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GlbN (Cyanoglobin) Is a Peripheral Membrane Protein That Is Restricted to Certain *Nostoc* spp.

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The *glbN* gene of *Nostoc commune* UTEX 584 is juxtaposed to *nifU* and *nifH*, and it encodes a 12-kDa monomeric hemoglobin that binds oxygen with high affinity. In *N. commune* UTEX 584, maximum accumulation of GlbN occurred in both the heterocysts and vegetative cells of nitrogen-fixing cultures when the rate of oxygen evolution was repressed to less than 25 μmol of O_2 mg of chlorophyll a^{-1} h^{-1}. Accumulation of GlbN coincided with maximum synthesis of NifH and ferredoxin $NADP^+$ oxidoreductase (PetH or FNR). A total of 41 strains of cyanobacteria, including 40 nitrogen fixers and representing 16 genera within all five sections of the cyanobacteria were screened for the presence of *glbN* or GlbN. *glbN* was present in five *Nostoc* strains in a single copy. Genomic DNAs from 11 other *Nostoc* and *Anabaena* strains, including *Anabaena* sp. strain PCC 7120, provided no hybridization signals with a *glbN* probe. A constitutively expressed, 18-kDa protein which cross-reacted strongly with GlbN antibodies was detected in four *Anabaena* and *Nostoc* strains and in *Trichodesmium thiebautii*. The *nifU-nifH* intergenic region of *Nostoc* sp. strain MUN 8820 was sequenced (1,229 bp) and was approximately 95% identical to the equivalent region in *N. commune* UTEX 584. Each strand of the DNA from the *nifU-nifH* intergenic regions of both strains has the potential to fold into secondary structures in which more than 50% of the bases are internally paired. Mobility shift assays confirmed that NtcA (BifA) bound a site in the *nifU-glbN* intergenic region of *N. commune* UTEX 584 approximately 100 bases upstream from the translation initiation site of *glbN*. This site showed extensive sequence similarity with the promoter region of *glnA* from *Synechococcus* sp. strain PCC 7942. In vivo, GlbN had a specific and prominent subcellular location around the periphery of the cytosolic face of the cell membrane, and the protein was found solely in the soluble fraction of cell extracts. Our hypothesis is that GlbN scavenges oxygen for and is a component of a membrane-associated microaerobically induced terminal cytochrome oxidase.

Gene expression in prokaryotic cells is influenced markedly by pO_2. Bacteria may use proteins which bind oxygen or which respond to changes in redox potential in order to coordinate their physiological responses during growth under oxic or anoxic conditions. Examples of bacterial heme proteins that can reversibly bind molecular oxygen include VHb in *Vitreoscilla* sp. (28), Hmp in *Escherichia coli* (56), FixL in *Rhizobium meliloti* (15), and GlbN (cyanoglobin) in the cyanobacterium *Nostoc commune* UTEX 584 (42). GlbN is a monomeric hemoglobin that binds oxygen reversibly, with high affinity, and with noncooperativity (54). The gene that encodes cyanoglobin, *glbN*, is positioned between *nifU* and *nifH*. These two *nif* genes are components of two contiguous operons in most cyanobacteria that have been characterized to date, and their products are essential for nitrogen fixation. However, the function of cyanoglobin in nitrogen fixation remains cryptic.

Some filamentous cyanobacteria, including *Nostoc* species, have the capacity to differentiate heterocysts when they are deprived of a source of fixed nitrogen. The structural and biochemical modifications of heterocysts contribute to the development of a cellular environment that is conducive to the sustained activity of nitrogenase under oxic growth conditions (19, 59). In a cell-free system, nitrogenase is rapidly inactivated in air. Certain of the modifications that arise during the differentiation of the cyanobacterial heterocyst may alleviate the effects of potentially toxic oxygen concentrations. These modifications include the loss of photosystem II function (19), the formation of a diffusion barrier with a reduced permeability to gases including oxygen (29, 40), attenuation of oxygen tension through the coupling of diffusion-limited kinetics and respiratory electron transport (39), and modification of nitrogenase to an O_2-insensitive form (51). The maturation of heterocysts can be blocked when mutations are introduced in genes that contribute to the developmental pathway (5, 58). One of these genes, *ntcA*, encodes a transcription factor that is known to positively regulate genes, such as *nirA* and *glnA*, whose expression is negatively regulated by ammonia (31).

A range of non-heterocyst-forming cyanobacteria including unicellular and filamentous forms also have the capacity for nitrogen fixation. These cyanobacteria employ a number of different, incompletely understood, strategies to protect their nitrogenases from inactivation by oxygen (11). Recently, evidence was found for a second functional Nif system, Nif2, which operates in the vegetative cells of *Anabaena variabilis* ATCC 29413 exclusively under anoxic conditions after the cells have been starved of combined nitrogen (50, 53). These find-

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ings underscore the diversity and complexity of nitrogenase synthesis and *nif* regulation in cyanobacteria.

To understand the function of cyanoglobin, we studied the factors that control the synthesis of this protein, determined its distribution in heterocyst-forming and non-heterocyst-forming (nitrogen-fixing) cyanobacteria, and investigated its cellular and subcellular location in *N. commune* UTEX 584.

MATERIALS AND METHODS

Cyanobacterial strains. Cyanobacteria were cultured in BG-11 or BG-11_O medium (46), Allen and Arnon medium (1), or Chu-10 (7) medium as described in the text and figure legends. Descriptions of the conditions of growth for *N. commune* UTEX 584 were provided elsewhere (43). Colonies of a strain of the marine cyanobacterium *Trichodesmium thiebautii* were collected in Atlantic waters 5 km South of Beaufort Inlet, N.C. The cells were fixing atmospheric nitrogen actively at the time of their collection (44).

Taxonomy. The cyanobacterial strains used are listed in Table 1. The strains are grouped according to the classification scheme of Rippka et al. (46). Further details of some of the strains used in this study are found in references 45 and 46, while reference 13 provides further information on more classical aspects of cyanobacterial taxonomy. The attributes that provide a means to discriminate unambiguously between a *Nostoc* or *Anabaena* form are unclear. For this reason, we simply retained the designations that were provided with the strains as we received them for study. For example, we retained the use of the epithet *Anabaena* sp. strain PCC 7120 but are aware that the alternative designation *Nostoc* sp. strain PCC 7120 has been proposed (45).

Growth of cyanobacteria. *N. commune* UTEX 584 and DRH1 and *Anabaena* sp. strain PCC 7120 were grown in 250 ml of liquid medium in 1-liter capacity (internal diameter, 5 cm) airlift fermentors at 32°C. Cultures were sparged continuously with sterile filtered 5% (vol/vol) CO_2 in air (oxic conditions) or with 5% (vol/vol) CO_2 in argon (microoxic conditions). With the use of the latter gas mixture, the growth medium was supplemented with 20× the concentration of Na_2CO_3 specified for BG-11_O medium. Irradiation was provided by banks of fluorescent 15-Watt strip lights (Sylvania). At the time of inoculation of media, the incident photon flux density was approximately 50 μmol of photons m^{-2} s^{-1} at the surface of the fermentors. As cultures became denser, the photon flux density was increased, in steps, such that at the termination of experiments the incident photon flux density was approximately 300 μmol of photons m^{-2} s^{-1}. Cultures of other strains were grown under similar conditions (see figure legends).

Induction of GlbN synthesis. Two methods were used to induce GlbN synthesis, and details are provided in the figure legends.

Nitrogenase activity and oxygen evolution. The growth stage of cells was monitored periodically during growth through light microscopy. Acetylene was generated with calcium carbide, and nitrogenase activity was measured by the acetylene reduction assay technique as described elsewhere (52). Respiratory oxygen uptake was not measured and was not considered upon calculation of the rates of oxygen evolution. The rates of ethylene generation and oxygen evolution were calculated by using purified standards of ethylene and oxygen, respectively. The rates were calculated on the basis of values of total protein, chlorophyll *a* content, and dry weight, which were determined by methods described elsewhere (48).

Isolation and purification of proteins. Protein extracts were obtained either by grinding cells under liquid nitrogen in the presence of Laemmli buffer (30) that had been modified through the addition of protease inhibitors (21) or by passing cells at least twice through a French pressure cell at 110 MPa. Purification of proteins through fast-protein liquid chromatography and subsequent analysis of native or denatured proteins followed published protocols (21, 42).

Cell fractionation. Cell wall and cytoplasmic membrane fractions were obtained from *N. commune* UTEX 584 (cell pellets, 50 g [wet weight]) by the method described by Olie and Potts (41). Purified heterocyst preparations were obtained from *A. variabilis* ATCC 29413 as described by Häfele et al. (17).

Purification of antisera. *glbN* was expressed in *E. coli* BL21 DE3(pGlbN) (42), and purified recombinant GlbN was used to raise polyclonal antibodies in rabbits. Three different procedures were used to purify the antiserum. In the first procedure, 1 ml of the antiserum was incubated at room temperature in a 35× 10-mm petri dish (Falcon) with 2-cm^2 nitrocellulose sheets that had been allowed to adsorb a cell lysate of *E. coli* BL21 DE3 (23). This antiserum is referred to as antiserum A and was used in Western analyses (see below). For immunocytochemical analyses, antiserum A was purified further by a modified form of the technique described by Gruber and Zingales (16). Antiserum A was diluted 1:50 in a mixture of 10 mM Tris-HCl (pH 7.5)–100 mM NaCl–1 mM EDTA (TBS) and 1% (wt/vol) bovine serum albumin and was then incubated with autoclave- and formalin-treated bacterial cells. One batch of antiserum A was treated with cell preparations from *E. coli* BL21 DE3(pTrc99A) only; this is referred to as antiserum B. A second batch of antiserum A was treated with cell preparations from both *E. coli* BL21 DE3(pTrc99A) and *Anabaena* sp. strain PCC 7120; this is referred to as antiserum C.

Western analyses. For analytical purposes, proteins were resolved under denaturing conditions in the presence of sodium dodecyl sulfate (SDS) in 0.75-mm-thick 15% (wt/vol) polyacrylamide gels with the use of a Mighty Small II SE 250 gel apparatus (Hoefer). Proteins were visualized using either Coomassie blue or silver staining (35). Western blotting (immunoblotting) was performed with either of two published protocols (34, 49). The concentrations of primary antibodies are given in the figure legends. The secondary antibodies were goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (affinity purified; Bio-Rad) used at a dilution of between 1:1,000 and 1:3,000 for up to 4 h at room temperature.

Southern analysis. Genomic DNA was obtained from the different cyanobacteria following the procedure described by Jackman and Mulligan (24) or that described by Xie and Potts (60). The conditions for transfer to Hybond (Amersham) or Gene Screen (NEN Research Products), preparation of digoxigenin-labelled or radioactively labelled probes, hybridization, washing, and probe detection were as described elsewhere (24, 60).

Cloning, nucleotide sequencing, and analysis. Restriction enzymes, other enzymes, and materials used in molecular cloning (obtained variously from New England Biolabs, Life Technologies, Boehringer Mannheim, Pharmacia, Promega, Stratagene, and U.S. Biochemicals), DNA manipulations, and analyses were performed according to standard procedures (4, 47). Analysis of nucleotide sequences was carried out with DNAlysis (W. Buikema, University of Chicago), MulFold, and LoopDLoop (14, 25, 26, 61).

Gel retardation assays. Plasmid pNDH1 (Table 1) (9) was digested with *Alu*I, and a 781-bp fragment, corresponding to bases 262 to 1042 described by Angeloni and Potts (2), was isolated and subcloned in pGEM-4 as pMVT1. The 781-bp fragment was then digested with *Dpn*I to generate a 445-bp fragment corresponding to bases 598 to 1042 (subcloned in pGEM-4 as pMVT2). Five picomoles (approximately 0.7 μg) of the 445-bp fragment was incubated with 25 μCi of [γ-^{32}P]dATP μl^{-1} in the presence of 10 U of T4 polynucleotide kinase in a reaction volume of 25 μl. Gel retardation assays were completed with the end-labelled 445-bp fragment (see Fig. 5A) and NtcA-enriched lysates according to the protocols described by Luque et al. (31) and Ausubel et al. (4).

Electron microscopy. General techniques for the embedding, fixing, and sectioning of cyanobacterial cells for electron microscopy and or immunocytochemical analysis were as described elsewhere (21, 22); further details are provided in figure legends.

Nucleotide sequences. The nucleotide sequence of a 1,229-bp region of *Nostoc* sp. strain 8820 containing *glbN* and the partial coding regions of *nifU* and *nifH* was deposited in the GenBank Nucleic Acid Database under accession number L47979. A 3,525-bp sequence containing the *nifU-glbN-nifD-nifH* region of *N. commune* UTEX 584 has been deposited under accession number L23514 (locus NOSMOFENIF [2]).

RESULTS

Synthesis of GlbN, NifH, and PetH by *N. commune* UTEX 584. We first studied the synthesis of GlbN, together with that of dinitrogenase reductase (NifH) and ferredoxin $NADP^+$ oxidoreductase (PetH or FNR), under oxic and microoxic conditions. NifH synthesis provides a marker for *nif* expression. PetH transfers electrons from NADPH to FdxH, the immediate electron donor to NifH (nitrogenase) in heterocysts (see reference 18).

When *N. commune* UTEX 584 was grown with 5% (vol/vol) CO_2 in air, either in continuous light or under a light-dark (12 h:12 h) cycle for periods of up to 276 h in BG-11_O, neither GlbN, NifH, nor PetH was detectable (FNR [Fig. 1]). Lysates from cells that were incubated for 60 h following microoxic stepdown provided visually conspicuous cross-reactions with NifH antibodies upon immunoblotting (Fig. 1). The increase in the amount of NifH in cells was accompanied by an increase in the capacity of the cells to reduce acetylene with rates of ethylene generation between 6.0 and 10.9 nmol of ethylene evolved μg of chlorophyll a^{-1} min^{-1}. GlbN was detectable—the reaction on the blots was weak and sometimes only just discernible to the eye—within approximately 24 h after the imposition of microoxic conditions. However, only extracts from cells that had been incubated under microoxic conditions for at least 108 h provided strong cross-reactions with GlbN- or PetH-specific antibodies (Fig. 1). These extracts contained the greatest amounts of NifH and contained additional polypeptides that cross-reacted with NifH. Further experiments confirmed that the latter polypeptides arose through degradation

TABLE 1. Strains and plasmids used[a]

Strain or plasmid	Description	*glbN*	GlbN	18-kDa protein	Source or reference
Section I					
Synechococcus sp. strain PCC 7942	Coccoid, nonheterocystous, does not fix N_2		−[b]	−	UK
Synechocystis sp. strain BO 8402	Isolated from the Bodensee by A. Ernst; coccoid, nonheterocystous, microaerobic N_2 fixation		−	−	UK
Section II					
Chroococcidiopsis sp.	Coccoid, nonfilamentous, microaerobic N_2 fixation; isolated by S. Scherer		−	−	UK
Section III					
Oscillatoria sp. strain PCC 7515	Filamentous, nonheterocystous, microaerobic; facultative photoheterotroph	−			PCC
Phormidium foveolarum B1462-1	Filamentous, nonheterocystous, microaerobic N_2 fixation; probably equivalent to strain B1442-1		−	−	UK
Phormidium foveolarum B1442-1	Filamentous, nonheterocystous, microaerobic N_2 fixation	−			R. Haselkorn
Pseudanabaena sp. strain PCC 7403	Filamentous, nonheterocystous, microaerobic N_2 fixation	−			PCC
Trichodesmium sp.	Filamentous, nonheterocystous, aerobic N_2 fixation; collected in Atlantic, 5 km south of Beaufort Inlet, N.C.		−	+[c]	H. Paerl
Section IV					
Anabaena sp. strain PCC 7120	Filamentous, heterocysts, aerobic N_2 fixation; =ATCC 27347 +ATCC 27893	−	−	+[c]	J. Elhai
Anabaena sp. strain PCC 7120	Filamentous, heterocysts, aerobic N_2 fixation; =ATCC 27347 =ATCC 27893	−			R. Haselkorn
Anabaena sp. strain PCC 7118	Filamentous, Het^- Nif^+ (microaerobic); =*Nostoc muscorum* =ATCC 27892		−	+[c]	ATCC
Anabaena sp. strain PCC 7119	Filamentous, heterocysts, aerobic N_2 fixation; =*Nostoc* sp. =ATCC 29151		−	+[c]	ATCC
Anabaena variabilis ATCC 29413	Filamentous, heterocysts, aerobic N_2 fixation; =PCC 7937	−	−	+[c]	UK
Anabaena sp. strain CH1	Filamentous, heterocysts, aerobic N_2 fixation		−	−	UK
Anabaena sp. strain CA	Filamentous, heterocysts, aerobic N_2 fixation; =ATCC 33047	−			R. Haselkorn
Anabaena sp. strain L31	Filamentous, heterocysts, aerobic N_2 fixation; from S. Apte-J. Thomas	−			R. Haselkorn
Anabaena sp. strain PCC 77S15	Filamentous, heterocysts, aerobic N_2 fixation; from C. Franche	+[d]			R. Haselkorn
Anabaena torulosa	Filamentous, heterocysts, aerobic N_2 fixation; from S. Apte-J. Thomas	−			R. Haselkorn
Anabaena azollae 1a	Filamentous, heterocysts, aerobic N_2 fixation	−			R. Haselkorn
Nodularia sp. strain PCC 73104	Filamentous, heterocysts, aerobic N_2 fixation; (slow growth); facultative heterotroph	+[d]			PCC
Cylindrospermum sp. strain PCC 7604	Filamentous, heterocysts, aerobic N_2 fixation	+[d]			PCC
Nostoc commune UTEX 584	Filamentous, heterocysts, aerobic N_2 fixation	+	+	+[c]	VPI
Nostoc sp. strain MUN 8820	Filamentous, heterocysts, aerobic N_2 fixation; isolated as minor contaminant from culture of *Oscillatoria tenuis* SAUG 1459-1	+			H. Böhme
Nostoc sp. strain 840215	Filamentous, heterocysts, aerobic N_2 fixation; origin unknown	+			R. Haselkorn
Nostoc sp. strain UCD 7801	Filamentous, heterocysts, aerobic N_2 fixation	+			J. Meeks
Nostoc sp. strain MACR2	Filamentous, heterocysts, aerobic N_2 fixation; isolated by J. Meeks	+[d]			R. Haselkorn
N. commune DRH1	Filamentous, heterocysts, aerobic N_2 fixation; isolated by Donna Hill		−	+[c]	VPI
Nostoc sp. strain ATCC 27896	Filamentous, heterocysts, aerobic N_2 fixation; =PCC 6310		−	−	C. P. Wolk
Nostoc sp. strain ATCC 27897	Filamentous, heterocysts, aerobic N_2 fixation	+			R. Haselkorn
Nostoc sp. strain ATCC 29107	Filamentous, heterocysts, aerobic N_2 fixation	−			R. Haselkorn
Nostoc sp. strain ATCC 29133	Filamentous, heterocysts, aerobic N_2 fixation; =PCC 73102		−	−	C. P. Wolk
Nostoc sp. strain 268	Filamentous, heterocysts, aerobic N_2 fixation; facultative heterotroph (on fructose); isolated by T. Vaara from the Baltic Sea		+[e]		K. Jäger
Nostoc gunnera 8001	Filamentous, heterocysts, aerobic N_2 fixation; cyanobiont of *Gunnera albocarpa*, isolated by J. Horstmann; collection of J. Meeks		−[e]		K. Jäger

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Description	*glbN*	GlbN	18-kDa protein	Source or reference
Nostoc commune SAUG 1453-3	Filamentous, heterocysts, aerobic N_2 fixation; may be same strain as *N. commune* UTEX 584; low frequency of heterocysts		+[e]		K. Jäger
Nostoc sp. strain PCC 9229	Filamentous, heterocysts, aerobic N_2 fixation		+[e]		K. Jäger
Scytonema sp. strain PCC 7110	Filamentous, heterocysts, aerobic N_2 fixation; distinct aerial growth; facultative heterotroph	+[d]			PCC
Tolypothrix sp. strain SAUG 1410-1	Filamentous, heterocysts, aerobic N_2 fixation; assignment questionable—received as *Calothrix membranaceae* from H. Böhme and subsequently classified as a *Tolypothrix* strain (45a)	+[d]			R. Haselkorn
Calothrix sp. strain PCC 7102	Filamentous, heterocysts, aerobic N_2 fixation	−			PCC
Section V					
Fischerella sp. strain PCC 7414	Filamentous, heterocysts, aerobic N_2 fixation	−			PCC
Fischerella sp. strain SAUG 1447-1	Filamentous, heterocysts, aerobic N_2 fixation; may be equivalent to PCC 7414		−	−	UK
Chlorogloeopsis sp. strain PCC 6912	Filamentous, heterocysts, aerobic N_2 fixation; facultative heterotroph	−			PCC
Recombinant plasmids					
pNDH1	2.4-kb *Eco*RI-*Hin*dIII fragment cloned in pBR322, containing *nifU-glbN-nifH* of *N. commune* UTEX 584 (GenBank accession number L23514)				9
pMVT1	781-bp *Alu*I fragment from pNDH1 cloned in pGEM-4				This study
pMVT2	445-bp *Dpn*I-*Alu*I fragment from pMVT1 cloned in pGEM-4 (putative promoter region of *glbN*)				This study
pCS126	964-bp *Msc*I-*Pvu*II fragment containing a truncated form of *Synechococcus* sp. strain PCC 7942 (*ntcA*) in pTrc99A; obtained from A. Herrero				31
pTrc99A	4.2-kb; Amp^r *lacI*^{q} P_{trc} *rrnBT1T2*; obtained from Pharmacia Biotech, Piscataway, N.J.				

[a] The classification scheme for strains proposed by Rippka et al. (46) was used. MUN, Memorial University of Newfoundland; UK, Universität Konstanz; PCC, Pasteur Culture Collection; VPI, Virginia Tech; ATCC, American Type Culture Collection.
[b] −, no immunorelated protein detected.
[c] +, immunorelated protein of 18 kDa detected.
[d] Weak hybridization under moderate stringency.
[e] Cells of *Nostoc* sp. strain 268, *Nostoc gunnera* 8001, *Nostoc commune* SAUG 1453-3, and *Nostoc* sp. strain PCC 9229 were grown in BG-11_O in airlift fermentors similar to those used in the present study, with GlbN induction by using sparging with argon. GlbN was detected through Western blotting with GlbN antiserum A used at a dilution of 1:7,000 (26a).

of NifH by an activity that was insensitive to the proteolytic inhibitors present in extraction buffers (data not shown).

GlbN and *glbN* homologs in *Anabaena* and *Nostoc* spp. No protein with a size equivalent to that of *N. commune* UTEX 584 GlbN was made by any cyanobacterial strain when tested under a wide range of growth conditions. When grown under conditions that induced synthesis of GlbN in *N. commune* UTEX 584, cell extracts from *Anabaena* sp. strains PCC 7120, PCC 7118, and ATCC 29413 and *N. commune* DRH1 each provided a specific, conspicuous cross-reaction with GlbN antiserum A (Fig. 2A and B). Extracts from cells of *Nostoc* strains ATCC 27896 and ATCC 29133, grown under the same conditions, provided no such cross-reaction (Fig. 2B). In each case in which a reaction was noted, it corresponded to a single polypeptide of 18 kDa (Fig. 2A and B). Careful inspection of Western blots suggested that the cross-reacting 18-kDa protein may be a doublet (e.g., Fig. 2A, lanes b, d, e, and i; Fig. 2C, lanes a, b, and c), but attempts to improve the resolution of the two bands were unsuccessful. In experiments that used different detergents and chaotropic agents, which were designed to extract and resolve proteins under different buffer conditions, no evidence was obtained to suggest that the reaction at 18 kDa reflected the association of a smaller protein with other proteins and/or membrane complexes.

Further studies of the synthesis of the 18-kDa protein in *A. variabilis* ATCC 29413 and *Anabaena* sp. strain 7119 (*Nostoc muscorum*) indicated that synthesis of this protein was constitutive, i.e., synthesis was not influenced either by pO_2, N source, or light or dark conditions in both strains (Fig. 2C). However, some variation was noted in the relative abundance of the 18-kDa protein in different experiments. For example, cultures of *N. commune* UTEX 584 grown in fermentors with controlled gassing contained no detectable protein of this size (Fig. 1). However, small-scale cultures, subjected to intermittent gassing, sometimes did (Fig. 2A, lane b). Experiments to characterize the amino acid sequence of the 18-kDa protein purified from *Anabaena* sp. strain PCC 7120 were unsuccessful because the N terminus was found to be blocked.

No cross-reactive proteins were detected in a broad range of heterocyst-forming and non-heterocyst-forming strains, including *Fischerella* sp. strain SAUG 1447-1, *Anabaena* sp. strain CHI, *Phormidium foveolarum* B1462-1, *Synechococcus* sp.

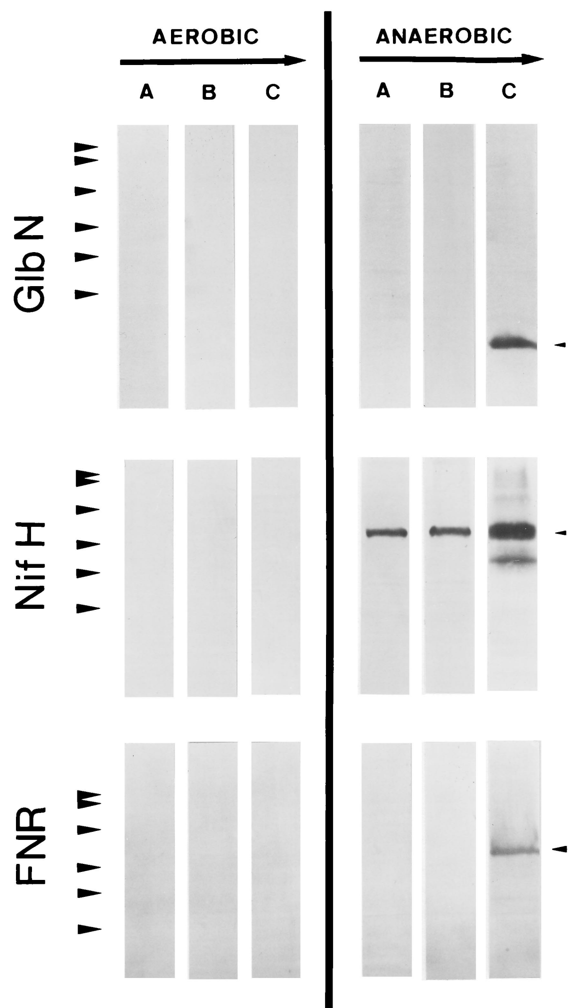

FIG. 1. GlbN, NifH, and PetH (FNR) accumulate in *N. commune* UTEX 584 cells following microoxic stepdown. Cultures of *N. commune* UTEX 584 were grown in fermentors at 32°C in BG-11_O. Two cultures were initially grown under oxic conditions; one was subjected to microoxic stepdown (5% [vol/vol] CO_2 in argon) after 168 h, while the other was maintained under oxic gas conditions. In this experiment, cultures were grown with a light-dark cycle (12 h:12 h). Cell lysates were prepared from both cultures 60 (lanes A), 84 (lanes B), or 108 (lanes C) h following the time at which one culture was subjected to microoxic stepdown. Approximately 10 μg of each extract was resolved in a 15% (wt/vol) SDS-PAGE gel. Replicate Western blots were developed with either GlbN antiserum A or PetH (FNR)- or NifH-specific antibodies at primary dilutions of 1:2,500, 1:800, and 1:800, respectively. The pattern of bands shown is representative of that observed in three separate trials, with cells grown under these conditions. Molecular mass markers in kilodaltons (18, 27, 32, 49, 80, and 108, in ascending order) are indicated to the left of each panel. Arrows indicate the major bands detected with the respective antisera.

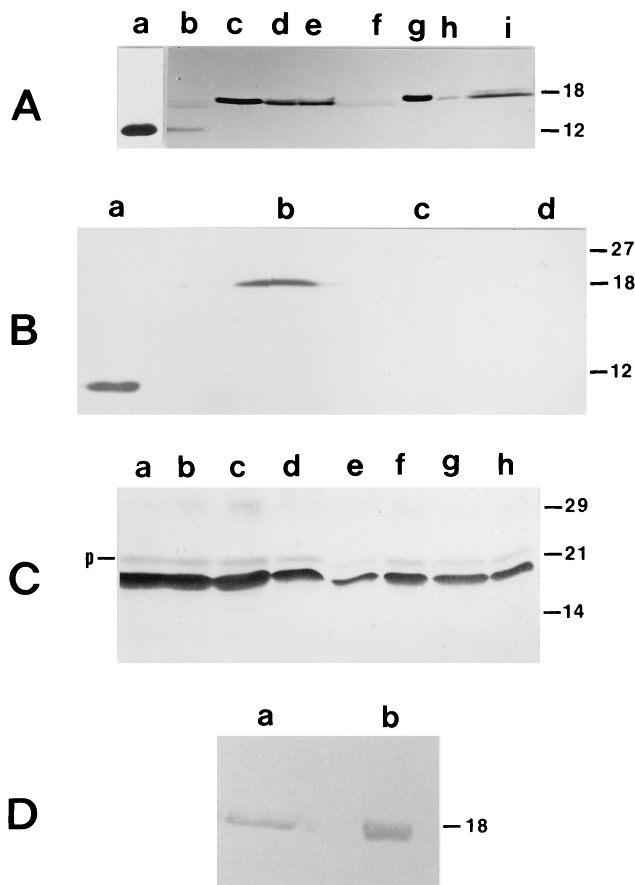

FIG. 2. *Anabaena* and *Nostoc* spp. contain a polypeptide that cross-reacts with GlbN-specific antibodies. (A) Protein extracts were prepared and resolved as described in the legend to Fig. 1. Western blots were developed with GlbN antiserum A at a primary dilution of 1:2,500. Western blots of total protein extracts from *N. commune* UTEX 584 (lanes b and f), *A. variabilis* ATCC 29413 (lanes c and g), *Anabaena* sp. strain PCC 7120 (lanes d and h), and *Anabaena* sp. strain PCC 7118 (lanes e and i) are shown. Strains were grown in BG-11_O under oxic gas conditions for approximately 168 h until they achieved logarithmic growth. Some aliquots of the cell suspensions were subjected to microoxic stepdown and incubated under microoxic conditions for 96 h (lanes b, c, d, and e); other aliquots of the cell suspensions were maintained under oxic conditions for 96 h (lanes f, g, h, and i). Lane a, 20 ng of purified recombinant GlbN. (B) Western blot of total protein extracts from *N. commune* DRH1 (lane b), *Nostoc* sp. strain ATCC 27896 (lane c) and *Nostoc* sp. strain ATCC 29133 (lane d). The conditions of growth and induction of GlbN synthesis were as in panel A. Lane a, 20 ng of purified recombinant GlbN. (C) Western blot of total protein extracts from *A. variabilis* ATCC 29413 (lanes a, b, c, and d) and *Anabaena* sp. strain PCC 7119 (lanes e, f, g, and h). In this experiment, cells were grown in fermentors under continuous light with Allen and Arnon media in the presence (lanes a and b and e and f) or absence (lanes c and d and g and h) of ammonia. Cells were washed and resuspended in fresh media, and aliquots of the cell suspension were transferred to 20-ml capacity glass bottles and incubated either under anoxic conditions for 16 h (lanes a and f and d and h) or under oxic conditions for 16 h (lanes b and e and c and g). A purified preparation of heterocysts from *A. variabilis* ATCC 29413 gave cross-reactions similar to those shown in lane c (data not shown). No heterocysts were observed in cultures grown in the presence of ammonia. All cultures grown in the absence of ammonia reduced acetylene. p (arrow), the position of pigmented phycobiliproteins (i.e., these are not signals due to immunoblotting). (D) Western blot of total protein extracts from colonies of *T. thiebautii* that actively fixed nitrogen in situ. Samples were prepared following treatment of the cells with guanidinium isothiocyanate (lane a) or by direct extraction of proteins in SDS-PAGE buffer (lane b).

strain 7942, and *Synechocystis* sp. strain BO 8402 (data not shown; Table 1). In contrast, colonies of a strain of the non-heterocyst-forming filamentous marine bloom former *T. thiebautii* that were collected in situ and which were actively fixing nitrogen did contain the 18-kDa protein (Fig. 2D). Like GlbN, the 18-kDa protein from *T. thiebautii* had a mobility in SDS-PAGE gels which was unaffected by any lipid-soluble components present in the cell extracts (Fig. 2D).

glbN is not present in the *nifU-nifH* intergenic region of the independent isolates, i.e., *Anabaena* sp. strain PCC 7120, *Anabaena* sp. strain L31, *Anabaena azollae* 1a, and *Plectonema boryanum* (12, 18, 24, 37, 38). However, in view of the finding of the 18-kDa protein which cross-reacted with GlbN antiserum, we investigated whether *glbN* was present in these and a variety of other nitrogen-fixing cyanobacteria by Southern hybridization using a *glbN* gene fragment from *Nostoc* sp.

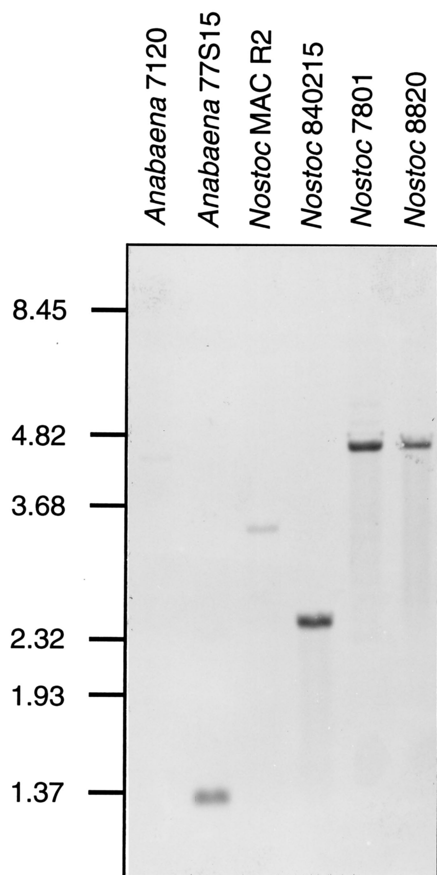

FIG. 3. Distribution of *glbN* in *Anabaena* and *Nostoc* strains. *Hin*dIII digests of genomic DNA from *Anabaena* sp. strain PCC 7120, *Anabaena* sp. strain PCC 77S15, *Nostoc* sp. strain MAC R2, *Nostoc* sp. strain 840215, *Nostoc* sp. strain UCD 7801, and *Nostoc* sp. strain 8820 were analyzed by Southern hybridization with a digoxigenin-labelled 270-bp *Alu*I-*glbN* fragment from *Nostoc* sp. strain 8820 as a probe. Bacteriophage lambda *Bst*EII fragments were used as size markers. Bound probe was detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody and then by colorimetric detection.

strain 8820 as a probe. A single strong signal was observed with genomic digests of *Nostoc* sp. strain 840215, *Nostoc* sp. strain UCD 7801, and *Nostoc* sp. strain 8820 (Fig. 3) and with *Nostoc* sp. strain ATCC 27897 (data not shown). The single band observed suggested that *glbN* was present in a single copy in these three strains, as it is in *N. commune* UTEX 584. Weakly hybridizing bands were detected in genomic digests of six strains of cyanobacteria of section IV (*Nostoc* sp. strain MAC R2, *Anabaena* sp. strain PCC 77S15, *Cylindrospermum* sp. strain PCC 7604, *Scytonema* sp. strain PCC 7110, *Tolypothrix* sp. strain SAUG 1410-1, and *Nodularia* sp. strain PCC 73104 [Table 1; Fig. 3]). Preliminary nucleotide sequence data indicate that no *glbN* gene is present between *nifU* and *nifH* in *Anabaena* sp. strain PCC 77S15 (36). No significant hybridization signals were detected either in *Anabaena* sp. strain 7120 (with separate isolates of this strain and when either radioactively or nonradioactively labelled probes were used), *Calothrix* sp. strain PCC 7102, or any strains from sections III or V (Table 1).

The *glbN* region in *Nostoc* sp. strain 8820. A 4.5-kb *Hin*dIII fragment containing the *nifH* gene was isolated from a size-directed library of *Nostoc* sp. strain 8820 *Hin*dIII fragments cloned in pUC18. The nucleotide sequence of a 1,229-bp region of this fragment was determined and compared with the equivalent region in *N. commune* UTEX 584. *Nostoc* sp. strain 8820 contains *glbN* positioned between *nifU* and *nifH* as in *N. commune* UTEX 584, and the coding regions of these genes are very similar in both strains: *nifU*, *glbN*, and *nifH* are 98.7, 95.5, and 98.2% identical, and NifU, GlbN, and NifH are 98.7, 95.8, and 97.3% identical, respectively.

The *glbN-nifH* and *nifU-glbN* intergenic regions of *N. commune* UTEX 584 contain a number of structural elements (2). The *glbN-nifH* regions of *Nostoc* sp. strain 8820 and *N. commune* UTEX 584 are almost identical in length (1-bp difference), with just 11 differences in 350 bp—an overall identity of 97%. All of the structural elements noted in the *glbN-nifH* region of *N. commune* UTEX 584 are present in the equivalent region of *Nostoc* sp. strain 8820. The following additional features and differences are annotated in the database entry: (i) a stem-loop structure (8 paired bases) (-11.9 kcal [1 cal = 4.184 J] mol^{-1}) which contains the upstream activating sequence noted by Angeloni and Potts (2), (ii) a secondary structure (17 paired bases [-18.6 kcal mol^{-1}]) formed by five copies of the short tandem repeat sequences (STRR) ACAAAG(A/G) (three copies are found in *N. commune* UTEX 584), and (iii) the highly iterated palindromic repeat (HIP1) sequence GCG ATCGC that forms part of a third possible secondary structure (24 paired bases [-23.4 kcal mol^{-1}]).

The *nifU-glbN* intergenic regions of the two strains differ both in their lengths and in their nucleotide sequences. The region is 226 bp in length in *Nostoc* sp. strain 8820 and 257 bp in length in *N. commune* UTEX 584. The overall sequence identity is 74.2% because of 190 bp that are identical between the two strains. Each *nifU-glbN* region has three distinct domains: (i) a conserved domain immediately following *nifU* that is 115 bp in length with 91.3% identity between the two strains, (ii) a conserved domain prior to *glbN* that is 86 bp in length with 83.7% identity between the two strains, and (iii) a central variable domain in which the major differences between the two strains occur.

The central domain is 25 bp in length in *Nostoc* sp. strain 8820 and 59 bp in length in *N. commune* UTEX 584 (positions 116 to 140 of *Nostoc* sp. strain 8820 and positions 115 to 173 of *N. commune* UTEX 584 [Fig. 4]). In *N. commune* UTEX 584, the domain includes two stretches of 14 and 20 nucleotides (nt) which are absent from *Nostoc* sp. strain 8820. Furthermore, in *N. commune* UTEX 584, the variable domain contains four copies of the STRR sequence AATTACG; no copies are found in *Nostoc* sp. strain 8820. Each single strand of the entire *nifU-glbN* intergenic region of the two strains is capable of forming an extensive secondary structure (Fig. 4). In *Nostoc* sp. strain 8820, 132 of 226 bases (58% of the total) are paired, with a free energy of -39.0 kcal mol^{-1}; in *N. commune* UTEX 584, 140 of 257 bases (54.5%) are paired, with a free energy of -44.0 kcal mol^{-1}. Apart from stem-loop structures, no such extensive secondary structure was detected in the *glbN-nifH* intergenic regions of *Nostoc* sp. strain 8820 or *N. commune* UTEX 584 or in the *nifU-nifH* intergenic region of *Anabaena* sp. strain PCC 7120.

Binding of NtcA to the 5′ region of *glbN*. The nucleotide sequence approximately 100 nt upstream of the *glbN* translation initiation codon in *N. commune* UTEX 584 showed similarity with the sequence of the NtcA-binding site in the putative *glnA* promoter of *Synechococcus* sp. strain 7942 (8, 31) (Fig. 5A). The similarity included the presence of 5′TAGG3′ at a position 22 bases downstream of the 5′TAC3′ element in the binding site but with the 5′GTA3′-5′TAC3′ elements of the binding-site motif separated by 7 (not 8) bp (Fig. 5A). Further similarities included a complete correspondence between the *N. commune* UTEX 584 half-site sequence 5′ATT

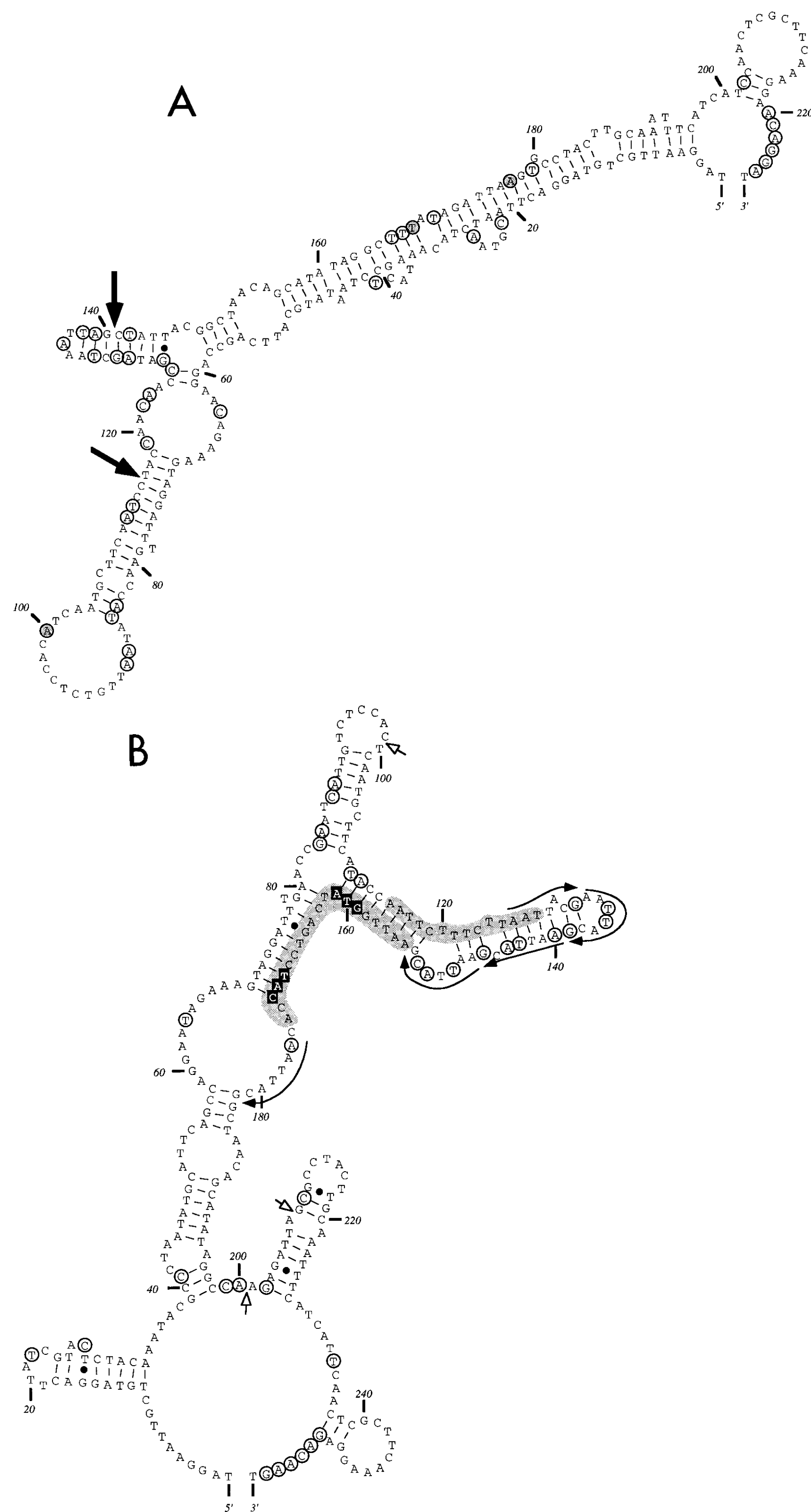

FIG. 4. The *nifU-glbN* intergenic regions of *Nostoc* sp. strain 8820 and *N. commune* UTEX 584 have extensive secondary structures. The nucleotide sequence of the *nifU-glbN* intergenic region of *Nostoc* sp. strain 8820 (A) and *N. commune* UTEX 584 (B) were folded by using the program Mulfold, displayed with the program loopDloop, and annotated by hand. Circled positions indicate differences in the two sequences. Circled and shaded bases in *Nostoc* sp. strain 8820 are not found in *N. commune* UTEX 584, but their positions are indicated by short open arrows for the latter. Large solid arrows in panel A indicate the sites of the large insertions in the *N. commune* UTEX 584 sequence which are indicated by shaded regions in panel B. The STRR sequence, AATTACG, is indicated by arrows. The NtcA recognition sequence is indicated by reverse type. Numbering starts with the first base of the intergenic region.

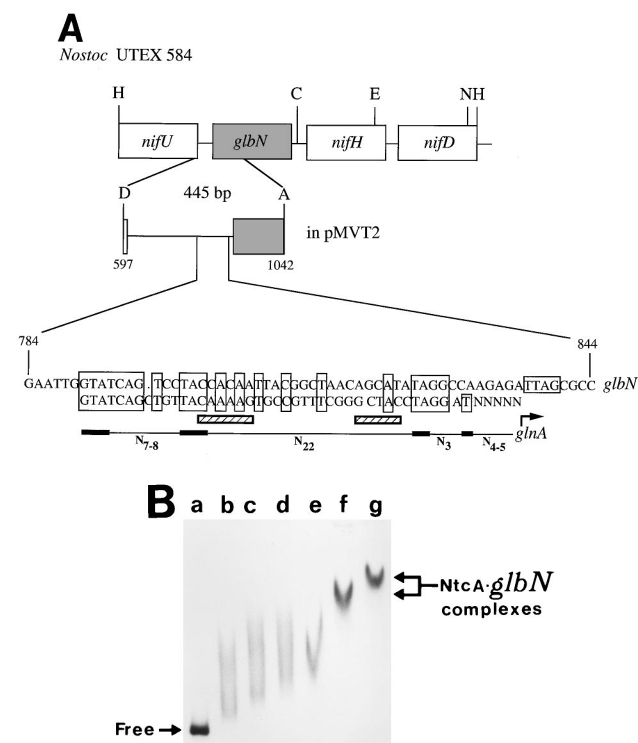

FIG. 5. The *nifU-glbN* intergenic region of *N. commune* UTEX 584 binds NtcA. (A) Restriction map of the *nifU-glbN* region of *N. commune* UTEX 584 showing the location of an NtcA-binding site upstream of *glbN*. Sites for restriction enzymes *Hin*dIII (H), *Cla*I (C), *Eco*RI (E), *Nhe*I (N), *Alu*I (A), and *Dpn*I (D) are indicated. The nucleotide sequence of the NtcA-binding site of *N. commune* UTEX 584 (upper sequence) is compared with that in the *glnA* region of *Synechococcus* sp. strain PCC 7942 (lower sequence). Identities between the two sequences are boxed. Conserved NtcA-binding site elements (31) are indicated by black lines together with the spacing of the nucleotides. The putative *glnA* promoter (8) in *Synechococcus* sp. strain PCC 7942 is indicated by hatched underlining; the transcription start point of *glnA* is indicated by an arrow. The rightmost TTAG box in the *glbN* sequence (positions 837 to 840) corresponds to a TTAG sequence very close to the transcription start point of *glnA* from *Anabaena* sp. strain PCC 7120. Base 844 lies 45 bases upstream from the A of the ATG translation initiation codon of *N. commune* UTEX 584 *glbN*. (B) *Synechococcus* sp. strain 7942 NtcA binds to the 445-bp intergenic *Dra*I-*Alu*I fragment. Signals in the autoradiogram derive from the ^{32}P-labelled 445-bp fragment from pMVT2 (see panel A). Each lane contains approximately 20,000 cpm of target DNA (lane a [DNA only]), together with either 1 (lane b), 6 (lane c), and 12 (lane d) μg of cell lysate from DH5α(pTrc99A) or 1.5 (lane e), 6 (lane f), and 12 (lane g) μg of cell lysate from DH5α(pCS126).

GGTA3′ and the NtcA half-site proximal to the coding region of *nir* (5′ATTGGTA3′) in *Synechococcus* sp. strain 7942 (31; Fig. 5A). No such sequence similarity could be detected in *Nostoc* sp. strain 8820.

Mobility shift experiments were carried out to determine whether NtcA could bind to the putative NtcA binding site. A single, well-resolved band, with a marked retardation in mobility, was resolved in reactions that contained between 6 to 12 μg of protein extract from *E. coli* BL21 DE3(pCS126) which were induced for synthesis of NtcA and a purified ^{32}P-labelled 445-bp DNA restriction fragment from the upstream region of *glbN* from *N. commune* UTEX 584 (Fig. 5A and B). This retardation was inhibited in reactions that were supplemented with between 100 and 300 ng of competitor DNA. With 300 ng of competitor DNA, the retardation of mobility and appearance of the signal were comparable to those shown in lane e of Fig. 5B. Footprint analyses confirmed that partially purified NtcA bound to the target DNA indicated in Fig. 5A (55). Nonspecific retardation of migration of the 445-bp fragment, visualized as a smear on autoradiograms, occurred with reactions the mixtures for which contained protein extracts from *E. coli* BL21 DE3(pTrc99A). As judged from the degree of smearing that was examined in multiple trials, this nonspecific retardation was saturated in reaction mixtures that contained approximately 0.5 ng of the target DNA fragment and 12 μg of protein extract.

Cellular and subcellular location of GlbN in *N. commune* UTEX 584. The intracellular location of cyanoglobin in *N. commune* UTEX 584 cells was examined by electron microscopy by immunogold labelling with three different GlbN antisera. Thin sections of cells that were incubated for 12 h following microoxic stepdown were labelled heavily when antiserum A was used (Fig. 6A). The deposition of particles was associated only with cells, and no (or only a very rare) labelling of the extracellular sheath was detected (Fig. 6A). Labelling was detected in heterocysts and vegetative cells in approximately equivalent amounts. The labelling pattern included a random distribution throughout the cytoplasm as well as a conspicuous deposition around the periphery of the cells (Fig. 6A). The latter represented approximately 66% of the total deposition in each cell. Sections of the same cells were labelled with fewer gold particles when GlbN antiserum B was used at the same dilution as antiserum A. The deposition of gold particles at the periphery of the cells was conspicuous and accounted for approximately 84% of the gold particles present (Fig. 6B). The amount of labelling was reduced still further when GlbN antiserum C was used at the same dilution (data not shown). In this case, the deposition of gold particles at the periphery of the cells accounted for approximately 90% of the total number of gold particles present. The depletion in titer of GlbN-specific antibodies in antiserum C was expected in view of the presence of the 18-kDa protein in *Anabaena* sp. strain PCC 7120 cell extracts that were used to cross-adsorb the serum (Materials and Methods). The use of serum that was preadsorbed with purified recombinant GlbN left sections that were completely devoid of gold particles—a result which was consistent with the lack of cross-reactivity when preimmune serum was used (data not shown).

The degree of labelling of cells that were grown under oxic conditions for 252 h was only slight when antiserum C was used (Fig. 7A) and was more or less equivalent to that seen for cells which had been incubated for 12 h following microoxic stepdown. In comparison, the use of antiserum C achieved an increase of greater than 1 order of magnitude in the gold labelling of cells grown under microoxic conditions for 84 h (Fig. 7B). Greater than 80% of the gold particles were located at and followed the contours of the cell membrane. The numbers of gold particles in these cells were even greater than those deposited in cells grown for 12 h under microoxic growth when they were labelled with antiserum A (i.e., compare Fig. 6A and 7B). These data are consistent with those from Western blotting that indicated GlbN was not detectable in aerobically grown cells while there was a marked induction of GlbN synthesis following microoxic stepdown and incubation (Fig. 1).

N. commune UTEX 584 cells were induced for GlbN synthesis, and the soluble, cytoplasmic membrane, cell wall, and outer membrane fractions were obtained (41). GlbN was identified exclusively in the soluble protein fraction.

DISCUSSION

Cyanoglobin is restricted to *Nostoc* spp. Fourteen strains that represented three genera of filamentous non-heterocyst-

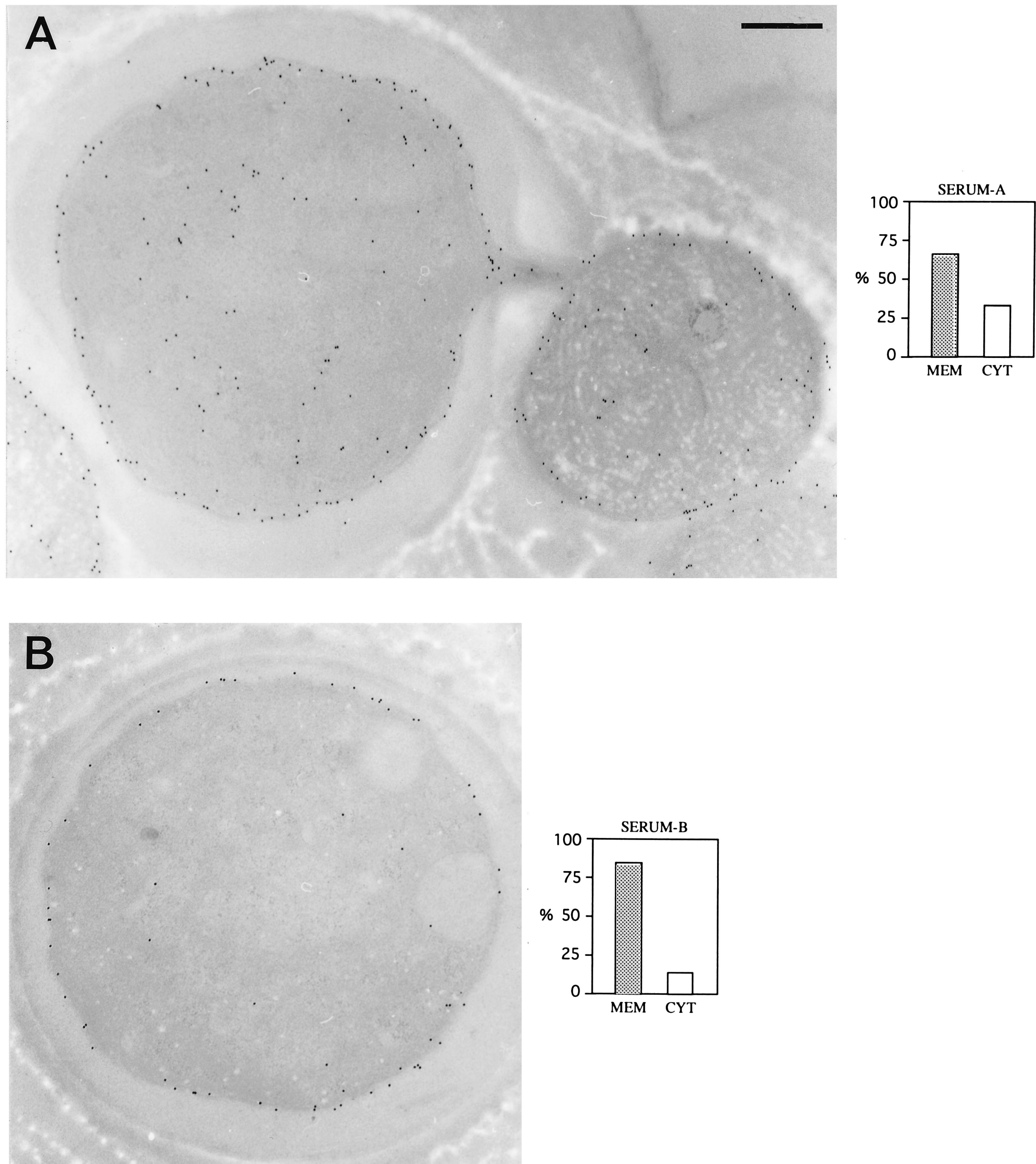

FIG. 6. GlbN is a peripheral membrane protein. Cells of *N. commune* UTEX 584 were grown in fermentors in BG-11_O under a 12 h:12 h light-dark cycle for 168 h under oxic conditions. Following microoxic stepdown, they were incubated for 12 h to induce GlbN synthesis. Ultrathin sections were prepared for transmission electron microscopy and were immunogold labelled with either GlbN antiserum A or antiserum B at a 1:500 dilution. The patterns shown are representative of those observed with multiple sections seen in multiple fields of view. (A) Immunogold labelling of a representative heterocyst (left) and vegetative cell (right) with antiserum A. (B) Immunogold labelling of a representative heterocyst with antiserum B. In each case, the total numbers of gold particles in three representative cells were counted. Histograms indicate the mean numbers of gold particles at the cell membrane (MEM) versus those within the cell (CYT), expressed as a percentage of the total. Scale bar, approximately 2 μm.

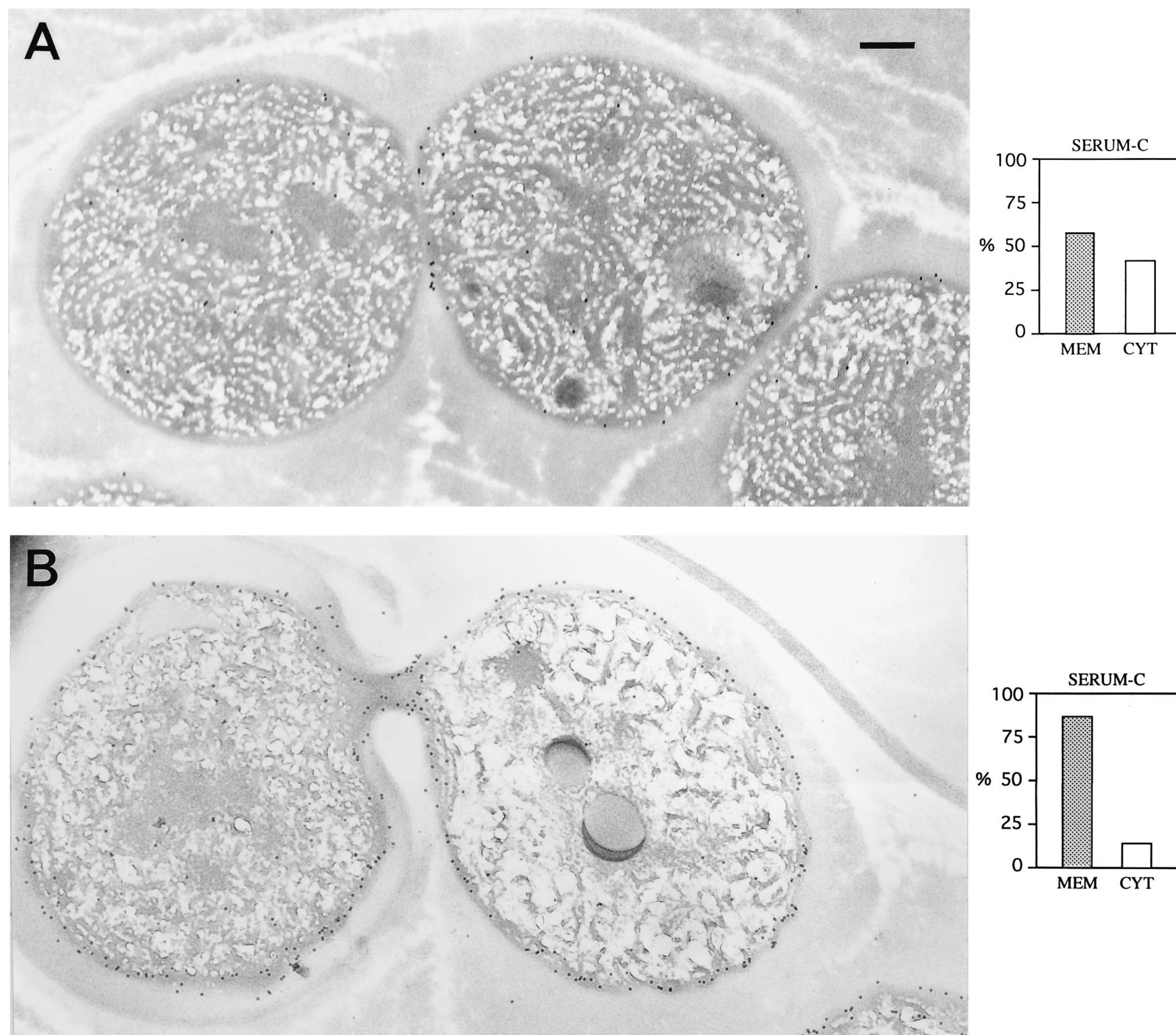

FIG. 7. GlbN synthesis is induced both in heterocysts and in vegetative cells specifically in response to microaerobic conditions. Cells of *N. commune* UTEX 584 were grown as described in the legend to Fig. 6 for either 252 h under oxic conditions (A) or for 168 h under oxic conditions and then for 84 h under microoxic conditions (B). Ultrathin sections were immunogold labelled with GlbN antiserum C. Scale bar, approximately 1 μm. See the legend to Fig. 6 for description of the histograms.

forming cyanobacteria (section III) and five genera of filamentous heterocyst-forming cyanobacteria (sections IV and V), including *Anabaena* sp. strain PCC 7120, provided no hybridization with a *Nostoc* sp. strain 8820 *glbN* probe. Western blotting with GlbN antibodies failed to identify any cross-reactive protein with a size equivalent to that of GlbN of *N. commune* UTEX 584 in three unicellular-coccoid cyanobacteria and two non-heterocyst-forming filamentous cyanobacteria (sections I, II, and III). If these data reflect the fact that GlbN homologs are absent from these strains, then cyanoglobin is restricted to a subset of *Nostoc* sp. strains in which *glbN* was identified, including *N. commune* UTEX 584, *Nostoc* sp. strain 8820, *Nostoc* sp. strain 840215, *Nostoc* sp. strain UCD 7801, and *Nostoc* sp. strain ATCC 27897. Such a restricted distribution of *glbN* suggests that the gene may provide a useful genotypic marker for taxonomic purposes.

Anabaena sp. strain PCC 7120, as well as 8 other *Anabaena*, *Nostoc*, and *Trichodesmium* strains, contained an 18-kDa polypeptide that cross-reacted with GlbN antibodies. However, the 18-kDa protein is unlikely to be cyanoglobin. First, it is significantly different in size from cyanoglobin. Second, it is synthesized constitutively, unlike cyanoglobin, whose synthesis is inducible. Third, *Anabaena* sp. strain PCC 7120 DNA provided no significant signals with a *glbN* probe. Nevertheless, the cross-reaction between the 18-kDa protein and GlbN antibodies did appear to be highly specific given the purity of the antiserum, the dilution of the serum (1:10,000) at which strong reactions were obtained in Western analyses, the capacity of *Anabaena* sp. strain PCC 7120 extracts to substantially deplete the titer of the GlbN antiserum, and the lack of cross-reactivity between the GlbN serum and myoglobin or leghemoglobin (54).

Expression of *glbN*. NtcA was originally identified in *Synechococcus* sp. strain 7942 as a positive regulator protein that

binds to the promoters of nitrogen-regulated genes such as the one that encodes nitrate reductase. Sequences hybridizing to *ntcA* were detected in a wide range of cyanobacteria. A homolog of NtcA, which was characterized in *Anabaena* sp. strain PCC 7120, was found to be synonymous with BifA (VF1), which binds to three adjacent sites in the upstream region of the *xisA* gene, which encodes a site-specific recombinase involved in the excision of an 11-kb DNA fragment from within *nifD* (6, 57). NtcA is also required for heterocyst differentiation in *Anabaena* sp. strain PCC 7120 (57). This protein clearly has multiple regulatory roles.

The similarity between the regions both upstream and downstream of *glbN* in *N. commune* UTEX 584 and *Nostoc* sp. strain 8820 is high, but it is surprising that *Nostoc* sp. strain 8820 apparently lacks the NtcA-binding site identified in the *nifU-glbN* intergenic region of *N. commune* UTEX 584. Although the mode of regulation of *glbN* expression by NtcA is not known at this time, we would have expected it to be consistent between strains which contain *glbN*; this may not be the case. It is not known whether the other strains in which *glbN* has been identified have NtcA-binding sites upstream of *glbN*.

Hemoproteins and nitrogen fixation. Cyanoglobin is a hemoprotein that binds oxygen reversibly and with high affinity (54). Hemoproteins have at least three known functions related to nitrogen fixation. First, the terminal (*d* type) cytochrome oxidases in branched electron transport systems remove O_2, thereby protecting nitrogenase (20). Second, leghemoglobins in plant cells facilitate O_2 flux to vigorously respiring *Rhizobium bacteroides* at a stabilized low O_2 tension (3). Third, hemoproteins, such as FixL (an oxygen-sensor and kinase) in *R. meliloti*, may have regulatory functions in nitrogen fixation (15). Specifically, for cyanobacteria, it has been suggested that hemoproteins in the cell wall and in the honeycomb membrane system of the heterocyst play a part in the binding of oxygen or protection of the nitrogenase complex (39). The subcellular location of these proteins in heterocysts was determined by their ability to oxidize 3,3′-diaminobenzidine in the dark. Cyanoglobin does oxidize 3,3′-diaminobenzidine (55), and it is also a hemoprotein which accumulates in the cell wall of heterocysts. However, the conditions under which cyanoglobin is synthesized, as well as the apparent lack of sequences which are homologous to *glbN* in a number of *Nostoc* and *Anabaena* spp., seem to exclude a general function for GlbN in the 3,3′-diaminobenzidine staining of the honeycomb region of heterocysts (10, 33, 39).

The recent finding of a second Nif system in *A. variabilis* ATCC 29413, which is induced under anoxic conditions and which is expressed both in heterocysts and in vegetative cells (50, 53), emphasizes how the role of oxygen in the regulation of nitrogen fixation is a complex one. It is unlikely that cyanoglobin plays a role in this system. Southern and Western analyses failed to identify any *glbN* or GlbN homologs in *A. variabilis* ATCC 29413. In addition, no *glbN* sequence was found in the *nif1*, *nif2*, *fdxH1*, or *fdxH2* gene clusters of this strain (32, 50, 53).

A hemoprotein that shares both some sequence similarity and possible functional similarity with cyanoglobin is the cytochrome oxidase (nitrite reductase) of *Pseudomonas perfectomarina* (data not shown). This is a periplasmic protein whose synthesis is induced by anoxic conditions and nitrite and is repressed by nitrate and inhibited by oxygen (27). Similarly, cyanoglobin is, as we have shown here, a peripheral membrane protein; its synthesis is induced by anoxic conditions and is repressed by nitrate and oxygen.

Function of cyanoglobin. Collectively, the data presented here suggest that the physiological function of cyanoglobin is connected with nitrogen fixation. First, GlbN was synthesized only in cells grown in the absence of combined nitrogen. Second, conditions which induced the greatest levels of GlbN synthesis provided cells with the greatest capacity for acetylene reduction. Third, there was a marked correlation between the accumulation of GlbN, NifH, and PetH. Fourth, in the two *Nostoc* strains for which sequence data are available, a single copy of *glbN* was present between two *nif* operons: immediately downstream of *nifU* and immediately upstream of *nifH*. Fifth, NtcA, a transcription factor which binds near the promoters of nitrogen-regulated genes and which is required for heterocyst differentiation (57), binds immediately upstream of *glbN* in *N. commune* UTEX 584.

Given the available data, a plausible role for cyanoglobin is that it scavenges oxygen and provides that oxygen to, and as a peripheral component of, a cytochrome oxidase complex when ATP generation and, thus, nitrogen fixation are limited. Experiments to confirm or discount this hypothesis are in progress.

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ADDENDUM IN PROOF

ORF slr2097 in the genome sequence of *Synechocystis* sp. strain PCC 6803 (Cyanobase; http://www.kazusa.or.jp/cyano/) is annotated as *glbN* based on sequence similarity between its putative product and protozoan myoglobins (49% identity). ORF slr2097 is not equivalent to *N. commune* 584 *glbN* because the two DNA sequences share only 51% identity and the slr2097 and *N. commune* 584 *glbN* products share only 38% identity. Any reference to "*glbN*" in *Synechocystis* sp. strain PCC 6803 will clearly cause confusion in view of the potential use of *N. commune* 584 *glbN* as a taxonomic marker. We recommend that ORF slr2097 be renamed. If the product of ORF slr2097 is a hemoprotein then it may represent a new example of the growing class of bacterial globins.

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