Microbiology (1998), **144**, 1537–1547 Printed in Great Britain

Characterization of the *glnB* gene product of *Nostoc punctiforme* strain ATCC 29133: *glnB* or the P_{II} protein may be essential

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Bacterial P_{II} 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_{II} antiserum. Modification of the purified *N. punctiforme* P_{II} protein by a *Synechococcus* 7942 P_{II} kinase was observed, but modified forms of P_{II} 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_{II} 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_{II} 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_{II} proteins function as negative effectors, but are regulated via distinct mechanisms. In response to nitrogen limitation, *E. coli* uridylylates P_{II} at tyrosine 51 (Atkinson *et al.*, 1994), while *Synechococcus* 7942 phosphorylates P_{II} at serine 49 (Forchhammer & Tandeau de Marsac, 1994, 1995b). In both cases, modification of the protein apparently interferes with the ability of P_{II} 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-

Abbreviations: Ap, ampicillin; ATCC, American Type Culture Collection; Cm, chloramphenicol; Em, erythromycin; GS, glutamine synthetase; Km, kanamycin; MCR, deficient in restriction of DNA containing methylated adenine and cytosine residues; Nm, neomycin; PCC, Pasteur Culture Collection.

The GenBank accession number for the sequence reported in this paper is AF017419.

ammonium uptake (Forchhammer & Tandeau de Marsac, 1995a). Similarly, an *E. coli glnB* null mutant constitutively overexpressed GS (Jiang *et al*., 1997a). The P_{II} 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 P_{II} 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 (Liotenberg *et al*., 1996). Alkaline phosphatase demodifies phosphoproteins and snake venom phosphodiesterase demodifies nucleotidylated proteins (uridylylation, adenylylation, ADP-ribosylation). Thus, the role of P_{II} in filamentous cyanobacteria has not been established. The goal of this work was to determine if the *N. punctiforme* P_{II} 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 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-^{32}P]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 *Afl*II–*Bgl*II 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 *Eco*RI fragment. The *Eco*RI fragment was subcloned into *Eco*RI-digested pBluescript KS^+. The resulting clone was then digested with *Eco*RV 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 *Kpn*I–*Pst*I fragment and ligating the fragment into *Kpn*I/*Pst*I-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 P_{II} protein was visualized using a rabbit polyclonal antiserum raised against the purified *Synechococcus* 7942 P_{II} 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*-iodophenol/luminol protocol (Ausubel *et al*., 1987).

Purification and *in vitro* modification of the *N. punctiforme* P_{II} 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* P_{II} was verified by immunoblotting and was visible by SDS-PAGE (data not shown). The *N. punctiforme* P_{II} 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 $MgCl_2$, 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 $MgCl_2$, 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 11·0 kb *glnB*-hybridizing *Xba*I fragment of cosmid p8C10 was subcloned into *Xba*I-digested pBluescript KS^+, generating pSCR317. Two *Hpa*I sites were found in the insert DNA, one of which was present in *glnB*. pSCR317 was partially digested with *Hpa*I and ligated to a gel-purified *Ecl*136II 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 Hpa*I 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

Strain or plasmid	Genotype/phenotype	Reference/source
***E. coli* strains**		
DH5α-MCR	Methylation-dependent restriction-defective derivative of *E. coli* DH5α for cloning	Cohen *et al.* (1994)
BD	*glnB glnD* double mutant derived from *E. coli* YMC10	Bueno *et al.* (1985)/Alex Ninfa
Cyanobacterial strains		
Synechococcus sp. PCC 7942/1	Small plasmid-cured strain	Rippka & Herdman (1992)
Nostoc punctiforme ATCC 29133 (PCC 73102)	Wild-type for this study	Rippka & Herdman (1992)
N. punctiforme derivatives		
UCD 311	*devR* transposon mutant strain; does not fix N_2 under oxic conditions	Campbell *et al.* (1996)
UCD 403	Nm^R Em^R Suc^S exconjugant of pSCR322; carries *glnB* and Ω::*npt*-interrupted *glnB*; P_{psbA}*npt* in Ω::*npt* transcribes anti-parallel to *glnB*	This study
UCD 404	Nm^R Em^R Suc^S exconjugant of pSCR322; carries *glnB* and Ω::*npt*-interrupted *glnB*; P_{psbA}*npt* in Ω::*npt* transcribes parallel to *glnB*	This study
UCD 404SR1–SR4	Nm^R Em^S Suc^R derivatives of UCD 404	This study
UCD 407	Nm^R Em^R Suc^S Ap^R, UCD 403 + pSCR304	This study
UCD 408	Nm^R Em^R Suc^S Ap^R, UCD 404 + pSCR304	This study
UCD 409–UCD 414	Nm^R Em^S Suc^R Ap^R, 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	This study
Plasmids		
pBluescript KS^+	Cloning vector	Stratagene
p8C10	*N. punctiforme* genomic cosmid clone including the *glnB* region	Cohen *et al.* (1994)
p20H11	*N. punctiforme* genomic cosmid clone including a rearranged *glnB* region with respect to the *N. punctiforme* genome	Cohen *et al.* (1994)
pRL271	Cyanobacterial suicide vector carrying *sacB*	Cai & Wolk (1990)/C. P. Wolk
pSCR9	Ω::*npt* source vector	Cohen & Meeks (1997)
pSCR202	*N. punctiforme*/*E. coli* shuttle vector	Campbell *et al.* (1996)
pSCR300	2·2 kb *Eco*RI *glnB*-hybridizing fragment in pBluescript	This study
pSCR301	686 bp *Eco*RV–*Eco*RI *glnB*-hybridizing fragment in pBluescript	This study
pSCR304	pSCR301 *Eco*RV–*Eco*RI fragment in pSCR202	This study
pSCR317	11·0 kb *Xba*I *glnB* fragment from cosmid p8C10 in pBluescript	This study
pSCR319	Ω::*npt* inserted in *glnB Hpa*I site in pSCR317; P_{psbA}*npt* transcribes anti-parallel to *glnB*	This study
pSCR320	Ω::*npt* inserted in *glnB Hpa*I site in pSCR317 P_{psbA}*npt* transcribes parallel to *glnB*	This study
pSCR321	12·7 kb *Xho*I–*Sst*I fragment of pSCR319 in pRL271	This study
pSCR322	12·7 kb *Xho*I–*Sst*I fragment of pSCR320 in pRL271	This study

ligating the larger *Xho*I–*Sst*I fragment of pSCR319 and pSCR320, respectively, to *Xho*I/*Sst*I-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 $1{\cdot}75 \times 10^{-13}$ 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 *Xba*I 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 P_{II}-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 P_{II} proteins are more similar to one another than to other P_{II} 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* P_{II} 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 P_{II} proteins.

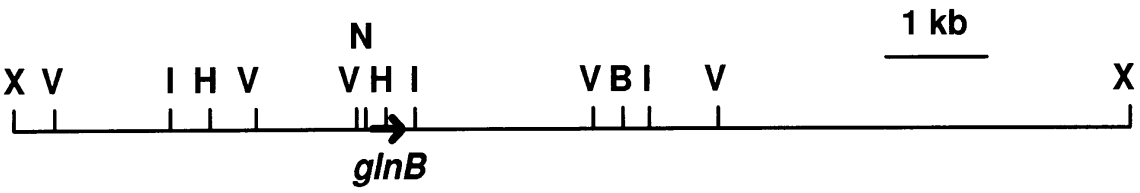
N. punctiforme total RNA extracted at time points following the removal of combined nitrogen was probed with the pSCR301 insert to verify that the cloned gene was expressed in *N. punctiforme* (Fig. 2). A single message of 470 nt which increased in abundance within 6 h following combined nitrogen removal 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* P_{II} 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 P_{II} antiserum shows strong cross-reactivity to a single band in *N. punctiforme* extracts in native PAGE which we designate the *N. punctiforme* P_{II} 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 P_{II} phosphorylation and independently confirms the results of Forchhammer & Tandeau de Marsac (1994). The multiple banding arises as a consequence of the homotrimeric structure of P_{II} and corresponds to one, two, or three phosphorylations per homotrimer. No modification of the P_{II} protein was evident in *N. punctiforme* growing with N_2, NO_3^- or NH_4^+ 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 P_{II} 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* P_{II} under non-

(a)



(b)

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                    1                                                  50
N. punctiforme      MKkVeaIIrP fKLDEvkiaL vnaGivGmtV sevrGfGrQK GqTErYRGse
  PCC 7601          MKkVeaIIrP fKLDEvkiaL vnaGivGmtV sevrGfGrQK GqTErYRGse
  PCC 7942          MKkIeaIIrP fKLDEvkiaL vnaGivGmtV sevrGfGrQK GqTErYRGse
  PCC 6301          MKkIeaIIrP fKLDEvkiaL vnaGivGmtV sevrGfGrQK GqTErYRGse
  PCC 6803          MKkVeaIIrP fKLDEvkiaL vnaGivGmtV sevrGfGrQK GqTErYRGse
E. coli GlnB        MKlVtvIIkP fKLEDvreaL ssiGiqGltV tevkGfGrQK GhaElYRGae
E. coli GlnK        MKkIdaIIkP fKLDDvreaL aevGitGmtV tevkGfGrQK GhTElYRGae

                    51                                                 100
N. punctiforme      ytVeflqKlK vEIvvdDnqv dmvvDkiiaa arTgeIGDGK IFIspVeqvr
  PCC 7601          ytVeflqKlK vEIvveDnqv dmvvDkiiaa arTgeIGDGK IFIspVeqvV
  PCC 7942          ytVeflqKlK lEIvveDaqv dtviDkivaa arpgeIGDGK IFVspVdqtI
  PCC 6301          ytVeflqKlK lEIvveDaqv dtviDkivaa arTgeIGDGK IFVspVdqtI
  PCC 6803          ytVeflqKlK iEIvvdEgqv dmvvDklvsa arTgeIGDGK IFIspVdsvV
E. coli GlnB        ysVnflpKvK iDVaiaDdql deviDivska ayTgkIGDGK IFVaelqrvI
E. coli GlnK        ymVdflpKvK iEIvvpDdiv dtcvDtiirt aqTgkIGDGK IFVfdVarvI

                    101         112      %Sim   %ID
N. punctiforme      eIRTGEknte Av        100    100
  PCC 7601          RIRTGEknte Av        100     98
  PCC 7942          RIRTGEknad Ai         95     87
  PCC 6301          RIRTGEknad Ai         96     88
  PCC 6803          RIRTGEkdte Ai         98     90
E. coli GlnB        RIRTGEadea Al         81     64
E. coli GlnK        RIRTGEedda Ai         79     55
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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 *Xba*I fragment in pBluescript KS^+. Restriction enzymes: B, *Bst*XI; H, *Hpa*I; I, *Eco*RI; N, *Nhe*I; V, *Eco*RV, X, *Xba*I. (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_{II}-like protein). Accession numbers are in parentheses. Filamentous cyanobacteria: PCC 7601, *Calothrix* 7601 (X97327); *N. punctiforme*, *Nostoc punctiforme*. Unicellular cyanobacteria: PCC 7942, *Synechococcus* 7942 (A39696); PCC 6301, *Synechococcus* sp. strain PCC 6301 (P80016); PCC 6803, *Synechocystis* sp. strain PCC 6803 (X97496). Serine 49 (the *Synechococcus* 7942 phosphorylation site) and tyrosine 51 (the *E. coli* uridylylation site), are underlined. Capitalized letters are consensus residues identified by a GCG PILEUP analysis with the following predicted protein sequences of P_{II} homologues in addition to those above: *Azospirillum brasilense glnB* (P21193) and *glnZ* (X92496), *Bacillus subtilis nrgB* (B36865), *Bradyrhizobium japonicum glnB* (A33600), *Clostridium longisporum glnB* (L49336), *Klebsiella pneumoniae glnB* (P11671), *Porphyra purpurea* chloroplast *glnB* (U38804), *Rhizobium leguminosarum glnB* (P09827), *Rhizobium meliloti glnB* (U50385), *Rhodobacter capsulatus glnB* (M28244), *Rhodobacter sphaeroides glnB* (X71659). The analysis was constrained to identify consensus residues based on conservation of similarity at 17 out of 18 sequences. Percentage similarity and identity are relative to the *N. punctiforme*-derived amino acid sequence.

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 P_{II} protein in *N. punctiforme* extracts (Fig. 4b). The even intensity of the P_{II} bands in *N. punctiforme* grown with different nitrogen sources implies that there is no strong nitrogen control over the level of P_{II} protein.

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

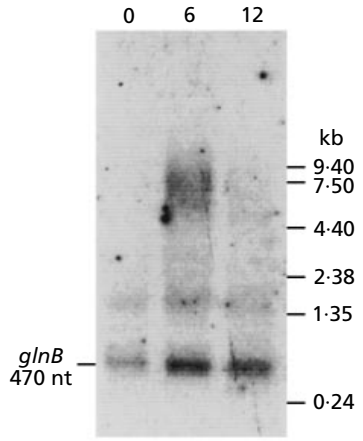

Fig. 2. Hybridization of a *glnB* probe to *N. punctiforme* total RNA. The lane numbers indicate time elapsed in hours after the removal of NH_4^+ from the medium. Ten micrograms of total RNA were loaded per lane. Positions of RNA markers are indicated. The probe was the *Eco*RI–*Eco*RV insert fragment of pSCR301.

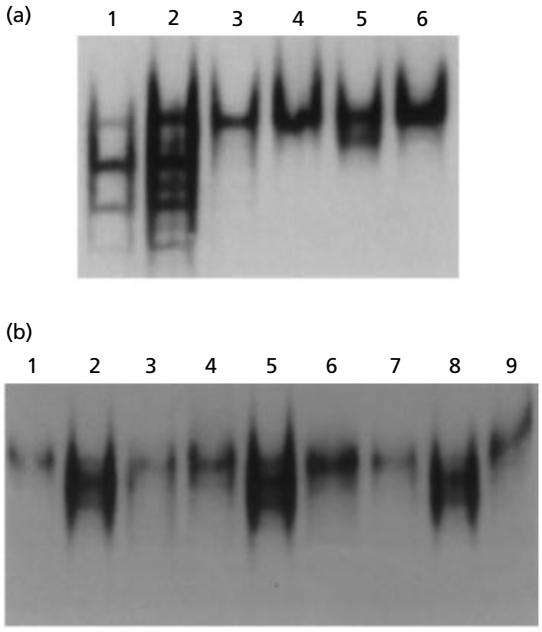

Fig. 3. *In vivo* P_{II} modification analysis by native PAGE immunoblotting. (a) *Synechococcus* 7942 extracts. Lanes 1–3, NH_4^+-grown culture deprived of combined nitrogen for 2 h; lanes 4–6, actively growing NH_4^+ culture. Lanes 1 and 4, no addition; lanes 2 and 5, snake venom phosphodiesterase 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.

(Forchhammer & Tandeau de Marsac, 1995b; K. Forchhammer & H. Dierks, unpublished results). Positive control reactions demonstrated the ATP and 2-

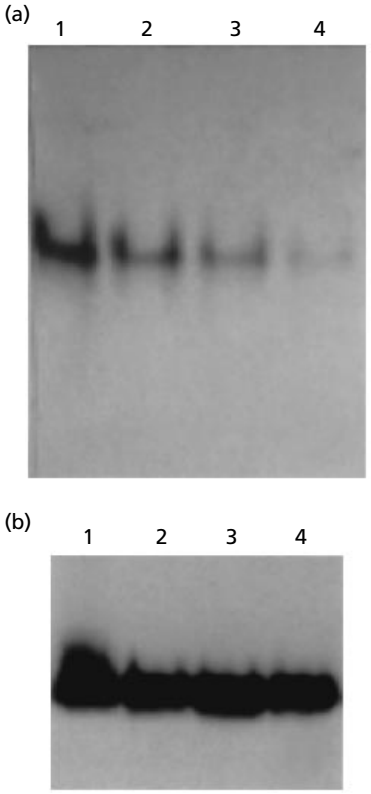

Fig. 4. Native PAGE characterization of purified *N. punctiforme* P_{II} protein. (a) Coomassie-stained native PAGE gel of purified *N. punctiforme* P_{II} protein expressed from pSCR301 in *E. coli* strain BD. Lanes: 1, 10·0 μg; 2, 5·0 μg; 3, 2·5 μg; 4, 1·3 μg. (b) Native PAGE immunoblot of purified *N. punctiforme* P_{II} protein from *E. coli* strain BD and *N. punctiforme* extracts. Lanes: 1, 120 ng P_{II}; 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 P_{II} is used as the substrate (Fig. 5a). When the *N. punctiforme* P_{II} 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_{II} protein exhibiting the same electrophoretic mobility as the modified *Synechococcus* 7942 P_{II} protein (Fig. 5b).

Insertional mutagenesis of *N. punctiforme glnB*

To determine the physiological role of P_{II} 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;

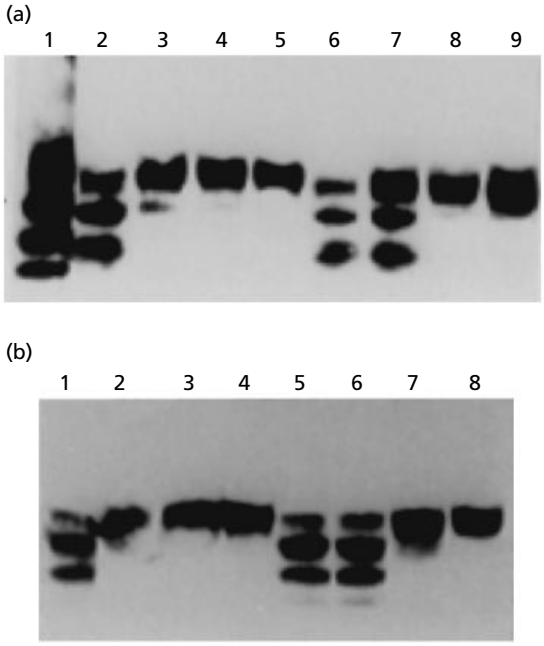

Fig. 5. *In vitro* kinase reactions with partially purified *Synechococcus* 7942 P_{II} kinase analysed by native PAGE. (a) *Synechococcus* 7942 P_{II} as substrate (40 ng). Lane 1, *Synechococcus* 7942 extract containing the unphosphorylated and three phosphorylated forms of P_{II}. Lanes 2–5, 0·5 mM 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* P_{II} 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.

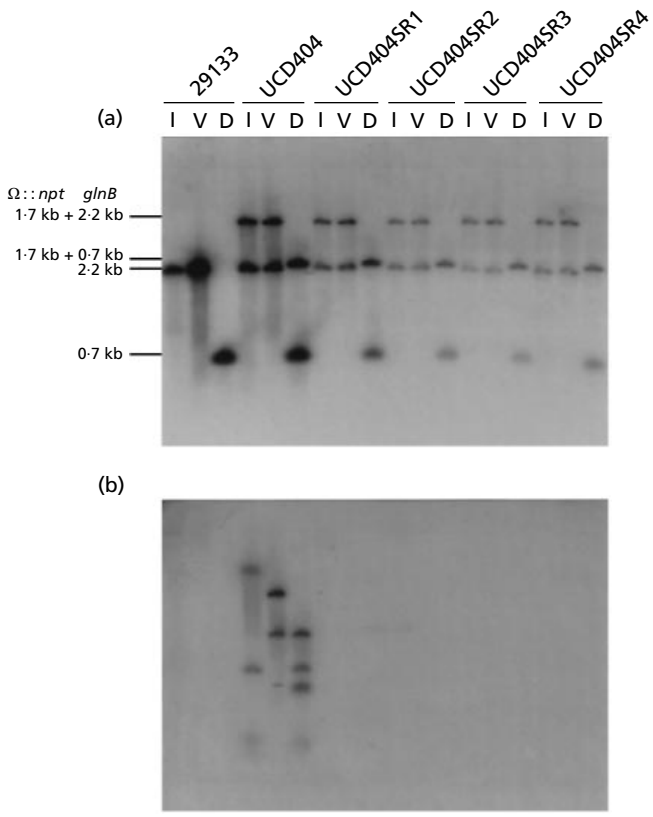

Fig. 6. Southern blot of total DNA from *N. punctiforme* (29133), strain UCD 404, and sucrose-resistant derivatives of strain UCD 404 (UCD 404SR1–4). DNA was digested with *Eco*RI (I), *Eco*RV (V) or *Eco*RI and *Eco*RV(D) followed by electrophoresis and blotting. (a) *N. punctiforme glnB* probe; (b) 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 8×10^{-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 Nm 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 *Xba*I-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 P_{psbA} 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: NO_3^- and NH_4^+, singly or in combination, as the nitrogen source during segregation and plating; supplementation with 0·05% (w/v) Casamino acids or 0·2% (w/v) glutamine during segregation or plating; and plating on $NH_4^+ + 50$ 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

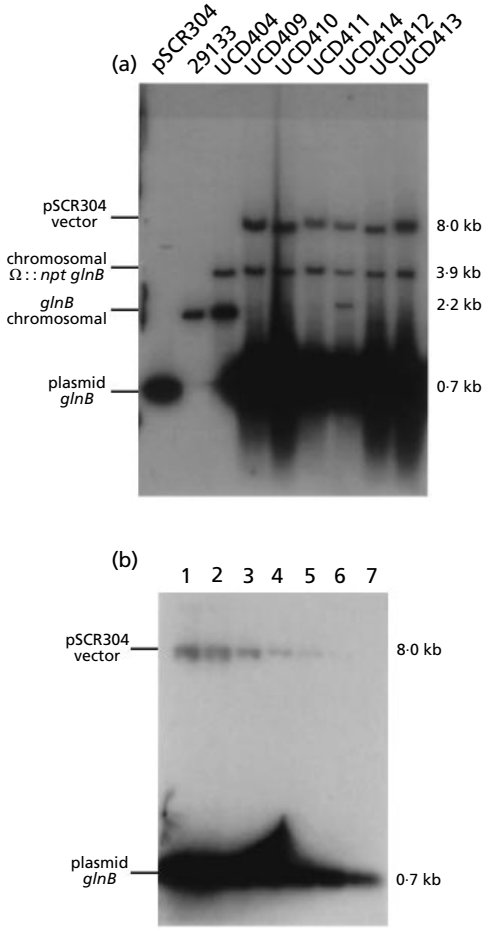

Fig. 7. Analysis of sucrose-resistant derivatives of strain UCD 407. (a) Southern blot of *Eco*RI-digested total DNA from *N. punctiforme* (29133), strain UCD 404, or sucrose-resistant derivatives of strain UCD 407 (UCD 403 + pSCR304) as strains UCD 410–UCD 414. Ten nanograms of purified pSCR304 digested with *Eco*RI was run as a control in lane 1. The blot was probed with *N. punctiforme glnB*. Positions of *glnB*, Ω::*npt*-inserted *glnB* and the plasmid-borne copy of *glnB* are indicated. (b) Serial twofold dilutions of *Eco*RI-digested pSCR304 probed with *N. punctiforme glnB*. Lanes: 1, 500 ng; 2, 250 ng; 3, 125 ng; 4, 63 ng; 5, 32 ng; 6, 16 ng; 7, 8 ng.

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 3×10^{-3} 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 8·0 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_{II} 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_{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 P_{II} protein varied depending on nitrogen source. In *N. punctiforme*, *in vivo* modification of P_{II} 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_{II} protein from residue 37 to 55 regulates interaction between P_{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 *E. coli* whereas it is a serine in *N. punctiforme* and all other known cyanobacterial P_{II} 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_{II} although target proteins directly interacting with P_{II} 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_{II}s may be capable of accepting both uridylyl and phosphoryl groups. The *Synechococcus* 7942 P_{II} 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_{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, 1995a) 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 *Anabaena* 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 caulinodans* (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 argue against polarity as an explanation 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} antiserum (Forchhammer & Tandeau de Marsac, 1994), unlike an *E. coli glnB* mutant (van Heeswijk *et al.*, 1995).

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.

ACKNOWLEDGEMENTS

This work was supported by the US National Science Foundation (grant IBN 95-14787), the Institut Pasteur and Centre National de la Recherche Scientifique (URA 1129). The authors would like to thank Alex Ninfa for the kind gift of *E. coli* strain BD, A. M. Castets for sequencing of the *Calothrix* 7601 *glnB* (accession number X97327), Enrique Flores for discussions on segregation, and E. L. Campbell, K. Hagen and F. Wong for critical reading of the manuscript.

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Received 5 November 1997; revised 4 March 1998; accepted 13 March 1998.