mrpA, a gene with roles in resistance to Na\textsuperscript{+} and adaptation to alkaline pH in the cyanobacterium \textit{Anabaena} sp. PCC7120

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Transposon mutagenesis of \textit{Anabaena} sp. PCC7120 led to the isolation of a mutant strain, PHB11, which grew poorly at pH values above 10. The mutant strain exhibited pronounced Na\textsuperscript{+} sensitivity; this sensitivity was higher under basic conditions. Mutant PHB11 also showed an inhibition of photosynthesis that was much more pronounced at alkaline pH. Reconstruction of the transposon mutation of PHB11 in the wild-type strain reproduced the phenotype of the original mutant. The wild-type version of the mutated gene was cloned and the mutation complemented. In mutant strain PHB11, the transposon had inserted within an ORF that is part of a seven-ORF operon with significant sequence similarity to a family of bacterial operons that are believed to code for a novel multiprotein cation/proton antiporter primarily involved in resistance to salt stress and adaptation to alkaline pH. The \textit{Anabaena} operon was denoted \textit{mrp} (multiple resistance and pH adaptation) following the nomenclature of the \textit{Bacillus subtilis} operon; the ORF mutated in PHB11 corresponded to \textit{mrpA}. Computer analysis suggested that all seven predicted \textit{Anabaena} Mrp proteins were highly hydrophobic with several transmembrane domains; in fact, the predicted protein sequences encoded by \textit{mrpA}, \textit{mrpB} and \textit{mrpC} showed significant similarity to hydrophobic subunits of the proton pumping NADH:ubiquinone oxidoreductase. \textit{In vivo} expression studies indicated that \textit{mrpA} is induced with increasing external Na\textsuperscript{+} concentrations and alkaline pH; \textit{mrpA} is also upregulated under inorganic carbon (Ci) limitation. The biological significance of a putative cyanobacterial Mrp complex is discussed.

INTRODUCTION

Cyanobacteria are the only prokaryotic organisms carrying out an oxygen-evolving photosynthesis. They are thought to have originated during the Precambrian era (more than $3 \times 10^9$ years ago) and as a group are known to survive a wide spectrum of environmental stresses, such as temperature shock, photooxidation, nutrient deficiency, pH changes, salinity and osmotic stress, and ultraviolet light. Thus, cyanobacteria appear to be a suitable system for analysing the active mechanism(s) developed in response to changing environmental conditions.

Regarding pH requirements, cyanobacteria have optimum growth between pH 7.5 and 11, being practically absent in habitats with pH values below 4 or 5 (Brock, 1973). There is still no satisfactory understanding of the molecular mechanisms underlying their preference for alkaline environments, although it should be noted that alkaline pH favours the formation of bicarbonate and that aquatic photosynthesizers are often limited by inorganic carbon (Ci) availability. Studies involving a range of organisms have identified both Na\textsuperscript{+}/H\textsuperscript{+} and K\textsuperscript{+}/H\textsuperscript{+} antiporters as major means of regulating internal pH (pH\textsubscript{in}) when the external pH is alkaline (Krulwich, 1995). However, the major Na\textsuperscript{+}-extruding mechanism in most bacterial cells is the Na\textsuperscript{+}/H\textsuperscript{+}
antiporter, which extrudes Na\(^+\) in exchange for H\(^+\) (Kruhlwich et al., 1994; Padan & Schuldiner, 1994). This process is driven by an electrochemical gradient of protons across the cytoplasmic membrane, which is established by the respiratory chain or the H\(^-\)-translocating ATPase. Thus, in bacteria, the Na\(^+\)/H\(^+\) antiporter plays a role in Na\(^+\) extrusion, pH homeostasis, cell-volume regulation and establishment of an electrical potential of Na\(^+\) (Padan & Schuldiner, 1996).

Na\(^+\) is an essential ion for most cyanobacteria, especially when they are grown at high external pH values (Allen & Arnon, 1955; Espie et al., 1988). Na\(^+\) is needed for the uptake of several inorganic nutrients (inorganic carbon, nitrate, phosphate) (Lara et al., 1993; Avendaño & Fernández-Valiente, 1994) and for photosynthetic electron transport at the O\(_2\)-evolving complex (Zhao & Brand, 1988).

While low concentrations of Na\(^+\) are required by cyanobacteria, higher concentrations of Na\(^+\) may be harmful. The salt tolerance of cyanobacteria varies widely, from low-salt-tolerance strains that tolerate a maximum of 0.5 M NaCl to strains from hypersaline environments that can tolerate up to 3 M NaCl. For successful salt adaptation at high salt concentrations, cyanobacteria actively extrude Na\(^+\), accumulate K\(^+\) and maintain internal ion concentrations comparable to cells grown under low-salt concentrations (Reed & Stewart, 1985). The extrusion of Na\(^+\) by cyanobacteria has been explained by Na\(^+\)/H\(^+\) proton exchange, since increased Na\(^+\)/H\(^+\) antiporter activities have been detected in salt-adapted cyanobacterial cells. These transporters use the proton motive force established by primary H\(^+\) pumps, such as H\(^-\)-ATPases or respiratory cytochrome oxidases. The main role of Na\(^+\)/H\(^+\) antiporters in ion export to achieve high salt tolerance has been clearly shown in Escherichia coli (Padan & Schuldiner, 1993). In contrast, from bioenergetic studies, the action of a primary Na\(^+\)-ATPase has been predicted to serve as the main source for active Na\(^+\) extrusion in cyanobacteria (Ritchie, 1992), since, at least under alkaline conditions, the adverse transmembrane pH gradient prevents the generation of a substantial proton motive force. However, although there are several potential Na\(^+\)-ATPases in the Synechocystis sp. PCC6803 genome, no Na\(^+\)-ATPase has been identified thus far in any cyanobacterium (Ritchie, 1998). Also, bioenergetic analysis reveals that, under physiological ranges of pH, Na\(^+\)-coupled secondary ion transport across membranes occurs in Synechococcus sp. PCC7942 (Ritchie, 1998), implying that there is an energy-consuming Na\(^+\)-efflux mechanism in cyanobacteria. Therefore, whether or not the predicted energy-dependent Na\(^+\)-efflux protein(s) actually exist, and the extent to which other cation ATPases may also affect Na\(^+\) sensitivity in cyanobacteria, remain to be established.

Most studies of salt adaptation have been performed in the moderately halotolerant cyanobacterium Synechocystis sp. PCC6803. Recently Wang et al. (2002) and Elanskaya et al. (2002), independently, have mutated five genes identified as encoding putative Na\(^+\)/H\(^+\) antiporters in the Synechocystis genome (Kaneko et al., 1996). Elanskaya et al. (2002) found that, with the exception of the NhaS3 mutant, which could not be segregated, none of the mutants showed a significant growth depression under high-salt and/or high-pH conditions. Wang et al. (2002) also found that NhaS3 may perform essential housekeeping functions for the survival of the organism, being critical for salt tolerance. Disruption of nhaS2 and nhaS4 demonstrated that both genes could be essential for the survival of cyanobacteria in freshwater habitats characterized by large fluctuations in salt concentration and pH.

No such studies have been undertaken in the heterocystous filamentous cyanobacterium Anabaena sp. PCC7120, a freshwater strain with low salt tolerance. In this work, we report the identification of a transposon-generated mutant of Anabaena sp. PCC7120, denoted PHB11, which exhibits pronounced Na\(^+\) sensitivity and which was initially isolated by its inability to grow at alkaline pH.

The transposon in PHB11 inserted within ORF all1838, which forms part of a putative seven-ORF operon (all1843–all1837) which in turn shows significant sequence similarity to a family of bacterial operons mainly involved in tolerance to high salt concentrations and in adaptation to alkaline pH (Hiramatsu et al., 1998; Putnoky et al., 1998; Ito et al., 1999; Kosono et al., 1999). The ORF mutated in PHB11 shows the highest similarity to ORFA of bacterial operons and has been denoted mrPA following the nomenclature of the Bacillus subtilis mrP (multiple resistance and pH adaptation) operon, which is the most extensively studied (Ito et al., 1999). The six remaining ORFs of the putative Anabaena operon were also denoted according to their highest similarity with the corresponding bacterial ORFs. The evidence presented indicates that Anabaena mrPA is involved, as are its bacterial counterparts, in Na\(^+\) tolerance, particularly at elevated pH. Interestingly, three genes of the putative Anabaena mrP operon, mrPA, mrPB and mrPD, code for predicted protein sequences that also show significant similarity to hydrophobic subunits of the proton-pumping NAD(P)H:ubiquinone oxidoreductase (complex I) found in mitochondria and eubacteria, which may suggest a potential relationship between complex I and the putative Anabaena MrP complex.

**METHODS**

**Strains and growth conditions.** Anabaena sp. strain PCC7120 and its derivatives (Table 1) were routinely grown at 28 °C in continuous warm white light (90 μE m\(^{-2}\) s\(^{-1}\)) on a rotary shaker in 50 ml medium AA/8 (Allen & Arnon, 1955) supplemented with nitrate (5 mM) and buffered with 25 mM HEPES, pH 7.5, in 125 ml Erlenmeyer flasks. To buffer media at pH 9 and above, Bistris propane (BTP) was used. Plasmid constructs (Table 1) were introduced into cyanobacterial strains by conjugations (Elhai & Wolk, 1988), and single and double recombinant strains were selected as described by Cai & Wolk (1990).

The different strains were grown in the presence of appropriate
antibiotics. Mutant PHB11 was grown in liquid AA/8 medium with 40 μg ml⁻¹ (final concentration) neomycin sulfate (Nm). Single recombinant SR2045-1B and double recombinant DR2045-6A were grown with 2 μg ml⁻¹ spectinomycin dihydrochloride (Sp) plus, respectively, 1 μg ml⁻¹ erythromycin (Em) or no additional antibiotic. Mutant PHB11 bearing plasmid PBG2041 was grown with 1 μg ml⁻¹ Em and 40 μg ml⁻¹ Nm.

Analytical methods. Culture density was determined spectrophotometrically at 750 nm. For dry-weight determinations, cells were collected, washed and dried at 70 °C for 24 h. For chlorophyll determinations, samples were extracted in methanol at 4 °C for 24 h in darkness. The chlorophyll content of the extract was estimated according to the spectrophotometric method of Marker (1972).

Preparation of thylakoid membranes. Thylakoid membrane isolation was performed using the method described by Mi et al. (1995) from cell suspensions exposed to pH 7.5-10.5. Cultures were harvested by centrifugation (20 000 g, 10 min). The cell pellet was washed twice and resuspended in 25% (v/v) glycerol, 10 mM MgCl₂, 10 mM NaCl, 20 mM sodium phosphate buffer, pH 7.5, and 1 mM PMSF. After 1 h on ice, cells were broken in a precooled French press (Simoamincos FA-078) at 147 000 kPa. Cell debris was removed by centrifugation; the supernatant was centrifuged at 100 000 g for 1 h at 4 °C. The pellet was resuspended in the same medium containing 25% (v/v) glycerol, 10 mM MgCl₂, 10 mM NaCl, 20 mM sodium phosphate buffer, pH 7-5, and 1 mM PMSF. Membranes were stored at −70 °C.

Measurement of photosynthetic activities. Oxygen evolution was measured at 30 °C under saturating white light (300 μmol photons m⁻² s⁻¹) with a Clark-type oxygen electrode (Hansatech).

Total photosynthetic flux was assayed as O₂ consumption using methyl viologen as artificial electron acceptor, as described previously (Lien, 1978).

Photosystem II capacity in isolated membranes (20 μg Chl ml⁻¹) (Chl, chlorophyll) was estimated as O₂ evolution in the presence of 2 mM phenyl-1,4-benzoquinone (PBQ), 10 mM CaCl₂ and 0-42 mM ferricyanide in saturating white light (300 μmol photons m⁻² s⁻¹).

Photosystem I activity was measured in isolated membranes (20 μg Chl

<p>| Table 1. Anabaena strains and plasmid constructs used in this study |
|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Derivation and/or salient characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>PCC7120</td>
<td>Wild-type</td>
<td>C. P. Wolk</td>
</tr>
<tr>
<td>PHB11</td>
<td>NmR</td>
<td>This work</td>
</tr>
<tr>
<td>PHB11</td>
<td>NmR EmR</td>
<td>This work</td>
</tr>
<tr>
<td>(pBG2041)</td>
<td>SpR SmR EmR product of single homologous recombination of plasmid pBG2045 with wild-type PCC7120</td>
<td>This work</td>
</tr>
<tr>
<td>SR2045-1B</td>
<td>SpR SmR EmR product of double homologous recombination of plasmid pBG2045 with wild-type PCC7120</td>
<td>This work</td>
</tr>
<tr>
<td>DR2045-6A</td>
<td>Cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>PCR 2.1 TOPO</td>
<td>Circularized EcoRV fragment from Anabaena sp. strain PHB11 that bears Tns-1063</td>
<td>This work</td>
</tr>
<tr>
<td>pBG2033</td>
<td>Anabaena DNA-containing Pstl-BamHI portion of pBG2033 fused to the Pstl-BamHI portion of pRL759D that contains oriV, bom, luxAB, and SmR SpR determinant</td>
<td>This work</td>
</tr>
<tr>
<td>pBG2038</td>
<td>728 pb PCR fragment bearing wild-type mrpA as its only ORF cloned in the PCR 2.1TOPO vector</td>
<td>This work</td>
</tr>
<tr>
<td>pBG2041</td>
<td>Wild-type mrpA on a BamHI-XhoI fragment from pBG2038, inserted into pRL1342 cut with BamHI and XhoI</td>
<td>This work</td>
</tr>
<tr>
<td>pBG2045</td>
<td>Product of ligation of pBG2037 linearized at EcoRV, with the FspI portion of pRL1075 that contains sacB and CmR EmR determinants</td>
<td>This work</td>
</tr>
<tr>
<td>pRL623</td>
<td>Helper plasmid bearing methylene genes M.Aval, M.Eco47II (whose product methylates AvalI sites), and M.EcoT22I (EcoT22I is an isoschizomer of AvaII)</td>
<td>Elhai et al. (1997)</td>
</tr>
<tr>
<td>pRL759D</td>
<td>One of a family of BLOS plasmids with bom, V. fischeri luxAB, oriV and SmR SpR determinant for in vitro replacement of all but the termini of a transposon</td>
<td>Black et al. (1993)</td>
</tr>
<tr>
<td>pRL1075</td>
<td>Contains a sacB-oriT (RK2) CmR EmR cassette that is separated from oriV by inverted palindromes</td>
<td>Black et al. (1993)</td>
</tr>
<tr>
<td>pRL1124</td>
<td>Km' derivative of pACY177 that bears the same methylene genes as pRL623</td>
<td>Cohen et al. (1998)</td>
</tr>
<tr>
<td>pRL1342</td>
<td>CmR EmR RSF1010-based plasmid</td>
<td>C. P. Wolk</td>
</tr>
</tbody>
</table>

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ml⁻¹) as O₂ consumption using sodium ascorbate (5 mM) and 2,6-dichlorophenol indophenol (DCPIP) as artificial electron donors and methyl viologen (0-13 mM) as artificial electron acceptor (Lien, 1978).

**Estimation of intracellular pH.** Intracellular pH was measured by electron spin resonance (ESR), mainly as described by Belkin et al. (1987). The nitroxide spin probes used were: 3-carbamoyl proxyl (neutral probe); 3-aminomethyl proxyl (basic probe) and 3-carboxy proxyl (acid probe). All assays were conducted using a Varian model E-12 EPR spectrometer. The samples consisted of a concentrated cell suspension (80 μg Chl ml⁻¹) to which the appropriate spin probe was added (final concentrations of 200 μM for the neutral probe and 100 μM for the basic and acid probes). When required, 1-75 M nickel tetraethylenepentaamine sulphate (NiTEPA) was added to quench the ESR signal emanating from probe molecules in the medium and thereby visualize only the intracellular signal. Ratios of internal to total probe concentrations were calculated from height ratios to the midfield lines of the quenched and unquenched samples. Samples were illuminated, when required, within the spectro-ratios to the midfield lines of the quenched and unquenched samples. Samples were illuminated, when required, within the spectrometer cavity with a fluence rate of 300 μmol photons m⁻² s⁻¹ from a cold white light from a fibre-optic system (KL1500 Electronic, Schott). Calculations of intracellular pH were made according to Belkin et al. (1987).

**Recovery of transposon-containing plasmids and construction of derivatives of these plasmids.** Plasmid pBG2033 (5.1-1063 and contiguous *Anabaena* sp. DNA) (Table 1) was obtained by digesting chromosomal DNA from mutant PHB11 with EcoRV and then by recircularization of the fragments with T4 DNA ligase and transfer to *E. coli* HB101 by electroporation. Colonies that grew on LB-agar plates with 50 μg/ml kanamycin sulphate (Km) were analysed further. Approximately 3-8 kb of *Anabaena* sp. strain PCC7120 genomic DNA was recovered in pBG2033.

In order to generate the PHB11 mutation in wild-type *Anabaena* sp. strain PCC7120 (see Table 1), most of the transposon was removed from pBG2032 by cutting with *PstI* and *BamHI*. The remaining 4-6 kb piece of DNA was ligated with pRL759D (Black et al., 1993) that had been cut with *PstI* and *BamHI*, generating pBG2037. Plasmid pRL1075, which bears the conditionally lethal gene *sacB*, which in turn allows for selection of double recombinant strains (Cai & Wolk, 1990), was cut with *FspI*, and a fragment of 5-6 kb was inserted into pBG2037 that had been cut with EcoRV, to generate pBG2045 (Table 1).

**Cloning of mrpA and assays of complementation of the mutant strain.** A PCR clone of wild-type *Anabaena* sp. strain PCC7120 DNA bracketing the transposon in mutant PHB11 was generated with the primers 5'-TGGCGGTTGCTATTCTTCTAGG-3' and 5'-TGGCGGAATTCGGCAGGACGA-3'. The resulting 728 bp PCR fragment was firstly cloned in the vector PCR2.1TOPO (Invitrogen) producing plasmid pBG2038 (Table 1). From pBG2038, the same fragment was cut and inserted between the *BamHI* and *Xhol* sites of pRL1342, a chloramphenicol- and erythromycin-resistant RSF1010-based plasmid (obtained from C. P. Wolk), generating plasmid pBG2041 (Table 1), which can replicate in *Anabaena* sp. strain PCC7120.

Plasmid pBG2041 was transferred, with pRL1342 as control, from *E. coli* to cells of mutant strain PHB11, as described by Wolk et al. (1984), using helper plasmids pDS4101 and pRL1124 (see Table 1) (Finnegan & Sherratt, 1982). Selection was made on Petri dishes of agar-solidified AA medium (Allen & Arnon, 1955) containing 10 μg Em ml⁻¹ and 200 μg Nm ml⁻¹.

The green colonies that appeared on the filters were further restreaked to plates of the same medium to assess their ability to grow at high pH and/or at high salt concentration.

**Southern analysis.** Southern analysis of chromosomal DNA made use of the Genius system (Boehringer Mannheim). DNA probes were labelled with digoxigenin-11-dUTP from random primers (DIG DNA Labelling Kit, Boehringer Mannheim).

**Sequence analysis.** Automated sequencing (ABI Prism 377 DNA Sequencer, Perkin-Elmer) was performed on fragments that were subcloned from pBG2033 and pBG2038. The initial sequencing from the ends of the transposon in pGB2033 was performed from specific primers for the left and right end of the transposon (Black et al., 1993). Sequence analysis was performed with the UW GCG package of the University of Wisconsin, Genetics Computer Group, version 7 (Devereux et al., 1984). Amino acid sequence analysis was performed with the DAS transmembrane prediction package (Proteomics tools) at the ExPASy Molecular Biology Server (http://www.expasy.org/) and Pfam (Protein Search Washington University in St Louis) (http://pfam.wustl.edu/) for conserved protein domains.

Database comparisons and alignments of the DNA and predicted protein sequences were performed by using the default settings of the algorithm developed by Altschul et al. (1997) at the National Center for Biotechnology Information (NCBI) with the BLAST network service programs (http://www.ncbi.nlm.nih.gov/blast/) and WU-BLAST from the European Bioinformatics Institute (EMBL-EBI) (http://www.ebi.ac.uk/blast2/index.html). Sequence alignments were performed with the CLUSTAL X program version 1.81 (Thompson et al., 1997).

**In vivo monitoring of the expression of mrpA.** The luciferase activity of aliquots from strains DR2045-6A and SR2045-1B exposed to increasing Na⁺ concentrations and/or alkaline pH was measured at specified times and used as a measure of the transcription of *mrpA*. *mrpA* expression was also measured under Ci limitation, which was applied by switching the aeration from 1-5% CO₂ in air (v/v) to air alone (350 ppm, Ci-limited) (Wang et al., 2004). The luminescence of both strains was measured with supplementation of exogenous aldehyde (0-01%, v/v, n-decyl aldehyde, 0-1%, v/v, Triton X-100). Luminescence was measured using a digital luminometer (Bio Orbit 1250 Luminometer). The luminometer was calibrated by setting the background counts to zero and the built-in standard photon source [a scaled ampoule of the isotope ¹³C, activity 0-26 μCi (9-62 kBg)] to 10 mV. Calculations made according to Hastings & Weber (1963) indicated that one unit (1 mV) corresponded to a light emission of 6·7×10⁶ quanta s⁻¹ from the vial.

**Nucleotide sequence accession number.** The sequence reported in this paper has been submitted to GenBank under accession no. AF239979.

**RESULTS AND DISCUSSION**

**Phenotype of mutant PHB11**

Transposon-bearing (i.e. antibiotic resistant) exconjugant colonies grown in AA plus nitrate medium buffered at pH 7-5 were transferred to Petri dishes with medium buffered at pH 10-5 (5 mM BTP). One colony that yellowed after several days under the basic pH conditions was selected for further study and denoted as PHB11. Southern analysis of PHB11 showed that only one copy of the transposon had inserted into the *Anabaena* genome within an EcoRV fragment of approximately 12 kb (data not shown).

To determine the function of the gene affected in the mutant, various physiological tests were carried out. First, a time-course study of the growth of the mutant in medium...
buffered at three selected external pH values, 7.5, 9 and 10.5, was undertaken (Fig. 1a–c). As shown in the figure (Fig. 1a), at pH 7.5 the growth curve of the mutant strain was similar to that of the wild-type, although in the long term (after 7 days of culture), the growth yield of the mutant was 10% less than that of the wild-type (Student’s t test, \( P < 0.05 \)). At pH 9 (Fig. 1b), the mutant strain showed 8% growth inhibition after 24 h (Student’s t test, \( P < 0.05 \)); after 7 days of culture the inhibition reached 16%. However, at pH 10.5 (Fig. 1c), the mutant strain showed impaired growth after only 24 h of culture (20% less growth, Student’s t test, \( P < 0.05 \)). After 10 to 11 days at an external pH of 10.5, mutant PHB11 bleached and died. Microscopic observations showed that under these basic conditions, filaments of the mutant strain appeared yellow, short and with distorted cells.

Photosynthesis was also examined in the mutant strain. As shown in Fig. 1(d–f), the photosynthetic activity was lower in the mutant strain than in the wild-type at the three selected external pHs, although the inhibition of photosynthesis was much more pronounced at pH 10.5 (Fig. 1f).

The observed effect of basic pH on photosynthesis of the mutant led us to measure the total photosynthetic flux after rapid alkalinization of the medium by suddenly shifting external pH from 7.5 to 9 and from 7.5 to 10.5 by addition of 0.05 M NaOH. The whole alkalinization process was tightly monitored with a pH electrode. After a pH shift from 7.5 to 9, the photosynthetic flux of the mutant strain was significantly inhibited, being around 45% that of the wild-type (39.77 ± 9.42 versus 87.79 ± 16.48 μmol O₂ mg Chl⁻¹ h⁻¹ for the wild-type; Student’s t test, \( P < 0.05 \)); after a pH shift from 7.5 to 10.5, the inhibition was around 95% of the wild-type strain value (5.89 ± 0.84 versus 110.27 ± 10.99 μmol O₂ mg Chl⁻¹ h⁻¹ for the wild-type; Student’s t test, \( P < 0.05 \)). The rapid and significant inhibition of the photosynthetic flux in the mutant strain after the pH change suggests that the mutated gene may be particularly important to cope with short-term stress.

The observed inhibition of photosynthesis may reflect an effect on the activity of the photosystems. To test the effect of the mutation on each photosystem, thylakoidal membranes of both strains were isolated from cells grown at pH 7.5.
and 10.5 and activities of photosystem I (PSI) and photosystem II (PSII) measured in the isolated membranes. The most significant effect was seen at pH 10.5, at which a 75% inhibition of PSI activity in the mutant strain (17.36 ± 3.11 versus 72.96 ± 1.53 μmol O₂ mg Chl⁻¹ h⁻¹ for the wild-type), and a 20% inhibition of PSI activity (131.26 ± 4.20 versus 164.27 ± 2.15 μmol O₂ mg Chl⁻¹ h⁻¹ for the wild-type) were recorded.

This initial phenotypic characterization indicated that the mutant had serious problems of adaptation to external pH values in the alkaline range; this may reflect difficulties of the mutant strain in controlling its intracellular pH. To check this, we used ESR spin-probe techniques (Belkin et al., 1987) to measure the intracellular pH values of the two strains exposed to increasing external pH values for 3 days.

As shown in Table 2, at alkaline external pH values, no significant differences in the intracellular pH values were found between the mutant strain (whose growth was already impaired after 3 days at high pH) and the wild-type.

The experiments already described were made with cultures grown with 3 mM NaCl in the growth medium, hereafter referred to as the Na⁺ standard concentration. Sodium is known to be involved in both the osmotic and the pH adaptation of bacterial cells (Booth, 1985) and is essential for many cyanobacteria, especially if they are growing at alkaline pH (Espie et al., 1988). To further analyse the role of the mutated gene in salt and pH tolerance, increasing concentrations of NaCl at external pHs of both 7.5 and 10.5 were tested. As shown in Table 3, at an external pH of 7.5, the generation time of the mutant strain was significantly higher (Student's t test, P < 0.05) than that of the wild-type at external NaCl concentrations above 25 mM. The inhibitory effect of NaCl on the growth of PHB11 became more pronounced at an external pH of 10.5, at which, at the standard Na⁺ concentration (as also evidenced in Fig. 1c), the generation time was 20% higher than that of the wild-type, increasing to 42% higher at 25 mM and to 65% at 50 mM (Student's t test, P < 0.001). At the highest external pH, the mutant strain did not grow above 50 mM NaCl, while the wild-type was able to grow, albeit very slowly, even at 150 mM NaCl. There was no comparable inhibition of growth by added K⁺ (data not shown). These results indicate that the mutant strain is sensitive to increasing Na⁺ concentrations and that this sensitivity is higher at alkaline pH.

Like most living cells, at high salt concentrations, cyanobacteria actively extrude Na⁺ to try to maintain internal ion concentrations comparable to those of cells grown under low salt concentrations. Hence, the observed sensitivity of the mutant strain may indicate difficulties in extruding Na⁺. In this regard, preliminary data suggest higher internal sodium in the mutant, particularly at higher pH. Since an increased level of Na⁺ could be cytotoxic, particularly at alkaline pH values (Krulwich et al., 1990), these results may explain the observed impairment of growth and photosynthesis (Fig. 1) and suggest a role for the mutated gene in Na⁺ extrusion that is particularly

### Table 2. Determination of intracellular pH values (pHᵢₑₓ) of *Anabaena* sp. strain PCC7120 wild-type and PHB11 mutant strain exposed during 3 days to external pH (pHₑₓ) values of 7.5, 9, 10.5 and 11

<table>
<thead>
<tr>
<th>pHₑₓ</th>
<th>Strain</th>
<th>pHᵢₑₓ</th>
</tr>
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<tbody>
<tr>
<td>7.5</td>
<td>Wild-type</td>
<td>6.93 ± 0.31</td>
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<td></td>
<td>PHB11</td>
<td>7.05 ± 0.07</td>
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<tr>
<td>9</td>
<td>Wild-type</td>
<td>7.12 ± 0.03</td>
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<td>PHB11</td>
<td>7.18 ± 0.09</td>
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<tr>
<td>10.5</td>
<td>Wild-type</td>
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<td></td>
<td>PHB11</td>
<td>7.10 ± 0.14</td>
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<tr>
<td>11</td>
<td>Wild-type</td>
<td>7.21 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>PHB11</td>
<td>7.18 ± 0.13</td>
</tr>
</tbody>
</table>

Standard errors were calculated from three independent experiments with duplicate samples.

### Table 3. Generation times of *Anabaena* sp. strain PCC7120 wild-type and PHB11 mutant strain exposed during 24 h to increasing Na⁺ concentrations, both at pH 7.5 and pH 10.5

Values shown are generation times (h). Standard errors were calculated from three independent experiments with duplicate samples. –, Growth not detected.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Na⁺ concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>pHₑₓ</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>25.03 ± 1.08</td>
</tr>
<tr>
<td></td>
<td>26.67 ± 4.38</td>
</tr>
<tr>
<td>pHₑₓ</td>
<td></td>
</tr>
<tr>
<td>10.5</td>
<td>23.41 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>27.09 ± 0.58</td>
</tr>
</tbody>
</table>

*Standard Na⁺ concentration in the growth medium.*
important at elevated external pH. However, measurement of internal pH (Table 2) indicates that the mutated gene may not have a significant involvement in internal pH regulation or, alternatively, that the activity of the single Na$^+/H^+$ antiporters may account for these results.

Within the full genome sequence of moderately halotolerant Synechocystis sp. PCC6803 are six putative genes encoding single Na$^+/H^+$ antiporters (Kaneko et al., 1996). Elanskaya et al. (2002) and Wang et al. (2002) have independently mutated five ORFs and have found that NhaS3 (both groups could not get a complete segregation of the mutant) performs housekeeping functions essential to the survival of the organism and which are critical for salt tolerance, especially under alkaline conditions, in this cyanobacterium.

Elanskaya et al. (2002) concluded that the other four antiporters were not clearly involved in salt tolerance or in growth at high pH, whilst Wang et al. (2002) reported that NhaS2 y NhaS4 might be essential for survival in freshwater habitats characterized by large fluctuations in salt concentration and pH. Unfortunately, no such mutagenesis study has been undertaken in Anabaena sp. PCC7120, for which analysis of the complete genome sequence also reveals the presence of five genes encoding putative single Na$^+/H^+$ antiporters (all1303, all2113, alr0252, alr0656 and all4832; http://www.kazusa.or.jp/cyano).}

Reconstruction of the mutation in mutant PHB11

To determine whether the phenotype of PHB11 was the result of insertion of the transposon rather than the result of a secondary mutation, the transposon insertion was reconstructed (Black et al., 1993). Transposon Tn5-1063 (7-8 kb) together with approximately 3-8 kb of contiguous Anabaena DNA was recovered from mutant PHB11 upon excision with EcoRV and circularization and transfer to E. coli by electroporation, producing plasmid pBG2033 (from which plasmid pBG2045 was constructed) (see Table 1 and Methods).

Southern blotting analysis of one strain, designated DR2045-6A, derived from presumptive recombination of pBG2045 with the wild-type strain Anabaena sp. PCC7120 showed that the original mutation had been reconstructed (data not shown). The phenotype of the double recombinant strain DR2045-6A also matched that of mutant strain PHB11 (data not shown). Strain DR2045-6A, as well as single recombinant strain SR2045-1B, were used for subsequent in vivo gene expression studies (see below).

Analysis of the gene interrupted by the transposon in strain PHB11

The transposon in strain PHB11 was found to interrupt an ORF of 579 bp (data not shown). The transposon had inserted 443 bp 3’ from the first ATG codon of the ORF, generating a 9 bp repeat (5’-CGCTATATG-3’).

The Tn5-interrupted ORF encodes a predicted protein of 193 amino acids with an expected molecular mass of 21.3 kDa and an expected pI of 5.51. Near the N-terminal domain, three predicted transmembrane domains are located (data not shown).

The ORF mutated in PHB11 corresponds to ORF all1838 of the Anabaena genome (http://www.kazusa.or.jp/cyano/). ORF all1838 is the sixth ORF of what seems to be a large transcriptional unit of seven ORFs. Each of the ORFs starts with ATG, except the first, which starts with GTG. A promoter-like sequence (−35 region and −10 region; http://www.softberry.com/berry.phtml) was found in the upstream region (data not shown). Neither a terminator-like nor a promoter-like sequence was found between the seven ORFs. Therefore, it seems that the seven ORFs comprise an operon.

The putative protein encoded by all1838 shows significant similarity (>50%) with a significant stretch of amino acids of a much larger protein (between 725 and 804 amino acids) identified as protein A of a putative seven-protein complex found in several bacterial species and involved in pH adaptation and Na$^+$ tolerance (Hiramatsu et al., 1998; Putnoky et al., 1998; Ito et al., 1999; Kosono et al., 1999). Supplementary Fig. S1, available with the online version of this paper at http://mic.sgmjournals.org, shows the sequence alignment. Besides the protein encoded by all1838, each of the remaining six genes products encoded by the putative cyanobacterial operon also shows significant similarity to the corresponding bacterial products. Furthermore, the seven putative Anabaena gene products are highly hydrophobic and show a similar number of predicted transmembrane helices to that reported for the homologous bacterial products (Hiramatsu et al., 1998; Putnoky et al., 1998; Ito et al., 1999; Kosono et al., 1999).

Details of the predicted similarities of the putative Anabaena gene products as well as several other characteristics, that is, coding positions, number of amino acid residues and number of predicted transmembrane helices, are available in Supplementary Table S1 with the online version of this paper at http://mic.sgmjournals.org.

The bacterial complex is believed to function as an unusual multicomponent Na$^+/K^+/H^+$ antiporter. This gene family was first discovered in alkaliphilic Bacillus sp. strain C-125 (Hamamoto et al., 1994); in that organism, it is required for pH homeostasis in an alkaline environment. A mutant derivative of the alkaliphilic Bacillus sp. is not able to grow at alkaline pH because of a mutation in the first of a four-ORF operon, suggesting that this gene cluster has an important role in pH adaptation. Soon afterwards, homologues of the alkalophilic Bacillus operon were described in three bacteria, Bacillus subtilis, Staphylococcus aureus and Rhizobium meliloti. In these three bacteria, the operon contains a whole set of seven genes (ORF A–G) and is thought to encode a multisubunit antiporter family. In Bacillus subtilis, Ito et al. (1999) denoted the operon as mnp (multiple resistance and pH adaptation) and demonstrated that the
operon is primarily involved in Na\(^+\) resistance, particularly at alkaline pH; simultaneously, Kosono et al. (1999) also described the same locus in *Bacillus subtilis*, but denoted it as *sha* (sodium/hydrogen antiporter). Hiramoto et al. (1998) had already described the operon in *Staphylococcus aureus*, denoting it *mnh* (multisubunit Na\(^+\)/H\(^+\) antiporter). The *mnh* operon complemented a Na\(^+\)/H\(^+\) antiporter-deficient *E. coli* strain, thereby suggesting that it may also function as a Na\(^+\)/H\(^+\) antiporter; the authors suggested that all seven genes (*mnhA–G*) were required for the antiporter activity, so that the Mnh antiporter probably forms a ion transport complex of substantial size. The *pha* (pH adaptation) locus of *Rhizobium meliloti* is required for invasion of nodule tissue to establish nitrogen-fixing symbiosis (Putnoky et al., 1998). *Pha* mutants show sensitivity to K\(^+\) but not to Na\(^+\) in their growth. It seems that the *pha* locus may encode a K\(^+\)/H\(^+\) antiporter that is involved in pH adaptation during the infection process.

Based on the observed similarities, we decided to denote the gene mutated in PHB11 as *mrpA* and the *Anabaena* operon as *mrp*, following the nomenclature of the *Bacillus subtilis* operon, for which the most extensive study of this loci of selected heterotrophic bacteria, *Anabaena* sp, PCC7120 and other cyanobacteria for which the genomes have been sequenced. The same gene arrangement found in *Anabaena* sp PCC7120 is also found in five other cyanobacteria: the unicellular *Synechocystis* sp. PCC6803, the unicellular thermophilic *Thermosynechococcus elongatus* BP-1 (http://www.kazusa.or.jp), the unicellular *Synechococcus elongatus* PCC7942, the marine filamentous *Trichodesmium erythraeum* and the heterocystous *Anabaena variabilis* (http://www.jgi.doe.gov). A characteristic feature of the *Synechocystis* operon is the presence of two extra 5′ ORFs that may have appeared as a result of gene duplication. However, the *mrp* operon was absent from the genomes of the thylakoid-less *Gloeobacter violaceus* (http://www.kazusa.or.jp), the marine unicellular *Synechococcus* WH8102, *Prochlorococcus marinus* strains MED4 and MIT9313 and the heterocystous symbiotic *Nostoc punctiforme* (http://www.jgi.doe.gov). Thus, the *mrp* operon does not seem to be of universal occurrence among cyanobacteria. The absence of the operon in the marine *Prochlorococcus* and *Synechococcus* strains could be explained by their small genome size and by the fact that, being marine, they probably have an elevated requirement for Na\(^+\); however, the filamentous *T. erythraeum* is marine but retains the operon, and *N. punctiforme* has probably one of the largest genomes among bacteria but apparently lacks the operon.

Another interesting feature is that three subunits of the putative *Anabaena* Mrp complex, MrpA, B and D, show significant similarity (more than 50 %) with subunits 2, 4, 5 or 6 of the eukaryotic proton pumping NADH : ubiquinone oxidoreductase (complex I); NdhB, NdhE, NdhF and NdhG of chloroplasts; Nad2, Nad4L, Nad5 and Nad6 of plant mitochondria NADH-dehydrogenase and NuoN, NuoK, NuoL and NuoJ of *E. coli* NADH-dehydrogenase 1 (see Supplementary Table S1).

In *E. coli*, complex I is assembled from 14 different subunits, seven of which are intrinsic membrane proteins (NuoA, NuoH, NuoJ–N) (Weidner et al., 1993). In mitochondria, complex I may contain as many as 40 different polypeptides. The 14 minimal subunits whose homologues make up the bacterial complex I can be subdivided in two groups: seven are peripheral or predominantly peripheral proteins including all subunits predicted to bind the known redox group; the remaining seven subunits are membrane intrinsic proteins encoded in the mitochondrial genome (ND1–6, ND4L; Friedrich et al., 1995). This membrane-embedded part of the complex is involved in proton translocation (Friedrich et al., 1995). At least 12 (NdhA–L) of the 14 minimal subunits of complex I have been found to be coded in the genome of the unicellular cyanobacterium *Synechocystis* sp. PCC6803 (Kaneko et al., 1996). Interestingly, Prommeenate et al. (2004) have found two additional Ndh subunits (encoded by slr1262 and slr1623) in this cyanobacterium. Most *ndh* genes are present as single copies in the *Synechocystis* genome. However, there are multiple copies of *ndhD* (six homologues) and *ndhF* (three homologues). In the *Anabaena* genome (Kaneko et al., 2001) there are 11 *ndh* genes (*ndhA–K*) with five copies of *ndhD* and three of *ndhF*, although it lacks the *ndhL* homologue of *Synechocystis*. However, *Anabaena* does contain an ORF (alr0751) that is not present in *Synechocystis* but that could code for a putative catalytic subunit homologous to NuoE of *E. coli*.

The fact that *ndhD* and *ndhF* are present as gene families suggests that several types of NDH-1 complex exist in cyanobacteria, each with different NdhD/NdhF subunits and with each potential complex having different functions (Klughammer et al., 1999; Ohkawa et al., 2000). The NdhD1/NdhD2 type of NDH-1 complex would be involved in the PSI-dependent cyclic electron transport pathway as well as in cellular respiration, while the NdhD3/NdhD4 NDH-1 complexes are essential for CO\(_2\) uptake (Ohkawa et al., 2000). Prommeenate et al. (2004) have found two large NDH-1 complexes, termed A (460 kDa) and B (330 kDa), with similar protein profiles by SDS-PAGE, by which they have identified hydrophilic as well as hydrophobic modules. However, NdhF3 was never found in the NDH-1 complexes. The authors argue that some of the annotated NdhD and NdhF subunits may actually have roles unrelated to NDH-1 function. More recently, Zhang et al. (2004) have also analysed the subunit composition and functional roles of the NDH-1 complexes of *Synechocystis*, and have found that the two larger NDH-1 complexes also lack the NdhD3 and NdhF3 subunits. In this regard, Putnoky et al. (1998) and
Hiramatsu et al. (1998) have suggested that the observed similarities between subunits of the bacterial mrp operons and hydrophobic subunits of Complex I point towards a common origin and function for both systems. The authors speculate that the membrane-embedded part of complex I evolved from an ancestral cation/proton antiporter and became specialized for concerted actions with the cytoplasmic subunits involved in electron transfer. Recently, Mathiesen & Hägerhäll (2003) have hypothesized that a multisubunit antiporter complex formed by MrpA (homologous to NuoL), MrpD (homologous to NuoM/N) and MrpC (which the authors have found homologous to NuoK) may have been recruited to the ancestral complex I, which would contain a NuoKLMN subunit module.

Cloning of the wild-type version of mrpA and complementation of the mutation

A 728 bp PCR fragment of Anabaena sp. PCC7120 DNA was shown by sequencing to contain the mrpA gene as the only ORF (data not shown). The sequence of the cloned gene was identical to that obtained from the transposon-mutagenized form of the fragment recovered on pBG2033 (Table 1; see Methods). The 728 bp fragment bearing the PCR-amplified wild-type mrpA was cloned into the RSF1010-based plasmid pRI1342, generating plasmid pBG2041, which can replicate in Anabaena. Plasmid pBG2041 was transferred by conjugation to mutant strain PHB11, and colonies resistant to erythromycin were selected. The complemented strain, denoted PHB11:pBG2041-6, behaved in the same way as the wild-type strain at both an external pH of 10-5 and an external pH of 10-5 supplemented with 50 mM Na⁺ (data not shown).

In vivo expression of mrpA

Transposon Tn5-1063 generates transcriptional fusions between the Vibrio fischeri luxA and luxB genes, which encode luciferase, and genes into which the transposon becomes inserted (Wolk et al., 1991), thus permitting the monitoring of gene expression in vivo, provided that the transposon is correctly oriented. However, the transposon in mutant PHB11 placed luxAB antiparallel to the gene mrpA (not shown). Plasmid pBG2037 (Table 1; see Methods) was constructed in order to reconstruct the mutation, placing luxAB parallel to the direction of transcription of mrpA. Single recombinant [Em⁺ Sp⁺ Sm⁺ (sucrose sensitive)] and double recombinant [Em⁺ Sp⁺ Sm⁺

![Graphs](http://mic.sgmjournals.org)
(sucrose resistant) strains were obtained (see above). Single recombinant SR2045-1B and double recombinant DR2045-6A were selected for the in vivo expression studies.

The in vivo expression of mrpA from cell suspensions of the single and double recombinant strains was first monitored as a function of time under increasing external Na⁺ concentrations at pH 7.5. As shown in Fig. 2(a, b), the pattern of expression of the gene in the double (a) as well as the single recombinant strain (b) is very similar. There is a clear induction of mrpA in the presence of increasing concentrations of Na⁺ in the medium; depending on the external Na⁺ concentration, the induction is maximal after 4–8 h incubation, with the highest level of induction at 100 mM Na⁺.

The second environmental condition tested was the combined effect of alkaline pH (pH 10.5) and increased Na⁺. In this case, there was a remarkable difference between the double recombinant (Fig. 2c) and the single recombinant strain (Fig. 2d). In the double recombinant, there is a clear induction of the expression of mrpA at relatively low external Na⁺ concentrations (3–25 mM). However, at higher Na⁺ concentrations there is no induction of expression, indicating that alkaline conditions combined with high Na⁺ concentrations affect the viability of the double recombinant strain, as already found with the original mutant strain PHB11. However, the pattern of induction of mrpA in the single recombinant strain, which retains a wild-type copy of the gene, is clearly different, with maximum levels of induction at the highest Na⁺ concentrations tested. The induction is maximal after around 1 h of exposure to basic pH, reaching luminescence values significantly higher than those found at pH 7.5. Therefore, expression of mrpA is upregulated in response to increased Na⁺ concentration in the culture medium. The level of induction is higher at alkaline pH values. Both the phenotypic results and the expression studies imply a role for mrpA, and probably for the whole operon, in resistance to Na⁺, particularly at alkaline pH, in this cyanobacterium.

Wang et al. (2004) reported that the transcription of two genes of Synechocystis sp. PCC6803 whose products resemble subunits 5 and 6 of NDH-1, subsequently named as ndhD5 and ndhD6, were upregulated in response to a CO₂ downshift. These genes were located in a large transcriptional unit that we have identified here as the Synechocystis mrp operon. We checked whether transcription of the Anabaena mrpA gene was also upregulated in response to a Ci downshift. Fig. 3 shows the pattern of mrpA expression in both the double (Fig. 3a) and single (Fig. 3b) recombinant strains after switching the aeration from 1.5% CO₂ in air (v/v) to air alone (350 ppm CO₂, Ci limited). As can be seen in the figure, the transcription of mrpA is induced twofold under Ci-limiting conditions in the double recombinant and threefold in the single recombinant strain. The results reported by Wang et al. (2004) together with ours suggest an involvement of the cyanobacterial mrp operon in low-Ci acclimation.

It is noticeable that the induction of mrpA under alkaline/Na⁺ stress and low Ci is very quick (Figs 2 and 3); this again suggests a relevant role for mrpA and probably the whole mrp operon in the response to short-term stress, whereas the growth data shown in Fig. 1 may indicate that, in the long term, compensatory mechanisms are induced that allow some growth of the mutant until it finally bleaches and dies.

Wang et al. (2004) also found that disruption of ndhR (Figge et al., 2001) activated the transcription of Synechocystis mrp genes. NdhR is a LysR-family regulator of Ci uptake, initially described as a transcriptional regulator for ndh genes in Synechocystis sp. PCC6803 (Figge et al., 2001). The T(N11)A sequence is often present in the promoter regions of genes controlled by LysR-type regulators in proteobacteria. Figge...
et al. (2001) already identified the TCAATG-(N10)-ATCAAT sequence as the consensus motif in Synechocystis sp. PCC6803. Three presumptive Ndhr-binding sites have been identified in the promoter region of the mrp operon in Synechocystis (Wang et al., 2004). We checked the promoter of the mrp operon of Anabaena and found eight presumptive Ndhr-binding sites (shown in Supplementary Fig. S3 with the online version of this paper at http://mic.sgmjournals.org). We searched for a homologue of ndhR in the Anabaena genome and found two possible candidates: all4986, which shows 69 % similarity to ndhR, and all3953, which shows 49 % similarity. All4986 is next to all4985, which putatively encodes sucrose synthase. All3953 is close to, although divergently transcribed from, genes ndhF (alr3956) and ndhD (alr3957), which are within a large transcript unit of nine ORFs (alr3954–alr3959, asr3959–asr3961).

Finally, with all this evidence, a major question arises regarding which is/are the specific role(s) of a putative Mrp complex in cyanobacteria. As has been proposed in heterotrophic bacteria (Hiramatsu et al., 1998), it may be a multicomponent Na+/H+ antiporter that is energized by electron transport through the subunits resembling hydrophobic components of complex I. However, additional experiments are required to clarify whether or not this system truly has a Na+/H+ antiporter activity in cyanobacteria and to determine which protein(s) in the complex function as the Na+/H+ antiporter, and the relationship, if any, between the subunits resembling the hydrophobic core of NDH-1 and NDH-1 itself. Also, if the Mrp complex is a true Na+/H+ antiporter, the Na+ gradient created may drive nutrient uptake, in other words, HCO3-, as suggested for Synechocystis (Wang et al., 2004). Also unresolved is the relationship, if any, between this multisubunit complex and the single Na+/H+ antiporters in Anabaena strain sp. PCC7120. A more complete mutational and biochemical analysis of this complicated and unexplored cyanobacterial locus is needed to fully resolve these issues.

ACKNOWLEDGEMENTS

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