Characterization of the \textit{glnB} gene product of \textit{Nostoc punctiforme} strain ATCC 29133: \textit{glnB} or the \( P_{\text{II}} \) protein may be essential

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Bacterial \( P_{\text{II}} \) proteins, encoded by \textit{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 \textit{glnB} gene was cloned from the filamentous heterocyst-forming cyanobacterium \textit{Nostoc punctiforme} strain ATCC 29133 (PCC 73102) by heterologous hybridization to a \textit{Synechococcus} sp. strain PCC 7942 gene fragment. Expression of the cloned gene was verified by hybridization to \textit{N. punctiforme} total RNA and a single cross-reactive polypeptide was observed in immunoblots of \textit{N. punctiforme} extracts probed with anti-\textit{Synechococcus} 7942 \( P_{\text{II}} \) antiserum. Modification of the purified \textit{N. punctiforme} \( P_{\text{II}} \) protein by a \textit{Synechococcus} 7942 \( P_{\text{II}} \) kinase was observed, but modified forms of \( P_{\text{II}} \) were not detected in extracts of \textit{N. punctiforme} from a variety of incubation conditions. The \textit{N. punctiforme} \textit{glnB} gene could not be disrupted by targeted gene replacement unless a second copy of \textit{glnB} was provided in trans, suggesting that the gene or gene product is essential for growth under the conditions tested.

Keywords: \textit{Nostoc punctiforme}, diazotrophic cyanobacterium, \textit{glnB} gene, \( P_{\text{II}} \) protein

INTRODUCTION

\textit{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 \textit{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 photo-synthetically produced and atmospheric oxygen. Heterocyst differentiation is under nitrogen control; while early acting genes such as \textit{hetR} and \textit{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 \textit{glnA}, between constitutive and induced levels in response to nitrogen source (Flores & Herrero, 1994), further implying the existence of nitrogen-dependent regulatory systems.

The \( P_{\text{II}} \) protein, encoded by the \textit{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 \textit{Synechococcus} sp. strain PCC 7942 and in \textit{Escherichia coli}, the \( P_{\text{II}} \) proteins function as negative effectors, but are regulated via distinct mechanisms. In response to nitrogen limitation, \textit{E. coli} uridylylates \( P_{\text{II}} \) at tyrosine 51 (Atkinson \textit{et al.}, 1994), while \textit{Synechococcus} 7942 phosphorylates \( P_{\text{II}} \) at serine 49 (Forchhammer & Tandeau de Marsac, 1994, 1995b). In both cases, modification of the protein apparently interferes with the ability of \( P_{\text{II}} \) to negatively influence regulatory targets. A \textit{Synechococcus} 7942 \textit{glnB} null mutant constitutively overexpressed GS and had defects in adaptation to the presence of ammonia and methyl-
ammonium uptake (Forchhammer & Tandeau de Marsac, 1993a). Similarly, an *E. coli* glnB null mutant constituted overexpressed GS (Jiang et al., 1997a). The PII protein has been proposed as a cellular differentiation regulator in *Calothrix* sp. strain PCC 7601 and strain PCC 7504 (Campbell et al., 1993; Tandeau de Marsac, 1994). Modified forms of PII 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 prolonged period of incubation under nitrogen-deprived conditions. Modified forms of PI have been proposed as a cellular 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 NH₄Cl or NH₄NO₃ (1:1 molar ratio of K⁺ and Na⁺ salts) or 1-25 mM NH₄Cl and 2-5 mM NO₃⁻. Nitrogen sources for *Synechococcus 7942* were 15 mM NO₃⁻ or 50 mM NH₄Cl; 30 mM NaHCO₃ was added to all cultures. *Synechococcus 7942* cultures. Selection for the *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 conjugations, 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 30 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 [α-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 ApⅠ-BglⅡI fragment of the *Synechococcus 7942* glnB gene was used as a probe to identify p20H11, a cosmid from a *N. punctiforme* random-sheared genomic library (Cohen et al., 1994) that carries the glnB gene on a 2.2 kb EcoRI fragment. The EcoRI 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 dideoxy 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-Protean 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/HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol in a Bio-Rad Mini-Transphor cell overnight. The PⅠ protein was visualized using a rabbit polyclonal antiserum raised against the purified *Synechococcus 7942* PⅠ protein as the primary antibody at a 1:20000 dilution. Horseradish peroxidase-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* PⅠ protein.** pSCR301 was used to transform *E. coli* strain BD, a glnBglnD double mutant kindly provided by Alex Ninfa (Bueno et al., 1985). Expression of *N. punctiforme* PⅠ was verified by immunoblotting and was visible by SDS-PAGE (data not shown). The *N. punctiforme* PⅠ protein was purified from *E. coli* strain BD carrying pSCR301 by the method of Forchhammer & Tandeau de Marsac (1994) except that the final DEAE-Sephacel 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/HCl pH 8.0, 60 mM NaCl, 5 mM MgCl₂, 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 MgCl₂, 5 mM DTT, 2 mM benzamidine, 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 insertionally inactivated *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 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 Eco136I 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>DH5a-MCR</td>
<td>Methylation-dependent restriction-defective derivative of <em>E. coli</em> DH5a for cloning</td>
<td>Cohen et al. (1994)</td>
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<tr>
<td>BD</td>
<td><em>glnB glnD</em> double mutant derived from <em>E. coli</em> YMC10</td>
<td>Bueno et al. (1985)/Alex Ninfa</td>
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<td><strong>Cyanobacterial strains</strong></td>
<td></td>
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<tr>
<td>Synechococcus sp. PCC 7942/1</td>
<td>Small plasmid-cured strain</td>
<td>Rippka &amp; Herdman (1992)</td>
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<tr>
<td>Nostoc punctiforme ATCC 29133 (PCC 73102)</td>
<td>Wild-type for this study</td>
<td>Rippka &amp; Herdman (1992)</td>
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<td><strong>N. punctiforme derivatives</strong></td>
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<tr>
<td>UCD 311</td>
<td><em>devR</em> transposon mutant strain; does not fix $\text{N}_2$ under oxic conditions</td>
<td>Campbell et al. (1996)</td>
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<td>UCD 403</td>
<td>$\text{Nm}^R \text{Em}^R \text{Suc}^R$ exconjugant of pSCR322; carries <em>glnB</em> and $\Omega::npt$-interrupted <em>glnB</em>; $\text{P}_{\text{pa}}::npt$ in $\Omega::npt$ transcribes anti-parallel to <em>glnB</em></td>
<td>This study</td>
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<td>UCD 404</td>
<td>$\text{Nm}^R \text{Em}^R \text{Suc}^R$ exconjugant of pSCR322; carries <em>glnB</em> and $\Omega::npt$-interrupted <em>glnB</em>; $\text{P}_{\text{pa}}::npt$ in $\Omega::npt$ transcribes parallel to <em>glnB</em></td>
<td>This study</td>
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<tr>
<td>UCD 404SR1–SR4</td>
<td>$\text{Nm}^R \text{Em}^R \text{Suc}^R$ derivatives of UCD 404</td>
<td>This study</td>
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<tr>
<td>UCD 407</td>
<td>$\text{Nm}^R \text{Em}^R \text{Suc}^R \text{Ap}^R$, UCD 403+pSCR304</td>
<td>This study</td>
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<tr>
<td>UCD 408</td>
<td>$\text{Nm}^R \text{Em}^R \text{Suc}^R \text{Ap}^R$, UCD 404+pSCR304</td>
<td>This study</td>
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<tr>
<td>UCD 409–UCD 414</td>
<td>$\text{Nm}^R \text{Em}^R \text{Suc}^R \text{Ap}^R$, sucrose-resistant derivatives of UCD 408; UCD 414 still maintains <em>glnB</em> and $\Omega::npt$ <em>glnB</em>; all others carry $\Omega::npt$-interrupted <em>glnB</em> in the chromosome and pSCR304</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pBluescript KS'</td>
<td>Cloning vector</td>
<td>Stratagene</td>
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<tr>
<td>p8C10</td>
<td><em>N. punctiforme</em> genomic cosmid clone including the <em>glnB</em> 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 <em>glnB</em> region with respect to the <em>N. punctiforme</em> genome</td>
<td>Cohen et al. (1994)</td>
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<tr>
<td>pRL271</td>
<td>Cyanobacterial suicide vector carrying <em>sacB</em></td>
<td>Cai &amp; Wolk (1990)/C. P. Wolk</td>
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<tr>
<td>pSCR9</td>
<td>$\Omega::npt$ source vector</td>
<td>Cohen &amp; Meeks (1997)</td>
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<tr>
<td>pSCR202</td>
<td><em>N. punctiforme</em>/<em>E. coli</em> shuttle vector</td>
<td>Campbell et al. (1996)</td>
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<tr>
<td>pSCR300</td>
<td>2.2 kb <em>EcoRI</em> <em>glnB</em>-hybridizing fragment in pBluescript</td>
<td>This study</td>
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<tr>
<td>pSCR301</td>
<td>686 bp <em>EcoRV</em>-<em>EcoRI</em> <em>glnB</em>-hybridizing fragment in pBluescript</td>
<td>This study</td>
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<tr>
<td>pSCR304</td>
<td>pSCR301 <em>EcoRV</em>-<em>EcoRI</em> fragment in pSCR202</td>
<td>This study</td>
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<tr>
<td>pSCR317</td>
<td>11.0 kb <em>XhoI</em> <em>glnB</em> fragment from cosmid p8C10 in pBluescript</td>
<td>This study</td>
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<tr>
<td>pSCR319</td>
<td>$\Omega::npt$ inserted in <em>glnB</em> <em>HpaI</em> site in pSCR317; $\text{P}_{\text{pa}}::npt$ transcribes anti-parallel to <em>glnB</em></td>
<td>This study</td>
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<tr>
<td>pSCR320</td>
<td>$\Omega::npt$ inserted in <em>glnB</em> <em>HpaI</em> site in pSCR317; $\text{P}_{\text{pa}}::npt$ transcribes parallel to <em>glnB</em></td>
<td>This study</td>
</tr>
<tr>
<td>pSCR321</td>
<td>12.7 kb <em>XhoI</em>–<em>SstI</em> fragment of pSCR319 in pRL271</td>
<td>This study</td>
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<tr>
<td>pSCR322</td>
<td>12.7 kb <em>XhoI</em>–<em>SstI</em> fragment of pSCR320 in pRL271</td>
<td>This study</td>
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lating the larger XhoI–SstI fragment of pSCR319 and pSCR320, respectively, to XhoI/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 ex-conjugants (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 P11-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 P11 proteins are more similar to one another than to other P11 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 P11 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 P11 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 P11 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 P11 antiserum shows strong cross-reactivity to a single band in N. punctiforme extracts in native PAGE which we designate the N. punctiforme P11 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 P11 phosphorylation and independently confirms the results of Forchhammer & Tandeau de Marsac (1994). The multiple banding arises as a consequence of the homotrimeric structure of P11 and corresponds to one, two, or three phosphorylations per homotrimer. No modification of the P11 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 activity in the crude phosphodiesterase preparation, such as proteases. 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 P11 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 P11 under non-
growth conditions; Synechococcus 7942 P_{II} is most strongly modified under nitrogen-starved, non-growth conditions.

These observations could imply that the N. punctiforme P_{II} protein is incapable of modification. This possibility was tested by first purifying the N. punctiforme P_{II} protein from E. coli strain BD carrying pSCR301 for use as substrate in a P_{II} 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_{II}. A Coomassie-stained native PAGE gel of purified N. punctiforme P_{II} 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_{II} protein migrated identically to PI, protein in extracts (Fig. 4b). The native PAGE gel of purified N. punctiforme P_{II} protein was used as the substrate for an in vitro phosphorylation reaction with a partially purified Synechococcus 7942 P_{II} kinase activity.
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 P$_i$ 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, 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 P$_i$ protein. (a) Coomassie-stained native PAGE gel of purified N. punctiforme P$_i$ protein expressed from pSCR301 in E. coli strain BD. Lanes: 1, 100 ng; 2, 50 ng; 3, 25 ng; 4, 13 ng. (b) Native PAGE immunoblot of purified N. punctiforme P$_i$ protein from E. coli strain BD and N. punctiforme extracts. Lanes: 1, 120 ng P$_i$; 2, 100 ng N$_2$-grown N. punctiforme extract; 3, 100 ng NO$_3^-$-grown N. punctiforme extract; 4, 100 ng NH$_4^+$-grown N. punctiforme extract.

oxoglutarate dependence of the reaction when purified Synechococcus 7942 P$_i$ is used as the substrate (Fig. 5a). When the N. punctiforme P$_i$ 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 P$_i$ protein exhibiting the same electrophoretic mobility as the modified Synechococcus 7942 P$_i$ protein (Fig. 5b).

Insertional mutagenesis of N. punctiforme glnB

To determine the physiological role of P$_i$ 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;
Fig. 5. In vitro kinase reactions with partially purified *Synechococcus* 7942 P₅₆ kinase analysed by native PAGE. (a) *Synechococcus* 7942 P₅₆ as substrate (40 ng). Lane 1, *Synechococcus* 7942 extract containing the unphosphorylated and three phosphorylated forms of P₅₆. 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* P₅₆ 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, 5.0 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.

Fig. 6. Southern blot of total DNA from *N. punctiforme* (2913), strain UCD 404, and sucrose-resistant derivatives of strain UCD 404 (UCD 404SR1–4). DNA was digested with EcoRI (I), EcoRV (V) or EcoRI and EcoRV(D) followed by electrophoresis and blotting. (a) *N. punctiforme* glnB probe; (b) pRL271 probe.

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 10⁻⁶ 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 N₅ 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). N₅- and sucrose-resistant, Em-sensitive colonies selected from strain UCD 404 unexpectedly showed the same genomic pattern of glnB hybridization as strain UCD 404. Based on their 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 P₅₆ 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 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...
that if the observed rearrangement is heavily favoured or independent of the glnB mutant phenotype then the presence of a plasmid carrying glnB should not affect the outcome of the sucrose selection.

pSCR304 carries the N. punctiforme glnB ORF in a cyanobacterial replicating vector encoding Ap resistance. pSCR304 was electroporated into strain UCD 403 and the resulting electroporant strain UCD 407 was segregated on Nm + Ap followed by plating for sucrose resistance. Sucrose-resistant strains were isolated at 3 \times 10^{-5} 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 glnB (data not shown). However, the presence of pSCR304 allowed 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 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 N. punctiforme was identified that encodes a predicted protein with high amino acid sequence identity to eubacterial P_I proteins. The cloned gene hybridized to a N. punctiforme mRNA which increased in abundance after the removal of ammonium from a N. punctiforme culture. The P_I 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 P_I protein varied depending on nitrogen source. In N. punctiforme, in vivo modification of P_I was not detected by native PAGE immunoblotting and a mutant with an interrupted glnB gene could not be isolated unless a second wild-type copy of the gene was present in trans. These two characteristics are in direct contrast to results with the Synechococcus 7942 and E. coli model systems and imply potential differences in the protein and its cellular role in N. punctiforme.

The solvent-exposed T-loop of the E. coli P_I protein from residue 37 to 55 regulates interaction between P_I 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 E. coli whereas it is a serine in N. punctiforme and all other known cyanobacterial P_I sequences. Serine 49 is phosphorylated under nitrogen-limiting conditions in Synechococcus 7942 (Forchhammer & Tandeau de Marsac, 1994). Thus, it appears that Synechococcus 7942 also uses the T-loop for regulating the interactions of P_I although target proteins directly interacting with P_I have not been identified in this system. Since the E. coli proteins lack serine 49, they may only be capable of accepting uridylyl groups while cyanobacterial P_I may be capable of accepting both uridylyl and phosphoryl groups. The Synechococcus 7942 P_I protein has been shown to be uridylylated in vivo in E. coli (Forchhammer & Hedler, 1997).

Analysis of the predicted N. punctiforme glnB protein sequence in light of recent structural and site-directed mutagenesis experiments with E. coli P_I (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* PII (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* PII protein was phosphorylated in vitro by a partially purified *Synechococcus* 7942 PII kinase to a similar extent as the homologous *Synechococcus* 7942 PII protein. Additionally, the same concentration dependence on 2-oxoglutarate (50 μM minimal and 200 μM saturation) for PII phosphorylation was observed with both the *N. punctiforme* and *Synechococcus* 7942 PII proteins. These observations are consistent with the *N. punctiforme* PII 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* PII protein were not identified in vivo following shifts in culture nitrogen status and various stress conditions. Since the *N. punctiforme* PII protein is capable of being phosphorylated, the remaining possible explanations for this result are that *N. punctiforme* does not possess a PII-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 PII were mixed with extracts of *N. punctiforme* (data not shown). Therefore, we can only conclude that either *N. punctiforme* lacks a PII modification system or the conditions under which it is active have not been identified. Biochemically separable, physiologically responsive, cognate PII kinase (Forchhammer & Tandeau de Marsac, 1995a) and PII-PO₄-phosphatase (Imler 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* glkB gene could not be replaced by an interrupted copy under a variety of nutrient-supplemented conditions unless a second functional copy of the glkB 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 glkB 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 glkB function is essential. This interpretation has precedence in studies of the icd gene encoding isocitrate dehydrogenase in *Anabaena* sp. strain PCC 7120 (Muro-Pastor & Florencio, 1994), although the phenomenon had not been previously observed in *N. punctiforme*. An extrachromosomal copy of glkB 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 glkB single recombinants can completely rearrange to a glkB mutant but cannot do so unless glkB is provided in trans. Thus, we hypothesize that the glkB gene, its mRNA and/or the unmodified PII protein may be essential to *N. punctiforme* under all of the growth conditions examined. Since an inability to generate glkB 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, glkB and glkA are transcriptionally linked and a glkB mutation by insertion would be expected to be lethal by polarity on glkA. Two observations argue against polarity as an explanation for the *N. punctiforme* results. First, glkB appears to be transcribed monocistronically and, secondly, the glkB ORF alone was sufficient to allow gene replacement to occur. It appears that there is no redundant function for PII in *N. punctiforme*. This does not rule out the presence of an alternative PII in *N. punctiforme* such as glkA 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 glkB homologue (Kaneko et al., 1996) and a *Synechococcus* 7942 glkB mutant shows no cross-reactivity to anti-PIII antiserum (Forchhammer & Tandeau de Marsac, 1994), unlike an *E. coli* glkB mutant (van Heeswijk et al., 1993).

The underlying biological rationale for the essential nature of the *N. punctiforme* glkB gene is not clear, but it may be related to the complex physiological and developmental pathways present in this organism. For example, *N. punctiforme* glkB 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 glkB and PII 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


Stewart, W. D. P. & Rowell, P. (1975). Effects of L-methionine-DL-
sulfoximine on the assimilation of newly fixed $\text{NH}_3$, acetylene reduction and heterocyst production in *Anabaena cylindrica*. Biochem Biophys Res Commun 65, 846–856.


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