Characterization of the glnB gene product of *Nostoc punctiforme* strain ATCC 29133: glnB or the P<sub>H</sub> protein may be essential

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Bacterial P<sub>H</sub> proteins, encoded by glnB genes, are central signalling molecules in nitrogen regulatory pathways and are modulated by post-translational modification in response to the cellular nitrogen status. The glnB gene was cloned from the filamentous heterocyst-forming cyanobacterium *Nostoc punctiforme* strain ATCC 29133 (PCC 73102) by heterologous hybridization to a *Synechococcus* sp. strain PCC 7942 gene fragment. Expression of the cloned gene was verified by hybridization to *N. punctiforme* total RNA and a single cross-reactive polypeptide was observed in immunoblots of *N. punctiforme* extracts probed with anti-*Synechococcus* 7942 P<sub>H</sub> antiserum. Modification of the purified *N. punctiforme* P<sub>H</sub> protein by a *Synechococcus* 7942 P<sub>H</sub> kinase was observed, but modified forms of P<sub>H</sub> were not detected in extracts of *N. punctiforme* from a variety of incubation conditions. The *N. punctiforme* glnB gene could not be disrupted by targeted gene replacement unless a second copy of glnB was provided in trans, suggesting that the gene or gene product is essential for growth under the conditions tested.

Keywords: *Nostoc punctiforme*, diazotrophic cyanobacterium, glnB gene, P<sub>H</sub> protein

**INTRODUCTION**

*Nostoc punctiforme* strain ATCC 29133 (PCC 73102) is a physiologically diverse filamentous cyanobacterium whose vegetative cells are capable of differentiating into heterocysts, akinetes and hormogonia filaments (Rippka *et al*., 1979). Cellular differentiation is initiated by environmental signals such as combined nitrogen limitation (heterocysts), phosphate starvation (akinetes), and replete nutrients or stress (hormogonia) (Tandeau de Marsac & Houmard, 1993). Heterocysts are the sites of nitrogen fixation and function to protect the oxygen-sensitive nitrogenase enzyme complex from photosynthetically produced and atmospheric oxygen. Heterocyst differentiation is under nitrogen control; while early acting genes such as *hetR* and *ntcA* have been identified, the signalling events for initiation of differentiation are as yet unknown (Wolk, 1996). Cyanobacteria regulate the synthesis of glutamine synthetase (GS), encoded by *glnA*, between constitutive and induced levels in response to nitrogen source (Flores & Herrero, 1994), further implying the existence of nitrogen-dependent regulatory systems.

The P<sub>H</sub> protein, encoded by the glnB gene, is the central signalling molecule and a very early step in nitrogen regulatory pathways in various eubacteria. In the unicellular obligately autotrophic cyanobacterium *Synechococcus* sp. strain PCC 7942 and in *Escherichia coli*, the P<sub>H</sub> proteins function as negative effectors, but are regulated via distinct mechanisms. In response to nitrogen limitation, *E. coli* uridylylates P<sub>H</sub> at tyrosine 51 (Atkinson *et al*., 1994), while *Synechococcus* 7942 phosphorylates P<sub>H</sub> at serine 49 (Forchhammer & Tandeau de Marsac, 1994, 1995b). In both cases, modification of the protein apparently interferes with the ability of P<sub>H</sub> to negatively influence regulatory targets. A *Synechococcus* 7942 glnB null mutant constitutively overexpressed GS and had defects in adaptation to the presence of ammonia and methyl-
ammonium uptake (Forchhammer & Tandeau de Marsac, 1995a). Similarly, an *E. coli* *glnB* null mutant constitutively overexpressed GS (Jiang et al., 1997a). The P_H protein has been proposed as a cellular differentiation regulator in *Calothrix* sp. strain PCC 7601 and strain PCC 7504 (Campbell Marsac, 1995a). Similarly, an ammonium uptake (Forchhammer

The PII protein has been proposed as a cellular constitutively overexpressed GS (Jiang et al., 1993; Tandeau de Marsac, 1994). Modified forms of P_H have been reported in cultures of *Calothrix* 7504 after a prolonged period of incubation under nitrogen-deprived conditions, but the modification was not sensitive to alkaline phosphatase or snake venom phosphodiesterase (Liottenberg et al., 1996). Alkaline phosphatase demodifies phosphoproteins and snake venom phosphodiesterase demodifies nucleotidylated proteins (uridylylation, adenylylation, ADP-ribosylation). Thus, the role of P_H in filamentous cyanobacteria has not been established. The goal of this work was to determine if the *N. punctiforme* P_H protein participates in pathways regulating cellular differentiation and/or nitrogen metabolism.

**METHODS**

**Cultures and media.** The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was cultured in Luria–Bertani (LB) broth for propagation and construction of plasmids. *N. punctiforme* and *Synechococcus* 7942 were cultured on plates in the full-strength medium of Allen & Arnon (1955) solidified with 1% (w/v) Bacto Noble Agar or in liquid in a fourfold dilution, buffered in both cases to pH 7.8 with 5.0 mM MOPS. Nitrogen sources for *N. punctiforme* were N_2, 2.5 mM NH_4Cl or 5.0 mM NO_3 (1:1 molar ratio of K^+ and Na^+ salts) or 1:25 mM NH_4Cl+2.5 mM NO_3. Nitrogen sources for *Synechococcus* 7942 were 15 mM NO_3 or 5.0 mM NH_4Cl; 30 mM NaHCO_3 was added to all *Synechococcus* 7942 cultures. Selection for the *Ω::npt* cassette used kanamycin (Km) at 50 µg ml\(^{-1}\) in *E. coli* and neomycin (Nm) at 30 µg ml\(^{-1}\) in cyanobacterial strains. *E. coli* strains carrying Ω::npt were grown at 30 °C except for triparental conjugal transfer, when they were grown at 37 °C. Ampicillin (Ap) resistance was selected at 100 µg ml\(^{-1}\) for *E. coli* and 10 µg ml\(^{-1}\) for *N. punctiforme*. Erythromycin (Em) resistance was selected at 15 µg ml\(^{-1}\) in *N. punctiforme*. Chloramphenicol (Cm) resistance was selected at 50 µg ml\(^{-1}\) in *E. coli*.

**DNA and RNA isolation and manipulations.** Small-scale plasmid DNA preparations from *E. coli* were performed by standard methods (Ausubel et al., 1987) and large-scale preparations by a commercial kit (Qiagen). DNA restriction enzymes were purchased from New England Biolabs or Gibco-BRL and used according to the manufacturer’s instructions. Southern and Northern hybridizations were performed with Gene Screen Plus membrane in 50% formamide hybridization buffers according to the manufacturer’s instructions (DuPont, NEN). Hybridization probes were labelled with [γ-^32P]dCTP by a random priming kit (Gibco-BRL). Preparation of total DNA and total RNA from *N. punctiforme* has been described elsewhere (Cohen et al., 1994; Summers et al., 1995).

**Cloning and sequencing of *N. punctiforme* *glnB*.** An AfIII–BglII fragment of the *Synechococcus* 7942 *glnB* gene was used as a probe to identify p20H11, a cosmid from a N. punctiforme genomic library (Cohen et al., 1994) that carries the *glnB* gene on a 2.2 kb EcoRII fragment. The EcoRII fragment was subcloned into EcoRI-digested *pBluescript* KS'. The resulting clone was then digested with EcoRV and religated to eliminate 1.5 kb of insert and generate pSCR301. The 686 bp insert in pSCR301 was sequenced on both strands by the dyeoxy method using primers complementary to *pBluescript*. The sequence was deposited in GenBank under accession number AF017419. pSCR304 was constructed by excising the insert in pSCR301 as a *KpnI–PstI* fragment and ligating the fragment into *KpnI*/*PstI*-digested pSCR202, a shuttle vector encoding Ap resistance (Summers et al., 1995). In this orientation *N. punctiforme* *glnB* is transcribed in an opposite direction to the lacZ promoter of pSCR202.

**Native and SDS-PAGE immunoblotting.** Native and SDS-PAGE immunoblotting were carried out according to Forchhammer & Tandeau de Marsac (1994) in a Bio-Rad Mini-Protein system. Native PAGE was performed with 6% stacking and 10% resolving gels, SDS-PAGE was performed with 6% stacking and 15% resolving gels. Separated proteins were transferred to nitrocellulose (Hybond ECL; Amersham) in 25 mM Tris/SCCO buffer pH 8.3, 192 mM glycine, 20% (v/v) methanol in a Bio-Rad Mini-Transphor cell overnight. The P_H protein was visualized using a rabbit polyclonal antiserum raised against the purified *Synechococcus* 7942 P_H protein as the primary antibody at a 1:20000 dilution. Horseradish-alkaline phosphatase conjugated goat anti-rabbit antibody obtained from Cappell Organon Teknika was used as the secondary antibody at a 1:10000 dilution. Bound secondary antibody was detected using a p-iophenol/luminol protocol (Ausubel et al., 1987).

**Purification and in vitro modification of the *N. punctiforme* P_H protein.** pSCR301 was used to transform *E. coli* strain BD, a *glnB*/*glnD* double mutant kindly provided by Alex Ninfa (Bueno et al., 1985). Expression of *N. punctiforme* P_H was verified by immunoblotting and was visible by SDS-PAGE (data not shown). The *N. punctiforme* P_H protein was purified from *E. coli* strain BD carrying pSCR301 by the method of Forchhammer & Tandeau de Marsac (1994) except that the final DEAE-Septachrom chromatography was performed twice to remove a low level of impurities still present after the first pass. Purified protein was stored frozen at −20 °C in 50 mM Tris/SCCO buffer pH 8.0, 60 mM NaCl, 5 mM MgCl_2, and 1 mM EDTA. The purified protein was used as substrate for a partially purified, kinase-active fraction of *Synechococcus* 7942 in a buffer containing 50 mM potassium phosphate pH 7.4, 50 mM KCl, 5 mM MgCl_2, 2 mM DTT, 2 mM benzamide, 0.5 mM EDTA in a total volume of 20 µl (Forchhammer & Tandeau de Marsac, 1995b). The small molecule effectors ATP and 2-oxoglutarate were added at the concentrations indicated in Fig. 5.

**Construction of insertionaly inactive *N. punctiforme glnB*.** Cosmid p20H11 was discovered to be rearranged relative to the *N. punctiforme* genome for *glnB* hybridizing fragments larger than 3 kb, so the pSCR301 insert was used as a probe to identify four additional cosmids bearing *N. punctiforme glnB*. Only one of these four cosmids, p8C10, had a restriction map consistent with respect to the *N. punctiforme* genomic *glnB* region when probed with the pSCR301 insert. An 11.0 kb *glnB*-hybridizing XbaI fragment of cosmid p8C10 was subcloned into XbaI-digested *pBluescript* KS', generating pSCR317. Two *HpaI* sites were found in the insert DNA, one of which was present in *glnB*. pSCR317 was partially digested with *HpaI* and ligated to a gel-purified EcoRII fragment of pSCR9 carrying the Ω::npt cassette (Cohen & Meeks, 1997). Transformants of *E. coli* strain DH5α-MCR were selected on Km and Ap. Two independent clones differing in the orientation of the Ω::npt cassette in the *glnB* *HpaI* site were identified by restriction analysis and called pSCR319 and pSCR320. pSCR321 and pSCR322 were constructed by
### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype/phenotype</th>
<th>Reference/source</th>
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<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
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<tr>
<td>DH5α-MCR</td>
<td>Methylation-dependent restriction-defective derivative of E. coli DH5α for cloning</td>
<td>Cohen et al. (1994)</td>
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<tr>
<td>BD</td>
<td>glnB glnD double mutant derived from E. coli YMC10</td>
<td>Bueno et al. (1985)/Alex Ninfa</td>
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<tr>
<td><strong>Cyanobacterial strains</strong></td>
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<tr>
<td><em>Synechococcus</em> sp. PCC 7942/1</td>
<td>Small plasmid-cured strain</td>
<td>Rippka &amp; Herdman (1992)</td>
</tr>
<tr>
<td><em>Nostoc punctiforme</em> ATCC 29133 (PCC 73102)</td>
<td>Wild-type for this study</td>
<td>Rippka &amp; Herdman (1992)</td>
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<td><em>N. punctiforme</em> derivatives</td>
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<tr>
<td>UCD 311</td>
<td>devR transposon mutant strain; does not fix N₂ under oxic conditions</td>
<td>Campbell et al. (1996)</td>
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<td>UCD 403</td>
<td>Nm&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt; Suc&lt;sup&gt;S&lt;/sup&gt; exconjugant of pSCR322; carries glnB and Ω::npt-interrupted glnB; P&lt;sub&gt;psbA&lt;/sub&gt;npt in Ω::npt transcribes anti-parallel to glnB</td>
<td>This study</td>
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<td>UCD 404</td>
<td>Nm&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt; Suc&lt;sup&gt;S&lt;/sup&gt; exconjugant of pSCR322; carries glnB and Ω::npt-interrupted glnB; P&lt;sub&gt;psbA&lt;/sub&gt;npt in Ω::npt transcribes parallel to glnB</td>
<td>This study</td>
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<tr>
<td>UCD 404SR1–SR4</td>
<td>Nm&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt; Suc&lt;sup&gt;S&lt;/sup&gt; derivatives of UCD 404</td>
<td>This study</td>
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<tr>
<td>UCD 407</td>
<td>Nm&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt; Suc&lt;sup&gt;S&lt;/sup&gt; Ap&lt;sup&gt;R&lt;/sup&gt;, UCD 403+pSCR304</td>
<td>This study</td>
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<tr>
<td>UCD 408</td>
<td>Nm&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt; Suc&lt;sup&gt;S&lt;/sup&gt; Ap&lt;sup&gt;R&lt;/sup&gt;, UCD 404+pSCR304</td>
<td>This study</td>
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<tr>
<td>UCD 409–UCD 414</td>
<td>Nm&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt; Suc&lt;sup&gt;S&lt;/sup&gt; Ap&lt;sup&gt;R&lt;/sup&gt;, sucrose-resistant derivatives of UCD 408; UCD 414 still maintains glnB and Ω::npt glnB; all others carry Ω::npt-interrupted glnB in the chromosome and pSCR304</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pBluescript KS&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Cloning vector</td>
<td>Stratagene</td>
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<td>p8C10</td>
<td><em>N. punctiforme</em> genomic cosmid clone including the glnB region</td>
<td>Cohen et al. (1994)</td>
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<tr>
<td>p20H11</td>
<td><em>N. punctiforme</em> genomic cosmid clone including a rearranged glnB region with respect to the <em>N. punctiforme</em> genome</td>
<td>Cohen et al. (1994)</td>
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<td>pRL271</td>
<td>Cyanobacterial suicide vector carrying sacB</td>
<td>Cai &amp; Wolk (1990)/C. P. Wolk</td>
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<tr>
<td>pSCR9</td>
<td>Ω::npt source vector</td>
<td>Cohen &amp; Meeks (1997)</td>
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<tr>
<td>pSCR202</td>
<td><em>N. punctiforme</em>/E. coli shuttle vector</td>
<td>Campbell et al. (1996)</td>
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<tr>
<td>pSCR300</td>
<td>2.2 kb EcoRI glnB-hybridizing fragment in pBluescript</td>
<td>This study</td>
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<td>pSCR301</td>
<td>686 bp EcoRV–EcoRI glnB-hybridizing fragment in pBluescript</td>
<td>This study</td>
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<tr>
<td>pSCR304</td>
<td>pSCR301 EcoRV–EcoRI fragment in pSCR202</td>
<td>This study</td>
</tr>
<tr>
<td>pSCR317</td>
<td>11.0 kb XhoI glnB fragment from cosmid in pBluescript</td>
<td>This study</td>
</tr>
<tr>
<td>pSCR319</td>
<td>Ω::npt inserted in glnB HpaI site in pSCR317; P&lt;sub&gt;psbA&lt;/sub&gt;npt transcribes anti-parallel to glnB</td>
<td>This study</td>
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<tr>
<td>pSCR320</td>
<td>Ω::npt inserted in glnB HpaI site in pSCR317; P&lt;sub&gt;psbA&lt;/sub&gt;npt transcribes parallel to glnB</td>
<td>This study</td>
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<tr>
<td>pSCR321</td>
<td>12.7 kb XhoI–SstI fragment of pSCR319 in pRL271</td>
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ligating the larger XbaI–SstI fragment of pSCR319 and pSCR320, respectively, to XbaI/SstI-digested pRL271, a sacB-based positive selection vector for targeted gene replacement in cyanobacteria (Cai & Wolk, 1990). E. coli cultures carrying pSCR321 and pSCR322 were screened to verify sucrose sensitivity and then conjugationally mobilized to N. punctiforme in triparental matings as described previously (Cohen et al., 1994). Nm-resistant, Em-resistant, sucrose-sensitive exconjugants (single recombinants) of pSCR321 and pSCR322 were named strain UCD 403 and strain UCD 404, respectively. Two independent isolates of each single recombinant were grown in medium containing Nm only to allow for gene replacement. Sucrose-resistant strains were isolated by plating strains on media containing Nm, 5% sucrose and various additions as noted in Results. Sucrose-resistant strains can arise by gene replacement, which eliminates pRL271 sequences, or by sacB inactivation. Strains that have eliminated pRL271 are Em sensitive, while sacB-inactivated strains are Em resistant (Cai & Wolk, 1990). The segregation period was varied in different experiments between 4 and 12 weeks, with transfers to fresh medium at 2–3 week intervals.

Single recombinant strains carrying glnB in trans were created by electroporation of strains UCD 403 and UCD 404 with pSCR304 selecting Nm, Em and Ap resistance and called UCD 407 and UCD 408, respectively. These strains were grown under Nm + Ap selection and plated for sucrose resistance. Sucrose-resistant strains were grown in Nm or Nm + Ap as appropriate and harvested for total DNA, which was then physically mapped by DNA:DNA hybridization using a probe internal to the N. punctiforme glnB generated by the polymerase chain reaction. For calculating the frequency of sucrose resistance, viable cell numbers were derived from chlorophyll a concentrations of cell suspensions immediately prior to plating using a conversion factor of 1.75 × 10⁻¹⁸ g chlorophyll a per viable cell (Cohen et al., 1994).

RESULTS

Cloning, sequencing and expression of the N. punctiforme glnB gene

Fig. 1(a) shows the physical map of the 11 kb XbaI fragment carrying the glnB region from N. punctiforme that was used for all subsequent gene inactivation experiments. Fig. 1(b) shows an alignment of the predicted N. punctiforme glnB amino acid sequence with the predicted amino acid sequences of E. coli glnB, E. coli glnK and glnB genes from three unicellular cyanobacteria and one filamentous cyanobacterium, Calothrix 7601. E. coli glnK encodes a P₁₈-like protein that is expressed under nitrogen-limiting conditions and whose precise function is unclear (van Heeswijk et al., 1995). N. punctiforme GlnB shows 98% amino acid identity with the Calothrix 7601 sequence. Differences are aspartate (N. punctiforme) vs glutamate (Calothrix 7601) at position 66, arginine (N. punctiforme) vs valine (Calothrix 7601) at position 100, and glutamate (N. punctiforme) vs arginine (Calothrix 7601) at position 101. Cyanobacterial P₃₈ proteins are more similar to one another than to other P₃₈ proteins particularly in the N-terminus including the potential modification sites for Synechococcus 7942 at serine 49 and for proteobacteria at tyrosine 51 (underlined in Fig. 1). The N. punctiforme P₃₈ protein shares 60% and 55% amino acid identity with E. coli glnB and glnK respectively. Both E. coli proteins have alanine at position 49 vs serine for cyanobacterial P₃₈ proteins.

N. punctiforme total RNA extracted at time points following the removal of combined nitrogen was probed with the pSCR301 insert to verify that the cloned gene was expressed in N. punctiforme (Fig. 2). A single message of 470 nt which increased in abundance within 6 h following combined nitrogen removal was observed. A 470 nt message could encode the 339 bp glnB ORF, but most likely not another ORF, implying monocistronic transcription.

Modification of the N. punctiforme P₃₈ protein

Fig. 3 shows native PAGE immunoblots of Synechococcus 7942 and N. punctiforme extracts isolated from cells incubated with different nitrogen sources. The anti-Synechococcus 7942 P₃₈ antiserum shows strong cross-reactivity to a single band in N. punctiforme extracts in native PAGE which we designate the N. punctiforme P₃₈ protein (Fig. 3b). A single 12 kDa cross-reactive band was also visualized in SDS-PAGE immunoblots (data not shown). In the Synechococcus 7942 extracts a nitrogen-starvation-dependent, alkaline-phosphatase-sensitive mobility shift to faster-migrating forms is seen (Fig. 3a). These control reactions indicate that the extraction and native PAGE procedure detects P₃₈ phosphorylation and independently confirms the results of Forchhammer & Tandeau de Marsac (1994). The multiple banding arises as a consequence of the homotrimeric structure of P₃₈ and corresponds to one, two, or three phosphorylations per homotrimer. No modification of the P₃₈ protein was evident in N. punctiforme growing with N₂, NO₃ or NH₄ as nitrogen sources (Fig. 3b). In all cases a faster-migrating band was seen when extracts were treated with snake venom phosphodiesterase regardless of the source of the extract; we interpret this as being a consequence of a contaminating enzyme or protease. However, no differences in electrophoretic mobility were observed in SDS-PAGE immunoblots between untreated and snake venom phosphodiesterase treated samples.

No growth or incubation condition was found that induced detectable P₃₈ modification in N. punctiforme. Other conditions examined included the following: different light regimes and electron-transport inhibitors in an attempt to change the oxidation–reduction status of the cell (Campbell et al., 1993); methionine sulfoximine (MSX) to inhibit nitrogen assimilation (Liotenberg et al., 1996; Stewart & Rowell, 1975); elevated temperature and salt concentrations as generalized stress; and a time course following the removal of combined nitrogen in wild-type and strain UCD 311 under oxic conditions. Strain UCD 311 (devR) cannot properly synthesize the heterocyst wall and thus cannot fix nitrogen when oxygen is present (Campbell et al., 1996). The MSX and strain UCD 311 experiments allowed examination of N. punctiforme P₃₈ under non-
growth conditions; *Synechococcus* 7942 P$_H$ is most strongly modified under nitrogen-starved, non-growth conditions.

These observations could imply that the *N. punctiforme* P$_H$ protein is incapable of modification. This possibility was tested by first purifying the *N. punctiforme* P$_H$ protein from *E. coli* strain BD carrying pSCR301 for use as substrate in a P$_H$ kinase reaction. The rationale of the strain BD background is to eliminate the potential for modification of the expressed protein by GlnD and contamination by *E. coli* P$_H$. A Coomassie-stained native PAGE gel of purified *N. punctiforme* P$_H$ indicates that a single protein was present at protein loadings up to 10 µg (Fig. 4a). Under native PAGE conditions, the purified *N. punctiforme* P$_H$ protein migrated identically to P$_H$ bands in *N. punctiforme* extracts (Fig. 4b). The even intensity of the P$_H$ bands in *N. punctiforme* indicates that a single protein was present at protein loadings up to 10 µg (Fig. 4a). Under native PAGE conditions, the purified *N. punctiforme* P$_H$ protein migrated identically to P$_H$ bands in *N. punctiforme* extracts (Fig. 4b). The even intensity of the P$_H$ bands in *N. punctiforme* indicates that there is no strong nitrogen control over the level of P$_H$ protein.

**Fig. 1.** Physical map and sequence analysis of *N. punctiforme* glnB. (a) Physical map of the *N. punctiforme* glnB region. The same map is observed when cosmid p8C10 or *N. punctiforme* genomic DNA is used as target for hybridization with a *N. punctiforme* glnB probe. The relative positions of restriction fragments that do not contain glnB were inferred by restriction mapping of pSCR317, which carries the depicted XbaI fragment in pBluescript KS+. Restriction enzymes: B, BstX I; H, HpaI; I, EcoRI; N, NheI; V, EcoRV, X, XbaI. (b) Alignment of predicted amino acid sequences for the products of cyanobacterial glnB genes and of *E. coli* glnB and glnK (GlnK is an *E. coli* P$_H$-like protein). Accession numbers are in parentheses. Filamentous cyanobacteria: *P. trichodes*, *C. linearis*, *G. subtulis*, *C. chloroplast*; *P. bursaria* (P09827), *R. meliloti* glnB (U50385), *R. capulatus* glnR (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulatus* glnB (B36865), *R. capsulaci
**Fig. 2.** Hybridization of a glnB probe to *N. punctiforme* total RNA. The lane numbers indicate time elapsed in hours after the removal of NH$_4^+$ from the medium. Ten micrograms of total RNA were loaded per lane. Positions of RNA markers are indicated. The probe was the EcoRI-EcoRV insert fragment of pSCR301.

**Fig. 3.** In vivo PI modification analysis by native PAGE immunoblotting. (a) *Synechococcus* 7942 extracts. Lanes 1–3, NH$_4^+$-grown culture deprived of combined nitrogen for 2 h; lanes 4–6, actively growing NH$_4^+$ culture. Lanes 1 and 4, no addition; lanes 2 and 5, snake venom phosphodiesterase treated; lanes 3 and 6, calf intestine alkaline phosphatase treated. (b) *N. punctiforme* extracts. Lanes 1–3, N$_2$-grown culture; lanes 4–6, NO$_3^-$-grown culture; lanes 7–9, NH$_4^+$-grown culture. Lanes 1, 4 and 7, no addition; lanes 2, 5 and 8, snake venom phosphodiesterase treated; lanes 3, 6 and 9, calf intestine alkaline phosphatase treated.

**Fig. 4.** Native PAGE characterization of purified *N. punctiforme* PI$_I$ protein. (a) Coomassie-stained native PAGE gel of purified *N. punctiforme* PI$_I$ protein expressed from pSCR301 in *E. coli* strain BD. Lanes: 1, 100 μg; 2, 50 μg; 3, 25 μg; 4, 13 μg. (b) Native PAGE immunoblot of purified *N. punctiforme* PI$_I$ protein from *E. coli* strain BD and *N. punctiforme* extracts. Lanes: 1, 120 ng PI$_I$; 2, 100 μg N$_2$-grown *N. punctiforme* extract; 3, 100 μg NO$_3^-$-grown *N. punctiforme* extract; 4, 100 μg NH$_4^+$-grown *N. punctiforme* extract.

oxoglutarate dependence of the reaction when purified *Synechococcus* 7942 PI$_I$ is used as the substrate (Fig. 5a). When the *N. punctiforme* PI$_I$ protein was used as substrate in the *Synechococcus* 7942 kinase assay, an identical ATP- and 2-oxoglutarate-dependent reaction could be observed, with the phosphorylated *N. punctiforme* PI$_I$ protein exhibiting the same electrophoretic mobility as the modified *Synechococcus* 7942 PI$_I$ protein (Fig. 5b).

**Insertional mutagenesis of *N. punctiforme* glnB**

To determine the physiological role of PI$_I$ in *N. punctiforme*, we attempted to construct a *N. punctiforme* glnB null mutant using a sacB-based positive selection system. In the presence of sucrose, sacB is induced and encodes levansucrase, which inhibits growth of a variety of Gram-negative bacteria, including cyanobacteria (Cai & Wolk, 1990). sacB positive selection has previously been used in gene-replacement experiments with *N. punctiforme* (Cohen et al., 1994; Forchhammer & Tandeau de Marsac, 1995b; K. Forchhammer & H. Dierks, unpublished results). Positive control reactions demonstrated the ATP and 2-
Fig. 5. In vitro kinase reactions with partially purified *Synechococcus* 7942 P₈ kinase analysed by native PAGE. (a) *Synechococcus* 7942 P₈ as substrate (40 ng). Lane 1, *Synechococcus* 7942 extract containing the unphosphorylated and three phosphorylated forms of P₈. Lanes 2–5, 0.5 mM 2-oxoglutarate (2-OG) plus: lane 2, 5.0 mM ATP; lane 3, 1.0 mM ATP; lane 4, 0.5 mM ATP; lane 5, 0.1 mM ATP. Lanes 6–9, 5 mM ATP plus: lane 6, 0.5 mM 2-OG; lane 7, 0.2 mM 2-OG; lane 8, 0.05 mM 2-OG; lane 9, 0 mM 2-OG. (b) *N. punctiforme* P₈ as substrate (40 ng). Lanes 1–4, 0.5 mM 2-OG plus: lane 1, 5.0 mM ATP; lane 2, 1.0 mM ATP; lane 3, 0.5 mM ATP; lane 4, 0.1 mM ATP. Lanes 5–8, 0.2 mM ATP plus: lane 5, 0.50 mM 2-OG; lane 6, 0.20 mM 2-OG; lane 7, 0.05 mM 2-OG; lane 8, 0 mM 2-OG.

Summers et al., 1995; Campbell et al., 1996). Sucrose-resistant derivatives of Ω::npt glnB single recombinant strain UCD 404 were isolated at a frequency of 8 x 10⁻⁵ per viable cell and >95% of these sucrose-resistant strains were Em sensitive. A subset of the Em-sensitive strains were grown in liquid under Nₜ selection for the preparation of genomic DNA for physical mapping.

*N. punctiforme* carries a single copy of *glnB* and strain UCD 404 carries the wild-type and a larger Ω::npt inserted copy of the *glnB* gene (Fig. 6a). Nm- and sucrose-resistant, Em-sensitive colonies selected from UCD 404 unexpectedly showed the same genomic pattern of *glnB* hybridization as strain UCD 404. Based on their Em sensitivity, strains UCD 404SR1 to SR4 should not contain vector DNA. The blot in Fig. 6(a) was stripped and reprobed with XbaI-digested vector, pRL271. The vector sequences present in strain UCD 404 were deleted when sucrose resistance was selected (Fig. 6b). This observation implies that a genomic rearrangement occurred which deleted vector sequences while maintaining both wild-type and Ω::npt-interrupted copies of *glnB*. Previous studies showed that sacB could be inactivated in the absence of gene replacement, and Em sensitivity has been used to separate sacB inactivation from gene replacement events (Buikema & Haselkorn, 1991; Cai & Wolk, 1990; Khyudakov & Wolk, 1996). Further analysis of the sucrose-resistant strains by physical mapping has not defined the nature of the rearrangement that occurred. The Pₚ₅₉₄ promoter of the Ω::npt cassette in UCD 404 transcribes parallel to *glnB*. The same series of experiments were carried out with strain UCD 403 carrying the Ω::npt cassette inserted in the opposite orientation and the same results were observed (data not shown).

Selection conditions examined in attempts to isolate a *glnB* null mutant included: NO₃⁻ and NH₄⁺, singly or in combination, as the nitrogen source during segregation and plating; supplementation with 0.05% (w/v) Cas-amino acids or 0.2% (w/v) glutamine during segregation or plating; and plating on NH₄⁺ + 50 mM fructose + 5% sucrose in the dark. Screening of a total of 55 independently isolated sucrose-resistant strains selected under different conditions failed to identify a *glnB* mutant. These results led to the hypothesis that the *glnB* gene may be essential. An alternative hypothesis is that the vector deletion is greatly favoured over gene replacement in strains UCD 403 or 404. We hypothesized

![Image](http://www.microbiologyresearch.org/figs/5.png)

![Image](http://www.microbiologyresearch.org/figs/6.png)
pSCR304 carries the \textit{N. punctiforme} \textit{glnB} ORF in a cyanobacterial replicating vector encoding \textit{Ap} resistance. pSCR304 was electroporated into strain UCD 403 and the resulting electroporant strain UCD 407 was segregated on \textit{Nm} + \textit{Ap} followed by plating for sucrose resistance. Sucrose-resistant strains were isolated at 3 \times 10^{-7} per viable cell, a 38-fold increase relative to the single recombinants without pSCR304. The presence of vector alone in single recombinants did not increase the frequency of sucrose resistance, and sucrose-resistant derivatives from such strains carry both wild-type and disrupted \textit{glnB} (data not shown). However, the presence of pSCR304 allowed \textit{glnB} replacement to occur in strain UCD 407 (Fig. 7a). The same result was observed in strain UCD 408, which is strain UCD 404 carrying pSCR304. The faint 80 kb band marked pSCR304 vector in Fig. 7(a) results from the non-specific hybridization of the \textit{glnB} probe to vector sequences and is only visible when large amounts of plasmid DNA are loaded (see Fig. 7b). Ten nanograms of pSCR304 were loaded in Fig. 7(a); thus no vector band is observed. The fact that it was necessary to load large amounts of total (plasmid + genomic) DNA from strains UCD 409 to UCD 414 in order to observe genomic hybridization gave rise to the observed vector band in Fig. 7(a).

**DISCUSSION**

A fragment of DNA cloned from \textit{N. punctiforme} was identified that encodes a predicted protein with high amino acid sequence identity to eubacterial \textit{P}_{\text{II}} proteins. The cloned gene hybridized to a \textit{N. punctiforme} mRNA which increased in abundance after the removal of ammonium from a \textit{N. punctiforme} culture. The \textit{P}_{\text{II}} protein level, when examined under steady-state growth conditions, did not mirror this trend, which could imply either that the observed mRNA increase was transient or that the turnover rate of the \textit{P}_{\text{II}} protein varied depending on nitrogen source. In \textit{N. punctiforme}, \textit{in vivo} modification of \textit{P}_{\text{II}} was not detected by native PAGE immunoblotting and a mutant with an interrupted \textit{glnB} gene could not be isolated unless a second wild-type copy of the gene was present \textit{in trans}. These two characteristics are in direct contrast to results with the \textit{Synechococcus} 7942 and \textit{E. coli} model systems and imply potential differences in the protein and its cellular role in \textit{N. punctiforme}.

The solvent-exposed T-loop of the \textit{E. coli} \textit{P}_{\text{II}} protein from residue 37 to 55 regulates interaction between \textit{P}_{\text{II}} and other proteins depending on the uridylylation state of tyrosine 51 (Carr et al., 1996; Jiang et al., 1997a, b). Residue 49, in the apex of the T-loop, is an alanine in \textit{E. coli} whereas it is a serine in \textit{N. punctiforme} and all other known cyanobacterial \textit{P}_{\text{II}} sequences. Serine 49 is phosphorylated under nitrogen-limiting conditions in \textit{Synechococcus} 7942 (Forchhammer & Tandeau de Marsac, 1994). Thus, it appears that \textit{Synechococcus} 7942 also uses the T-loop for regulating the interactions of \textit{P}_{\text{II}} although target proteins directly interacting with \textit{P}_{\text{II}} have not been identified in this system. Since the \textit{E. coli} proteins lack serine 49, they may only be capable of accepting uridylyl groups while cyanobacterial \textit{P}_{\text{II}} may be capable of accepting both uridylyl and phosphoryl groups. The \textit{Synechococcus} 7942 \textit{P}_{\text{II}} protein has been shown to be uridylylated \textit{in vivo} in \textit{E. coli} (Forchhammer & Hedler, 1997).

Analysis of the predicted \textit{N. punctiforme} \textit{glnB} protein sequence in light of recent structural and site-directed mutagenesis experiments with \textit{E. coli} \textit{P}_{\text{II}} (Carr et al., 1996; Jiang et al., 1997a, b) shows that conserved
regions are maintained in the *N. punctiforme* protein with the exception of positions 100 and 101. Positions 100 and 101 are at the junction between β-5 and the C-loop and the side chain of arginine 101 lines the cleft between the B- and C-loops in *E. coli* P_{II} (Carr et al., 1996). Arginine 101 is conserved among other cyanobacteria, but is replaced by a glutamate in *N. punctiforme*, while position 100 is changed from isoleucine or valine to arginine in *N. punctiforme*. This change might be expected to affect small molecule effector binding, which apparently involves the cleft between the T-, B- and C-loops (Jiang et al., 1997a). However, *N. punctiforme* P_{II} was phosphorylated *in vitro* by a partially purified *Synechococcus* 7942 P_{II} kinase to a similar extent as the homologous *Synechococcus* 7942 P_{II} protein. Additionally, the same concentration dependence on 2-oxoglutarate (50 μM minimal and 200 μM saturation) for P_{II} phosphorylation was observed with both the *N. punctiforme* and *Synechococcus* 7942 P_{II} proteins. These observations are consistent with the *N. punctiforme* P_{II} protein having similar ligand-binding properties to those determined for the *Synechococcus* 7942 protein (Forchhammer & Hedler, 1997).

In contrast to the *in vitro* phosphorylation results, phosphorylated or uridylylated forms of the *N. punctiforme* P_{II} protein were not identified *in vivo* following shifts in culture nitrogen status and various stress conditions. Since the *N. punctiforme* P_{II} protein is capable of being phosphorylated, the remaining possible explanations for this result are that *N. punctiforme* does not possess a P_{II}-modification activity, possesses low activity, or possesses a strong demodification activity. Substantial demodification activity was not detected when extracts of *Synechococcus* 7942 containing modified forms of P_{II} were mixed with extracts of *N. punctiforme* (data not shown). Therefore, we can only conclude that either *N. punctiforme* lacks a P_{II} modification system or the conditions under which it is active have not been identified. Biochemically separable, physiologically responsive, cognate P_{II}-kinase (Forchhammer & Tandeau de Marsac, 1993a) and P_{II}-PO_{4} phosphatase (Irmler et al., 1997) activities have been identified in *Synechococcus* 7942. When the genes encoding these activities become available, and if sufficiently conserved, their presence in *N. punctiforme* could be determined, which may clarify the current results.

The *N. punctiforme glnB* gene could not be replaced by an interrupted copy under a variety of nutrient-supplemented conditions unless a second functional copy of the *glnB* gene was supplied *in trans*. A sacB-based positive selection system was utilized, but sucrose and Nm resistance was initially generated in *N. punctiforme* by deletion of the vector without complete replacement of the wild-type *glnB* with the insertionally inactivated gene. Sucrose resistance without gene replacement will occur due to insertion or point mutations in *sacB* (Buikema & Haselkorn, 1991; Cai & Wolk, 1990; Khyudakov & Wolk, 1996), but these types of mutations were not observed here. These sucrose- and Nm-resistant clones of *N. punctiforme* most likely reflect a mixture of mutant and wild-type chromosomes produced by resolution of multiple single recombinant chromosomes to either mutant or wild-type which cannot be segregated if *glnB* function is essential. This interpretation has precedence in studies of the *icd* gene encoding isocitrate dehydrogenase in *Anaabaena* sp. strain PCC 7120 (Muro-Pastor & Florencio, 1994), although the phenomenon had not been previously observed in *N. punctiforme*. An extrachromosomal copy of *glnB* allowed for direct gene replacement to occur at a much higher frequency than was observed for the deletion of the vector, pRL271. This result implies that *glnB* single recombinants can completely rearrange to a *glnB* mutant but cannot do so unless *glnB* is provided *in trans*. Thus, we hypothesize that the *glnB* gene, its mRNA and/or the unmodified P_{II} protein may be essential to *N. punctiforme* under all of the growth conditions examined. Since an inability to generate *glnB* null mutants has also been observed in *Azotobacter vinelandii* (C. Kennedy, personal communication) and *Rhodospirillum rubrum* (Johansson & Nordlund, 1997), *N. punctiforme* is not unique in this characteristic.

In *Azospirillum brasilense* (de Zamaroczy et al., 1993), *Azorhizobium caulodans* (Michel-Reydellet et al., 1997), *R. rubrum* (Johansson & Nordlund, 1997) and certain other eubacteria, *glnB* and *glnA* are transcriptionally linked and a *glnB* mutation by insertion would be expected to be lethal by polarity on *glnA*. Two observations suggest a possible role for the *N. punctiforme* results. First, *glnB* appears to be transcribed monocistronically and, secondly, the *glnB* ORF alone was sufficient to allow gene replacement to occur. It appears that there is no redundant function for P_{II} in *N. punctiforme*. This does not rule out the presence of an alternative P_{II} in *N. punctiforme* such as *glnK* in *E. coli*; however, we detected no other hybridization bands under low-stringency Southern hybridization conditions in wild-type *N. punctiforme*, the genome sequence of *Synechocystis* PCC 6803 contains a single *glnB* homologue (Kaneko et al., 1996) and a *Synechococcus* 7942 *glnB* mutant shows no cross-reactivity to anti-P_{II} antisera (Forchhammer & Tandeau de Marsac, 1994), unlike an *E. coli glnB* mutant (van Heeswijk et al., 1993).

The underlying biological rationale for the essential nature of the *N. punctiforme glnB* gene is not clear, but it may be related to the complex physiological and developmental pathways present in this organism. For example, *N. punctiforme glnB* mutants may be lethal due to the stimulation of a differentiation pathway leading to non-growth states such as heterocysts or hormogonia. While *N. punctiforme* was isolated as a symbiont in the cycad *Macrozamia* sp., it is assumed to grow and compete in the soil as a free-living population prior to infection of plants and, thus, would be subject to the same environmental selection as other soil microorganisms. Characterization of *glnB* and P_{II} in other cyanobacteria in addition to *Synechococcus* 7942, Calo-
thrix 7601 and N. punctiforme may clarify its apparently divergent role in the growth of cyanobacteria.

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REFERENCES


sulfoximine on the assimilation of newly fixed NH$_3$, acetylene reduction and heterocyst production in *Anabaena cylindrica*. Biochem Biophys Res Commun 65, 846–856.


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