (24), with (DBMn) or without (DMN) 2-mercaptoethanol, and frozen until purity of the culture was verified. The thawed suspension was centrifuged at $20,000 \times g$ for 15 min. The supernatant fraction was treated on ice with 0.5% (wt/vol) streptomycin sulfate (Sigma) for 20 min with stirring and centrifuged at $12,000 \times g$ for 15 min. The supernatant fraction was kept on ice and treated sequentially with ammonium sulfate at 40, 60, and 75% saturation, with stirring, for 45 min at each concentration. The solutions were centrifuged as before, and the pellet fractions were assayed for GS activity. The majority of the activity was found in the 60% saturation pellet. This fraction was suspended in 1 M ammonium sulfate in DBMn buffer and then loaded on and eluted from a Reactive Blue 2-Sepharose CL-6B (Sigma) column as described previously (24). The active fractions were pooled, concentrated, and eluted from a Bio-Sil TSK-400 high-pressure liquid chromatography (HPLC) column (Bio-Rad Laboratories, Richmond, Calif.) with 0.1 M Na₂SO₄ and 20 mM NaH₂PO₄, pH 6.8, at 0.8 ml min⁻¹ with an IBM model 9533 chromatography system (IBM Instruments, Danbury, Conn.). A representative purification procedure is presented in Table 1. Electrophoretic separations

of the proteins in various steps of the purification procedure and Western blots of the purified GS are shown in Fig. 1 and 2. Antiserum was prepared in rabbits by standard protocols (13).

Electrophoresis. Proteins were separated by electrophoresis in 10% (wt/vol) polyacrylamide gels with 0.1% (wt/vol) sodium lauryl sulfate (SDS) in 375 mM Tris hydrochloride, pH 8.8. Samples were boiled in 3% (wt/vol) SDS–5% (vol/vol) 2-mercaptoethanol in 188 mM Tris hydrochloride, pH 8.8, to denature proteins before electrophoresis and loaded onto a 7.5% (wt/vol) acrylamide stacking gel. Electrophoresis was done for 4 h at 25 mA in 17 mM Tris hydrochloride–200 mM glycine–0.1% SDS. The separated proteins were visualized by staining with Coomassie blue G or electrophoretically transferred to nitrocellulose (Western blot [4]). The transfer to nitrocellulose was for a minimum of 1,000 mA-h. Portions of the nitrocellulose were stained with amido black to check the efficiency of transfer. The remainder of the nitrocellulose was incubated with 3% (wt/vol) bovine serum albumin to block any additional binding sites. The nitrocellulose was then incubated with rabbit anti-GS antiserum, reacted with goat anti-rabbit antiserum labeled with horseradish peroxidase (Kirkegaard and Perry Labs Inc., Gaithersburg, Md.), and developed with hydrogen peroxide and diaminobenzidine tetrahydrochloride (Kirkegaard and Perry).

Immunoelectrophoresis gels (rocket gels [39]) were 0.5 or 1% (wt/vol) agarose in 50 mM Tris acetate, pH 8.2. Proteins were loaded into wells in a strip containing no antiserum and then transferred into the gel containing rabbit anti-GS antiserum by electrophoresis at 200 V for 12 to 17 h. The gels were washed extensively in 0.1 M NaCl and stained with Coomassie blue G. The rockets were quantitated by integrating the areas under the cross-reactive peaks, and the results for crude extracts were compared with standards of purified GS.

ELISA. The amount of GS protein present in *Nostoc* sp. strain 7801 extracts was determined by using serial dilutions of the above-purified GS as standards in an enzyme-linked immunosorbent assay (ELISA [8]) and comparing these with dilutions of crude extracts from symbiotic or free-living *Nostoc* sp. strain 7801. Purified GS or crude protein extract was bound to polystyrene plates (Gilford Instruments, Oberlin, Ohio), specific rabbit antiserum was reacted with the antigen, goat anti-rabbit immunoglobulin G (IgG) labeled with horseradish peroxidase (Kirkegaard and Perry) was allowed to react with the rabbit antiserum, and 2,2'-azino-di-(3-ethyl-benzthiazoline sulfonate) (Kirkegaard and Perry) substrate was mixed with hydrogen peroxide and reacted with the peroxidase. Absorbance readings at 405 nm were taken every 5 min for 30 min with a Gilford enzyme immunoassay analyzer. The standard curve was prepared by plotting the rate of product formation against the known amount of GS purified from *Nostoc* sp. strain 7801. The standard curve of this ELISA system was linear between 1 and 50 ng of GS protein.

GS activities and antigen concentrations were assayed and normalized to the amount of protein in the supernatant fractions after centrifugation at $20,000 \times g$ for 15 min. Protein was determined by the method of Lowry et al. (17) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

In vitro GS activity. The specific activity of GS, assayed with saturating substrate concentrations, in crude extracts of

TABLE 2. Activity and amount of GS in free-living and symbiotic *Nostoc* sp. strain 7801^a

Growth state	GS sp act (U/mg of protein)		Amount of GS (μ g/mg of protein)	
	N ₂ -grown	NH ₄ ⁺ -grown	N ₂ -grown	NH ₄ ⁺ -grown
Free-living	0.85 $\pm$ 0.05	0.46 $\pm$ 0.05 (54)	68 $\pm$ 9	25 $\pm$ 6 (37)
Symbiotic	0.27 $\pm$ 0.08 (32)	0.22 $\pm$ 0.01 (26)	50 $\pm$ 13 (74)	44 $\pm$ 3 (65)

^a The amount of GS was determined by ELISA with rabbit antiserum to GS purified from *Nostoc* sp. strain 7801. Numbers in parentheses are the percentage of the value for free-living, N₂-grown *Nostoc*. For free-living cultures, values are means $\pm$ SE of 6 (NH₄⁺) or 28 (N₂) experiments; for symbiotic cultures, values are means $\pm$ SE of 3 (NH₄⁺) or 8 (N₂) experiments.

symbiotically grown *Nostoc* sp. strain 7801 was three- to fourfold lower than the activity in extracts of free-living cultures (Table 2). The difference was of the same magnitude as when the rates of in vivo ammonium assimilation by free-living and symbiotic *Nostoc* sp. strain 7801 are compared (19). This result is evidence that the low rate of in vivo ammonium assimilation by symbiotic *Nostoc* sp. strain 7801 is not a consequence of GS substrate limitation. To distinguish transcriptional and posttranscriptional control of GS from a posttranslational mechanism, the enzyme was purified for subsequent immunological analysis.

Purification of GS. The yield and degree of purification of GS from *Nostoc* sp. strain 7801 (Table 1) was nearly identical to the results obtained with *Anabaena* sp. strain 7120 (24). The final fraction was more than 95% pure GS according to densitometer tracings of denaturing protein electrophoresis gels (Fig. 1) and Western blot analysis with rabbit antiserum generated against GS (Fig. 2). The two minor high-molecular-weight bands that cross-reacted in homologous Western blots of crude extracts (Fig. 2, lane 5) were also present when antiserum to GS, highly purified from *E. coli* (3), was used (Fig. 2, lane 3). There was also an

FIG. 1. Electrophoretic analysis of GS purification. Lanes: 1 and 6, molecular weight standards; 2, crude extract (0.5 U); 3, 60% saturated ammonium sulfate pellet fraction (3.5 U); 4, blue-Sepharose column peak (3.5 U); 5, TSK400 column peak (3.0 U). The arrow indicates the GS subunit.

FIG. 2. Western blot analysis of GS and antiserum used in these experiments. Lanes 1 and 2 contain crude extracts of free-living (250 μ g of protein) and symbiotic (200 μ g of protein) *Nostoc* sp. strain 7801, respectively, reacted with preimmune serum; lane 3 contains a crude extract of free-living *Nostoc* sp. strain 7801 reacted with antiserum specific to *E. coli* GS (courtesy of Paul Baumann); lanes 4, 5, and 6 were reacted with antiserum to *Nostoc* sp. strain 7801 GS and contain crude extract of symbiotic (lane 4, 200 μ g of protein) and free-living (lane 5, 250 μ g of protein) *Nostoc* sp. strain 7801 and purified GS from *Nostoc* sp. strain 7801 (lane 6, 35 μ g of protein). Arrows indicate areas of cross-reaction. Three to four units of GS activity were used in each lane.

additional minor band seen with antiserum to *E. coli* GS (Fig. 2, lane 3). Western blots were done six times, and these minor bands varied with each blot. We conclude that these minor bands are most likely aggregates of GS subunits. The subunit molecular weight of *Nostoc* GS was calculated from electrophoretic migration as 55,000; within experimental error, this is the same value as determined for the enzyme from *Anabaena* sp. strain 7120 (24), *Anabaena cylindrica* (32), and *Anacystis nidulans* (10).

Immunological assays. The results of rocket immunoelectrophoresis (Fig. 3) and ELISA (Table 2) with antiserum generated against *Nostoc* sp. strain 7801 GS gave concentrations of 51 (rocket) to 68 (ELISA) μ g of GS per mg of protein in N₂-grown, free-living *Nostoc* sp. strain 7801. In N₂-grown, symbiotic *Nostoc* sp. strain 7801, the values were 35 (rocket) and 50 (ELISA) μ g of GS per mg of protein. The standard controls in ELISA experiments were preimmune serum and linearity with varying concentrations of crude extract. The differences in GS protein between free-living and symbiotic *Nostoc* strains were substantially less than the differences in GS catalytic activity. The results imply that, relative to total soluble protein, synthesis of the GS protein is not highly regulated in this symbiotic *Nostoc* sp. and that

FIG. 3. Immunoelectrophoresis rocket gel. Lanes: 1 and 2, crude extract from symbiotic, N₂-grown *Nostoc* sp. strain 7801; 3 and 4, purified GS; 5 and 6, crude extract from free-living, N₂-grown *Nostoc* sp. strain 7801 (0.5% agarose); 1 and 5, 500 μ g of protein; 2 and 6, 300 μ g of protein; 3 and 4, 24 and 12 μ g of purified GS, respectively. Integration of these peak areas gave values for free-living, N₂-grown *Nostoc* sp. strain 7801 of 54 μ g of GS per mg of protein and symbiotic *Nostoc* sp. strain 7801 of 31 μ g of GS per mg of protein. The mean values calculated from three immunoelectrophoresis gels were 51 and 35 μ g of GS per mg of protein for free-living N₂-grown and symbiotic *Nostoc* sp. strain 7801, respectively.

TABLE 3. Additional control experiments for ELISA

Organism for test antigen	Growth state or strain	Antiserum to GS from:	Amount of GS ($\mu\text{g}/\text{mg}$ of total protein) ^a
<i>Nostoc</i> sp.	Free-living (N ₂)	<i>E. coli</i>	77 $\pm$ 17
	Free-living (NH ₄ ⁺)	<i>E. coli</i>	34 $\pm$ 5
	Symbiotic (N ₂)	<i>E. coli</i>	36 $\pm$ 4
<i>S. typhimurium</i>	TA831	<i>Nostoc</i>	5.9 $\pm$ 0.9
	SK35	<i>Nostoc</i>	0.9 $\pm$ 0.2
<i>A. punctatus</i>	Symbiont-free	<i>Nostoc</i>	8.9 $\pm$ 1.7
<i>Nostoc</i> sp.	Free-living (N ₂)	Preimmune	0

^a Values expressed as mean $\pm$ SE for three or four experiments.

the catalytic activity of GS under these growth conditions is controlled by a posttranslational mechanism.

These data contrast with reports of stringent transcriptional control of GS in the *Anabaena* symbiont of *Azolla* (22, 36) and *Nostoc* spp. in certain lichens (36). Therefore, we devised additional control experiments to test our preliminary conclusion. One control was the use of antiserum to GS purified from *E. coli* (3) in ELISA experiments, while retaining GS purified from *Nostoc* sp. strain 7801 as the standard (Table 3). A matched-pair Student's *t* test with a single-tailed distribution was used to compare results with antiserum against GS purified from *E. coli* with those for GS from *Nostoc* sp. strain 7801. The statistical analysis indicated that there were no significant ($P < 0.05$) differences in the amount of GS in either free-living N₂-grown or symbiotic N₂-grown *Nostoc* sp. strain 7801 with the two different antisera. Therefore, the GS values derived for free-living N₂-grown and for symbiotic N₂-grown *Nostoc* sp. strain 7801 from the two preparations of antiserum were combined. The resulting mean ($\pm$ standard error [SE]) values were 71 $\pm$ 11 and 46 $\pm$ 11 μg of GS per mg of protein for N₂-grown free-living and symbiotic strains, respectively. A statistical analysis identical to that above indicated no significant difference in the amount of GS protein in symbiotic N₂-grown and free-living N₂-grown *Nostoc* sp. strain 7801.

As a further test to demonstrate specific immunological cross-reaction to GS, we examined a mutant strain of *S. typhimurium* (SK35) that has a complete deletion of the GS structural gene and its parental strain (TA831) in our ELISA (Table 3). The parental strain had 5.9 μg of GS per mg of protein, compared with 0.9 μg of GS per mg of protein in extracts of the mutant. The 17% cross-reactivity of protein in the mutant strain relative to the wild-type strain was similar to the cross-reactivity of *A. punctatus* tissue grown without *Nostoc* spp. compared with the symbiotic *Nostoc* sp. strain 7801 value (8.9 versus 68 μg of GS per mg of protein, 13% cross-reaction). Thus, nonspecific cross-reactivity was only about 15% and cannot account for the high and similar values of GS protein we detected in free-living and symbiotic *Nostoc* sp. strain 7801.

Regulation of GS expression and correlation with heterocyst differentiation. The additional experiments confirmed our initial conclusion of posttranslational control of GS expression in symbiotic *Nostoc* sp. strain 7801. At present, we have no information on the nature of the control mechanism. Although GS can be covalently modified (adenylylated) in other gram-negative bacteria (14), there is no precedent for covalent modification of any cyanobacterial GS (9). Thioredoxin modulation of enzyme activity may occur (25).

The in vitro GS activity of a variety of N₂-fixing cyanobacteria has been shown to be about twofold lower in

cultures grown with NH₄⁺ than with N₂ as the sole nitrogen source (6, 21, 23, 30). We confirmed a similar decrease in GS activity of NH₄⁺-grown *Nostoc* sp. strain 7801 and showed that it was accompanied by a decrease in the amount of GS antigen (Table 2). Tumer et al. (37) identified at least two promoter sequences for transcription of the GS structural gene in *Anabaena* sp. strain 7120. One promoter is similar to the consensus *E. coli* promoter sequence and appears to function in *Anabaena* sp. strain 7120 under NH₄⁺ growth conditions. A second promoter has a sequence similar to that of the unique *Anabaena* sp. strain 7120 nitrogenase (*nif*) promoter. The *Anabaena nif*-like promoter is thought to function in elevated transcription of GS under N₂-fixing conditions (37). The difference in concentration of GS antigen from N₂- and NH₄⁺-grown *Nostoc* sp. strain 7801 appears to support the hypothesis of transcriptional control in cyanobacteria.

Growth of N₂-fixing cyanobacteria in the presence of NH₄⁺ has long been known to cause repression of heterocyst differentiation and nitrogenase synthesis (12, 40). However, if GS activity is inhibited by L-methionine-DL-sulfoximine, heterocysts form and nitrogenase is expressed in the presence of NH₄⁺ (35). This observation led to the idea that the high heterocyst frequency in symbiotic cyanobacteria may be a consequence of decreased GS activity (12, 31, 36). Treatment of *Anthoceros-Nostoc* cultures with NH₄⁺ results in a decline in both nitrogenase activity and heterocyst frequency of the associated *Nostoc* sp. strain 7801 (7). However, the specific activity and amount of GS were similar in *Nostoc* sp. strain 7801 from N₂-grown and NH₄⁺-treated symbiotic associations (Table 2). We conclude from these data that GS and its regulation do not play a direct role in heterocyst differentiation under symbiotic growth conditions. This conclusion is consistent with a recent report that GS expression does not vary in symbiotic *Nostoc* spp. along a developmental gradient of increasing heterocyst frequency in the coralloid roots of cycads (16).

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