The signal transducer $P_{\text{II}}$ and bicarbonate acquisition in *Prochlorococcus marinus* PCC 9511, a marine cyanobacterium naturally deficient in nitrate and nitrite assimilation

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The amino acid sequence of the signal transducer $P_{\text{II}}$ (GlnB) of the oceanic photosynthetic prokaryote *Prochlorococcus marinus* strain PCC 9511 displays a typical cyanobacterial signature and is phylogenetically related to all known cyanobacterial *glnB* genes, but forms a distinct subclade with two other marine cyanobacteria. $P_{\text{II}}$ of *P. marinus* was not phosphorylated under the conditions tested, despite its highly conserved primary amino acid sequence, including the seryl residue at position 49, the site for the phosphorylation of the protein in the cyanobacterium *Synechococcus* PCC 7942. Moreover, *P. marinus* lacks nitrate and nitrite reductase activities and does not take up nitrate and nitrite. This strain, however, expresses a low- and a high-affinity transport system for inorganic carbon ($C_i$; $K_{m,\text{app}}$ 240 and 4 $\mu$M, respectively), a result consistent with the unphosphorylated form of $P_{\text{II}}$ acting as a sensor for the control of $C_i$ acquisition, as proposed for the cyanobacterium *Synechocystis* PCC 6803. The present data are discussed in relation to the genetic information provided by the *P. marinus* MED4 genome sequence.

**Keywords**: Prochlorales, *glnB* and *glnK* genes, lack of $P_{\text{II}}$ phosphorylation, inorganic carbon uptake, *Prochlorococcus marinus* MED4

INTRODUCTION

The discovery about 10 years ago of *Prochlorococcus* spp., the smallest (0.5–0.7 $\mu$m) prokaryotic oxygenic phototrophs, has changed our general view of community structure and productivity in the world’s oceans (Partensky *et al.*, 1999). With up to $4 \times 10^4$ cells ml$^{-1}$, they are the most abundant marine photosynthetic organisms and they dominate in oligotrophic areas of oceans where nitrogen and phosphorus are often limiting. Although *Prochlorococcus* spp. lack phycobilisomes and synthesize divinyl derivatives of chlorophyll $a$ and $b$ as major photosynthetic pigments, they are assigned to the same phylum as cyanobacteria (Urbach *et al.*, 1998; Turner, 1997).

The *P_{\text{II}}* protein (*glnB* gene product) is one of the most widespread signal transducers in the control of nitrogen metabolism (Arcondéguy *et al.*, 2001; Ninfa & Arkinson, 2000; Merrick & Edwards, 1995). It has been found in archaea and bacteria, as well as in algae and plants. In *Escherichia coli*, the paradigm for *P_{\text{II}}* structure and function in bacteria, *P_{\text{II}}* is modified by uridylylation at a conserved Y51 residue, located at the tip of a solvent-exposed loop (Cheah *et al.*, 1994; Jaggi *et al.*, 1996). In contrast, the *P_{\text{II}}* protein of the cyanobacterium *Synechococcus* sp. PCC 7942 is phosphorylated at residue S49 (Forchhammer & Tande de Marsac, 1994). In both organisms, the trimeric *P_{\text{II}}* protein binds the small effectors ATP and 2-oxoglutarate in a synergistic manner (Forchhammer & Hedler, 1997; Jiang & Ninfa, 1999; Kamberov *et al.*, 1995). However, the *P_{\text{II}}* signal transduction pathway has diverged during evolution. In *E. coli*, the main signal for cellular nitrogen status is glutamine, the level of which is sensed by the

**Abbreviations**: $C_i$, inorganic carbon; MSX, L-methionine-$\delta$-sulfoximine.

The GenBank accession number for the *glnB* gene sequence reported in this paper is AJ271089.
uridylyl-transferase/uridylyl-removing (UTase/UR) enzyme complex (Jiang et al., 1998a, b; Reitzer, 1996). PII transduces the perceived signal to proteins that modulate glutamine synthetase activity and control the expression of genes regulated by nitrogen availability (Jiang et al., 1998b, c; Jiang & Ninfa, 1999). In cyanobacteria, as exemplified by *Synechococcus* sp. PCC 7942, a protein serine kinase and a phosphatase regulate the phosphorylation state of PII (Forchhammer, 1999; Irmler et al., 1997). Furthermore, 2-oxoglutarate rather than glutamate may serve as the main signal for the cellular status of nitrogen. High levels of 2-oxoglutarate favor the phosphorylation of PII, and low levels lead to its dephosphorylation (Forchhammer, 1999; Forchhammer & Tandeau de Marsac, 1995a; Irmler et al., 1997; Muro-Pastor et al., 2001). In the presence of the unphosphorylated form of PII, under an ammonium regime, an inhibition of nitrate and nitrite uptake is observed, whereas in the absence of ammonium the phospho-PII, liganded to an effector (probably 2-oxoglutarate), alleviates this inhibition (Lee et al., 1998, 1999). In *Synechocystis* sp. PCC 6803, the redox state of the cells may act as a trigger for PII phosphorylation (Hisbergues et al., 1999). Moreover, under a high inorganic carbon (C) regime, the PII system relieves inhibition of nitrate uptake and inhibits the high affinity transport system for bicarbonate. While it is now established that in two unicellular freshwater cyanobacteria, *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803, the role of PII is to co-ordinate nitrogen and carbon metabolism, in filamentous strains the role of this protein remains unclear (Hanson et al., 1998; Liotenberg et al., 1996).

A second PII protein, termed GlnK, has been identified in *E. coli* (van Heeswijk et al., 1996). PII and GlnK are structurally similar, but in part functionally distinct proteins (Atkinson & Ninfa, 1998). A PII-like protein has also been identified in the chloroplast genome of the red alga *Porphyra purpurea* (Reith & Munholland, 1993) and of higher plants such as *Arabidopsis thaliana* and *Ricinus communis* (Hsieh et al., 1998). In eukaryotes, PII may serve as part of a complex signal transduction network involved in the recognition of the status of carbon and nitrogen, as has been proposed for PII in cyanobacteria (Forchhammer & Tandeau de Marsac, 1995b; Hisbergues et al., 1999).

We report here the characterization of the PII protein (*glnB* gene product) and some aspects of carbon and nitrogen assimilation of the marine cyanobacterium *Prochlorococcus marinus* PCC 9511, which grows in the presence of ammonium or urea, but not with nitrate or nitrite. During the course of the present work, the genome sequence of *P. marinus* MED4, a strain identical at the subspecies level to *P. marinus* PCC 9511 (Lalou et al., 2002; Rippka et al., 2000), was completed. This provided a means to confirm the biochemical results obtained with *P. marinus* PCC 9511 and helped us to obtain deeper insights into the putative role of PII in a strain not yet amenable to genetic studies.

**METHODS**

**Strain and culture conditions.** The axenic strain *Prochlorococcus marinus* Chisholm et al. 1992 subsp. *pastoris* strain PCC 9511T (hereafter designated *P. marinus* PCC 9511) was grown at 18–20 °C in liquid medium PCR-Tu without agitation as described by Rippka et al. (2000). *(NH₄)₂SO₄* (400 μM) was used as the source of nitrogen. HEPEs, pH 7.5 (1 mM) and NaHCO₃ (2 mM) were added after autoclaving. White light was supplied by fluorescent tubes (True Lite 29; Duro-Lite). The photosynthetic photon flux density was 20 μmol quanta m⁻² s⁻¹ (measured with a LICOR LI-185B quantum/radiometer/photometer equipped with an LI-190SB quantum sensor), with a light/dark cycle of 14/10 h. Strains *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803 were grown as described by Lee et al. (1998).

Plasmids were maintained in *E. coli* strain DH5α Mcr−. Recombinant *E. coli* strains were grown at 37 °C in Luria–Berry medium supplemented with 100 μg ampicillin ml⁻¹.

**Nucleic acid methods.** DNA was extracted from pooled pellets corresponding to a total culture volume of 1 l and an OD₅₅₀ of about 0.15–0.17. After two washes in 10 ml NET solution (6 M Tris/HCl, pH 8, 1 M NaCl and 100 mM Na₂EDTA, pH 8), the cells lysed following resuspension in 1 ml 10 mM Tris/HCl, 20 mM EDTA, pH 8. Two extractions were performed with phenol/chloroform (1:1, v/v) followed by two with chloroform/isoamylalcohol (24:1, v/v). DNA was ethanol-precipitated, washed once with 70% (v/v) ethanol, air-dried and resuspended in 10 mM Tris/HCl, 0.1 mM EDTA, pH 8.

DNA gel electrophoresis, blotting and hybridizations were carried out as described by Damerval et al. (1989). Prehybridization (4 h) and hybridization (16 h) experiments were performed at 65 and 55 °C, respectively. An internal HindIII–BsaB1 fragment (236 bp) of the *glnB* gene from *Calothrix* sp. PCC 7601 (accession no. X97327) was used as a probe. The probe was labelled with [α-³²P]dATP (110 Tbk mmol⁻¹) by using a Megaprime random labelling kit (Amersham).

A partial library was constructed by ligating XbaI DNA fragments of approximately 4 kb into the dephosphorylated pBluescript SK− vector as described by Sambrook et al. (1989). Ligated DNA was transformed by electroporation (Bio-Rad) into *E. coli* strain DH5α Mcr− (Dower et al., 1988). The recombinant plasmid DNA from the clone carrying the proper insert was purified with the QIA filters Qiagen kit (ref. 12262) and sequenced on both strands (Genome Express).

**PII analysis.** Cultures of *P. marinus* PCC 9511 were grown to an OD₅₅₀ of about 0.15−0.17. Cells were harvested by centrifugation at 12000 g for 15 min at 18 °C and resuspended to an OD₅₅₀ of approximately 1.5 in the medium used for the experimental tests, containing 400 μM (NH₄)₂SO₄, no nitrogen source, 100 μM NaNO₃ without CO₂ enrichment or 100 μM Na₂SO₄ with 3% (v/v) CO₂. After 18 h incubation under these experimental conditions, cell suspensions were concentrated approximately 10 times by centrifugation and incubated for an additional period of 6 h before proceeding to the preparation of the cell-free extracts as described by Forchhammer & Tandeau de Marsac (1994). For PII analyses of *Synechocystis* sp. PCC 7942 and *Synechocystis* sp. PCC 6803, cell extracts were prepared from experimental cultures incubated as described by Lee et al. (1998). Protein content in cell-free extracts was estimated by using the Bio-Rad protein assay with BSA (Sigma A-9647) as standard. Native PAGE, immunoblotting and detection with *Synechococcus* sp. PCC 7942 PII antisera were carried out as described by...
Forkhammer & Tandeau de Marsac (1994). $P_{\text{II}}$ was visualized using the ECL detection system of Amersham in which skim dried milk, used in the blocking reagent, was replaced by BSA and the solutions for primary and secondary antibodies contained 0.25% (v/v) Tween (Sigma P-4675) and BSA at final concentrations of 3 and 1% (w/v), respectively, in 150 mM NaCl, 15 mM Tris/HCl, 1 mM EDTA, pH 7.5.

**Chlorophyll determination.** The concentration of chlorophyll $a$ and $a_2$ were determined spectroscopically from methanolic extracts using the extinction coefficient of chlorophyll $a$ at 665 nm (74-5; Mackinney, 1941).

**Enzyme assays.** Nitrate and nitrite reductase activities were determined as described by Lee et al. (2000).

The nitrate uptake assay was performed as described by Lee et al. (1998), except that cells were resuspended in standard N-free growth medium and incubated at 20 °C under a photosynthetic photon flux density of 400 µmol quanta m$^{-2}$ s$^{-1}$.

For the bicarbonate uptake assay, cells were resuspended in artificial seawater (Turks Island Salt Solution; Merck Index no. 9954; Rippka et al., 2000) buffered with 50 mM HEPES, pH 8, to a concentration of 10–12 µg chlorophyll $a_2$ ml$^{-1}$ (equivalent to an OD$\text{_665}$ = 1). The concentrated cell suspension was preincubated in a Clark electrode, under a photosynthetic photon flux density of 400 µmol quanta m$^{-2}$ s$^{-1}$, until carbon storage was exhausted as shown by the levelling off of the O$_2$ emission rate. Bicarbonate uptake activity was then measured as described by Bédou et al. (1995) on cells incubated under 70 µmol quanta m$^{-2}$ s$^{-1}$.

**Phylogenetic analysis.** Distance analysis (neighbour-joining) and maximum-parsimony methods were performed using the PHYLIP package (version 3.57c) (Felsenstein, 1988). The Dayhoff option was employed to compute evolutionary distances. Bootstrap analyses (100 replicates; Felsenstein, 1985) were performed for both analyses.

**In silico analysis of the $P$. marinus MED4 genome.** The search for orthologues in the genome of strain MED4 (http://genome.ornl.gov) was performed by using the Synechocystis sp. PCC 6803 genome database (CyanoBase; http://www.kazusa.or.jp/cyano/) and TBLASTN version 2.2.1. Sequences from other micro-organisms were extracted from GenBank or SWISS-PROT.

**RESULTS AND DISCUSSION**

**Characterization of the $glnB$ gene**

As in other prokaryotes and in the plastidial genome of the red alga *Porphyra purpurea*, the $glnB$ gene of *P. marinus* PCC 9511 is 339 nt long. The gene is preceded by putative Shine–Dalgarno (AGAGA) and NtcA-binding (GTA-N$\text{_2}$-AAC) (Herrero et al., 2001) sequences, located 5 and 290 nt from the translation start codon ATG, respectively. The transcriptional effector NtcA may therefore regulate the expression of the $glnB$ gene as shown for *Synechococcus* sp. PCC 7942 (Lee et al., 1999). The gene ends with a TGA stop codon and encodes a polypeptide with a calculated molecular mass of 12314.22 Da. Fig. 1A shows an alignment of the predicted amino acid sequence of the $glnB$ gene of *P. marinus* PCC 9511 with those of other cyanobacterial representatives and of some proteobacteria that have a high degree of sequence similarity. As expected (Rippka et al., 2000), the $glnB$ gene product of *P. marinus* PCC 9511 is 100% identical to that of *P. marinus* MED4. It shows an overall identity of 74–83% with the other cyanobacterial amino acid sequences, while it presents only 39–59% identity with $P_{\text{II}}$ proteins of other prokaryotes and photosynthetic eukaryotes (Fig. 1A and data not shown). Moreover, it displays typical cyanobacterial amino acid signatures, including an S49 residue (Fig. 1A) that is also conserved in the plastidial proteins (data not shown). This amino acid is the site of phosphorylation in the $P_{\text{II}}$ protein of *Synechococcus* sp. PCC 7942 (Forkhammer & Tandeau de Marsac, 1994). The *E. coli* $P_{\text{II}}$ protein, in which an alanyl residue occupies position 49, is uridylylated on a Y51 residue. Uridylylation of a Y51 residue has also been shown for the $P_{\text{II}}$ protein of *Synechococcus* sp. PCC 7942, but only if expressed in *E. coli* (Forkhammer & Hedler, 1997). Such a modification is not expected to occur in the $P_{\text{II}}$ protein of *P. marinus* PCC 9511, since like in *Arabidopsis thaliana* and *Ricinus communis*, this tyrosyl residue is replaced by a phenylalanine (Hsieh et al., 1998).

The overall topology of the neighbour-joining tree of the $P_{\text{II}}$ proteins from bacteria, archaea and cyanobacteria is conserved in trees inferred by parsimony analysis (data not shown). As shown in Fig. 1(B), the cyanobacterial lineage is distant from the bacterial $P_{\text{II}}$ used as an outgroup, and the $P_{\text{II}}$ of the two *Prochlorococcus* isolates and the oceanic *Synechococcus* strain WH 8102 form a distinct subclade within the cyanobacterial $P_{\text{II}}$ radiation.

In contrast to proteobacteria, the presence of additional $glnB$-like genes has not been reported in cyanobacteria so far. In agreement, only one band was detected in Southern hybridizations using the $glnB$ gene of *P. marinus* PCC 9511 as probe, irrespective of the restriction enzymes employed (data not shown) and, like in other cyanobacterial genomes, no equivalent of $glnK$ was found in strain MED4.

**Analysis of $P_{\text{II}}$ modification**

The isoforms of the $P_{\text{II}}$ protein can be separated by electrophoresis on native PAGE. The molecular masses of the $P_{\text{II}}$ proteins from *P. marinus* PCC 9511, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 are nearly identical (12314-18, 12397-38 and 12391-36 Da, respectively), but their isoelectric points differ (5-28, 6-33 and 7-95, respectively) leading to the difference in electrophoretic mobility observed in Fig. 2 (http://kr.expasy.org/tools/).

Immunoblots of extracts of *P. marinus* PCC 9511 cells, grown with either ammonium or urea, or incubated for 24 h with nitrate or no source of nitrogen, with or without enrichment with 3% (v/v) CO$_2$, showed only one protein that gave a cross-reaction with the antibodies raised against $P_{\text{II}}$ from *Synechococcus* sp. PCC 7942 (Fig. 2 and data not shown). The mobility of this protein remained unchanged after treatment with alkaline phosphatase or snake venom phosphodiesterase, indicating that it corresponded to the unmodified form of $P_{\text{II}}$ (data not shown). Moreover, phosphorylation
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Fig. 1. (A) Alignment of the predicted amino acid sequences of GlnB (P\textsubscript{II}) of cyanobacteria and some proteobacteria, and of GlnK from \textit{Escherichia coli}. The S49 and Y51 residues are indicated in bold letters. Percentages of similarity (S) and identity (I) are relative to the amino acid sequence of \textit{P. marinus}. Amino acid signatures shared by all cyanobacteria and not present in bacterial P\textsubscript{II} are marked with asterisks. (B) Rooted neighbour-joining tree of the cyanobacterial GlnB sequences available with \textit{Aquifex aeolicus} GlnB (AE000674) used as an outgroup. Cyanobacterial genes: \textit{P. marinus} PCC 9511 (this work), \textit{Prochlorococcus} sp. WH 8102, \textit{Synechocystis} PCC 6803, \textit{Calothrix} PCC 7601, \textit{Synechococcus} PCC 7002, \textit{Nostoc} PCC 73102, \textit{Anaabaena} PCC 7120, \textit{Synechococcus} PCC 7942. Cyanobacterial signature \textit{Sinorhizobium melloti} \textit{Aquifex aeolicus} \textit{Escherichia coli} \textit{Herbaspirillum seropedicae} \textit{Escherichia coli} GlnK.

was not detected when cells of \textit{P. marinus} PCC 9511 were incubated with ammonia in the presence of l-methionine-d-l-sulfoximine (MSX), an inhibitor of glutamine synthetase (data not shown).

The lack of \textit{in vivo} modification of P\textsubscript{II} in \textit{P. marinus} PCC 9511 is in contrast to the results obtained with the unicellular freshwater strains \textit{Synechococcus} sp. PCC 7942 (Forchhammer & Tandeau de Marsac, 1994).
Fig. 2. Immunoblot of cell-free extracts of Prochlorococcus sp. PCC 7942, Synechocystis sp. PCC 6803 and P. marinus PCC 9511. P₀, P₁, P₁₀ and P₁₁ indicate isoforms of P₁₀ carrying zero, one, two and three phosphate groups, respectively. NH₄⁺ cells grown in the presence of (NH₄)₂SO₄ (400 µM); –N, cells exposed to nitrogen starvation for 24 h; NO₃⁻ and NO₃⁻ + CO₂ incubation of cells for 24 h in the presence of NaNO₃ (100 µM) without CO₂ enrichment or with 3% (v/v) CO₂, respectively. Cell-free extracts of Synechococcus sp. PCC 7942 and Synechocystis sp. PCC 6803 were obtained from cultures grown with nitrate (17.6 mM). Each lane contains 20 µg total soluble protein ml⁻¹, except the lane corresponding to ammonium-grown cells (40 µg ml⁻¹).

Lack of nitrate and nitrite assimilation

The preferred sources of nitrogen in cultures of P. marinus PCC 9511 are urea and ammonia, whereas no growth is observed with nitrate, a nitrogen source commonly used by other cyanobacteria (Rippka et al., 2000). Accordingly, no nitrate and nitrite reductase activities were detected. Nitrate uptake was also insignificant [0.5 ± 0.1 nmol min⁻¹ (mg chlorophyll a)⁻¹ compared to 151 ± 15 nmol min⁻¹ (mg chlorophyll a)⁻¹ for Synechococcus sp. PCC 7942].

In strain MED4, the genes for the reductases and the uptake system for nitrate and nitrite are lacking (Table 2). The same holds true for ureTP, a gene encoding a novel type of nitrate/nitrite permease first described for the coastal cyanobacterium Synechococcus sp. PCC 7002 (Sakamoto et al., 1999). P. marinus strains MED4 and PCC 9511 are therefore naturally deficient for assimilation of these nitrogen sources. In contrast, the ure genes encoding the urease complex that have been

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* ORFs designated sll-, slr- and ssl- are from Synechocystis sp. PCC 6803 (http://www.kazusa.or.jp/cyano/). AF001333 is the GenBank accession number for genes from Synechococcus sp. PCC 7942. For data on ure genes, see Palinska et al. (2000).
Table 2. ORFs of Synechocystis PCC 6803 not detected in the P. marinus MED4 genome

Data are taken from the Synechocystis PCC 6803 genome (http://www.kazusa.or.jp/cyano/) unless otherwise indicated. References: 1, Shi et al. (1998); 2, Leonard et al. (1998); 3, Irmler & Forchhammer (2001); 4, Shibata et al. (2001); 5, Bonfil et al. (1998); 6, Omata et al. (2001).

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![Figure 3](image-url) Uptake of HCO<sub>3</sub> by P. marinus PCC 9511 cells. (A) S, Uptake measured in the presence of increasing concentrations of HCO<sub>3</sub> from 2.5 to 500 µM; V, HCO<sub>3</sub> uptake in nmol·min<sup>−1</sup>·(mg chl)<sup>−1</sup>. (B) The Lineweaver-Burk plot reveals two apparent K<sub>m</sub> values of 240 and 4 µM. with the 2-oxoglutarate-P<sub>II</sub> complex inhibiting, directly or indirectly, the high affinity C<sub>i</sub> transport system independently of the phosphorylation state of P<sub>II</sub>. Such a control of C<sub>i</sub> acquisition may also be exerted in P. marinus PCC 9511, incapable of P<sub>II</sub> phosphorylation.

A search for genes involved in C<sub>i</sub> acquisition in the genome of strain MED4 revealed no orthologues of cupA and cupB, encoding components of an inducible and a constitutive system for CO<sub>2</sub> uptake in Synechocystis sp. PCC 6803, respectively (Table 2). In addition, no orthologues of ictB, encoding a putative Na<sup>+</sup>/HCO<sub>3</sub> symporter, and of the cnp genes encoding an ABC-type transport system for bicarbonate ions, which is induced under CO<sub>2</sub>-limited conditions, were detected (Table 2). In contrast, genes with high similarities to cynABD and cynS were found (Table 1). In Synechococcus sp. PCC 7942 the cynABD genes encode an ABC-type transporter for cyanate and cynS encodes a cyanase that degrades cyanate to CO<sub>3</sub> and ammonium using bicarbonate as a second substrate (Harano et al., 1997). The CynABD transport system might therefore be utilized for the transport of both bicarbonate and cyanate as proposed by Harano et al. (1997). At this stage, however, one cannot exclude that another bicarbonate transporter either not yet identified or very divergent from those known so far might be operating in this subspecies of P. marinus strains.

Concluding remarks

In both Synechococcus PCC 7942 and Synechocystis sp. PCC 6803, the signal transducer P<sub>II</sub> coordinates both nitrogen and carbon metabolism (Hisbergues et al., 1999; Lee et al., 2000). The nitrate and nitrite assimilation pathway is absent in P. marinus MED4, a subspecies identical to PCC 9511 (Rippka et al., 2000).
Moreover, the number of putative serine/threonine kinases and phosphatases is limited. Finally, if PII exerts a regulatory function in members of Prochlorococcus, its interplay will need to proceed without post-translational modification of the protein.

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tion of the signal transducer P$_{II}$ (GlnB) and influences its phosphorylation level in response to nitrogen and carbon supplies in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J Bacteriol* 181, 2697–2702.


