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The *clpP* multigene family for the ATP-dependent Clp protease in the cyanobacterium *Synechococcus*

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In the cyanobacterium *Synechococcus* sp. strain PCC 7942 a multigene family of three different isozymes encodes the proteolytic subunit ClpP of the ATP-dependent Clp protease. In contrast to the monocistronic *clpPI* gene, *clpPII* and *clpPIII* are part of two bicistronic operons with *clpX* and *clpR*, respectively. Unlike most bacterial Clp proteins, the *Synechococcus* ClpP2, ClpP3, ClpR and ClpX proteins were not highly inducible by high temperatures, or by other stresses such as cold, high light or oxidation, although slower gradual rises occurred for all four proteins during high light, and for ClpP3, ClpR and ClpX at low temperature. Attempts to inactivate the *clpPII*, *clpIII*, *clpR* or *clpX* genes were only successful for *clpPII*, suggesting the others are essential for *Synechococcus* cell viability. The Δ*clpPII* mutant exhibited no significant phenotypic changes from the wild-type, including no change in ClpX content. Despite the apparent bicistronic arrangement of both *clpPII-clpX* and *clpR-clpPIII*, all four genes primarily produce monocistronic transcripts, although polycistronic transcripts were detected. Mapping of 5′ ends for the *clpX* and *clpPIII* monocistronic transcripts revealed promoters situated within the 3′ region of *clpPII* and *clpR*, respectively. Transcriptional and translational studies further showed differences in the expression and regulation between the *clpP-clpR-clpX* genes. Inactivation of *clpPI* caused a significant decrease in ClpP2 protein concomitant to small increases in both ClpP3 and ClpR. Inactivation of *clpPII* resulted in a large rise in *clpPI* transcripts but to a lesser extent in ClpP1 protein. Similar small increases in ClpP3, ClpR and ClpX proteins also occurred in Δ*clpPII*. These results highlight the regulatory complexity of these multiple *clp* genes and their functional importance in cyanobacteria.

Keywords: cyanobacteria, gene expression, protein regulation, proteolysis, stress

INTRODUCTION

Molecular chaperones and energy-dependent proteases are two vital contributors to cell homeostasis. Without an effective system constantly controlling and monitoring protein quality, many cellular processes would eventually cease, leading ultimately to cell death. Chaperones perform many roles, facilitating processes such as protein folding, assembly and membrane transport, while also enabling protein stabilization, renaturation and resolubilization under various adverse growth conditions. Targeted degradation by energy-dependent proteases is equally important by regulating the availability of regulatory proteins and removing non-functional but potentially harmful polypeptides arising from misfolding, denaturation or aggregation (reviewed by Gottesman, 1996). Many of these proteases incorporate chaperone activity, whereby substrates targeted for degradation require unfolding prior to proteolysis. One of the best-studied ATP-dependent proteases in *Escherichia coli* are the Clp proteases, which consist of regulatory ATPase/chaperone (ClpA, ClpX, ClpY) and proteolytic (ClpP, ClpQ) subunits.

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Abbreviations: Chl, chlorophyll; MBP, maltose-binding protein.

The GenBank accession numbers for the sequences of *clpPII-clpX* and *clpR-clpPIII* reported in this paper are U92039 and AJ132005, respectively.

Clp proteases have an overall conserved architecture that resembles the cytosolic 26S proteasome in eukaryotes. Two apposed annuli of the proteolytic subunit, heptameric rings for ClpP and hexameric for ClpQ, form a central cavity housing the proteolytic active sites (Rohrwild *et al*., 1997; Wang *et al*., 1997). Narrow axial pores that only unfolded polypeptides can traverse restrict access to the inner cavity. Flanking the proteolytic complex are single hexameric rings of the ATPase/chaperone subunit, in *E. coli* being ClpA and/or ClpX with ClpP (Grimaud *et al*., 1998), and ClpY with ClpQ (Rohrwild *et al*., 1997). Substrate recognition is conferred by the chaperone subunit, which, after binding, unfolds the protein substrate and enables its transfer into the proteolytic chamber (Singh *et al*., 2000). Once inside, the protein substrate is efficiently degraded to small peptide fragments that later diffuse out.

Clp proteins are widely distributed in nature and are found in all eubacteria, plants and mammals. Although ClpP is common to all these organisms, the type and number of ATPase/chaperone subunits vary. ClpA and ClpX are now known as members of the Clp/Hsp100 family of chaperones. This family is divided into two basic groups, with members of the first (ClpA–E) being characterized by having two distinct ATP-binding domains, whilst those of the second (ClpX, ClpY) have only one. Although ClpX is ubiquitous, ClpA appears restricted to Gram-negative eubacteria like *E. coli*. Instead of ClpA, Gram-positive bacteria, cyanobacteria and plant chloroplasts commonly have ClpC, with additional types also occurring in most Gram-positive bacteria (ClpE) and higher plants (ClpD) (reviewed by Porankiewicz *et al*., 1999).

Clp proteases in *E. coli* degrade a variety of substrates, including SsrA-tagged polypeptides as part of the protein quality control system for removal of unstable or misfolded proteins subsequent to translation (Gottesman *et al*., 1998). Despite such roles, however, loss of ClpP in *E. coli* produces no obvious phenotypic changes. In contrast, more diverse and crucial roles for Clp proteins occur in Gram-positive bacteria, cyanobacteria and plants. In *Bacillus subtilis*, for example, proteins like ClpC, ClpX and ClpP are vital for resistance to many stresses, and for many cellular and developmental processes such as cell division, motility, sporulation and genetic competence (reviewed by Porankiewicz *et al*., 1999). The ClpXP protease is also mainly responsible for degradation of SsrA-tagged proteins in *B. subtilis* like the homologous protease in *E. coli* (Wiegert & Schumann, 2001). ClpC and ClpP functions are equally essential in cyanobacteria and plants, as shown by various genetic studies (Shanklin *et al*., 1995; Clarke & Eriksson, 1996; Clarke *et al*., 1998; Shikanai *et al*., 2001).

Cyanobacteria are a diverse group of eubacteria that can be found in nearly all habitats. They constitute one of the largest and most ecologically important bacterial groups, being one of the major contributors to biomass accumulation on Earth. Cyanobacteria are also the only prokaryotes that perform oxygenic photosynthesis like algae and higher plants, and are generally considered the progenitors of plastid evolution, according to the endosymbiotic theory. With such attributes, cyanobacteria have served as valuable model organisms for investigating many cellular processes common to photosynthetic organisms. One feature common, but not exclusive to cyanobacteria and plants is the presence of multiple ClpP isomers. These photosynthetic organisms furthermore have the ClpR variant of ClpP that lacks the three active site amino acids representative of ClpP proteases and whose function remains unclear (Clarke, 1999). To date, little is known about the regulation and functional importance of the ClpP and ClpR proteins in cyanobacteria. Previously, we described the *clpPI* gene in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (*Synechococcus*) and its importance for stress acclimation (Clarke *et al*., 1998). We now identify the remaining Clp proteins in this cyanobacterium and examine their complex regulatory characteristics under a range of physiological conditions.

METHODS

Culture conditions. All *Synechococcus* strains were grown on solid or in liquid BG-11 (Clarke *et al*., 1995). Liquid cultures were grown in 80 ml glass tubes in 37 °C water-baths with continuous light of 70 μmol photons m^{-2} s^{-1} and bubbled with 5 % CO_2 in air (standard growth conditions). Cells in exponential growth phase with a chlorophyll (Chl) concentration of 2·5–3·5 μg ml^{-1} were used for all experiments. Mutant strains were maintained on solid plates and in liquid cultures supplied with 5 μg kanamycin (Δ*clpPI*) or chloramphenicol (Δ*clpPII*) ml^{-1} to maintain selection. No antibiotic, however, was added to the experimental cultures to eliminate the possibility of antibiotic-induced phenotypic changes.

Cloning and sequencing of *clp* genes. The *clpPII-clpX* and *clpR-clpPIII* operons from *Synechococcus* were identified using degenerate oligonucleotides specific for highly conserved domains within known ClpX and ClpP3 homologues, respectively. Each primer was 27–35 bases long and included *Eco*RI restriction sites at the 5′ ends to facilitate cloning. Primers for *clpX* were 5′-GTIGAATTCGTIGCIGTITA(CT)-AA(CT)CA(CT)TA(CT)AA and 5′-AT(CT)TTGAATTCI-GC(CT)TG(CT)TGIACICC(CT)TCICC, and for *clpPIII* were 5′-CCIGAATTCCA(AG)TA(CT)GA(AG)(AC)GITG-GATIGA(CT)ATITA and 5′-GCCGAATTCIGG(CT)TG-(AG)TGIATCATIAT. The expected 200 (*clpX*) and 260 (*clpPIII*) bp fragments were PCR-amplified from *Synechococcus* genomic DNA, cloned into the plasmid pUC19 and verified by DNA sequencing. Fragments were used as specific DNA probes to isolate clones containing the complete *clpPII-clpX* and *clpR-clpPIII* operons from partial *Synechococcus* genomic libraries constructed from 8–10 kb *Sac*I and 2·5–4 kb *Hin*dIII restriction fragments in pUC19, respectively. Clones were isolated by colony hybridization as described previously (Eriksson & Clarke, 1996). DNA sequencing was done using the ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit (Perkin Elmer), analysed on an automated sequencer (ABI377; Perkin Elmer) and viewed with AutoAssembler computer software.

Construction of *clpPII*-inactivation plasmid and transformation. The Δ*clpPII* plasmid was made by replacing a 218 bp

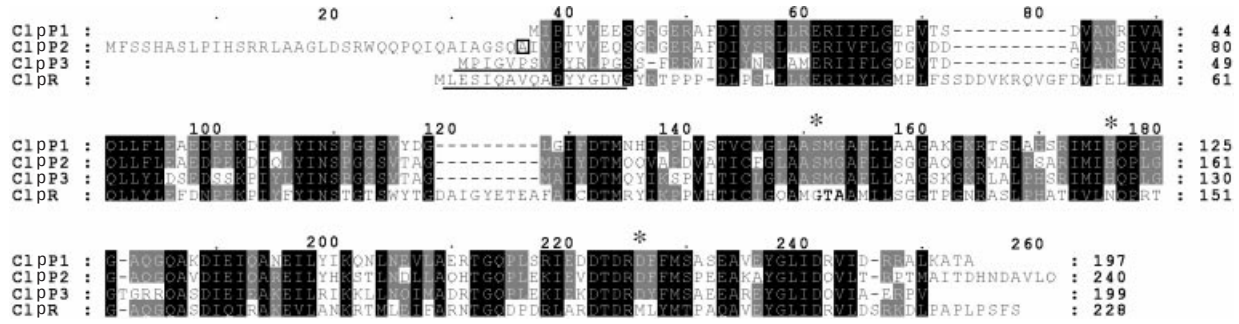

Fig. 1. Sequence alignment of *Synechococcus* ClpP and ClpR proteins. Amino acid sequences were aligned using the PILEUP program from the UWGCG package. Amino acids identical for all (black) or three (grey) of the four Clp proteins are shaded as indicated. Gaps are shown as dashes, while asterisks indicate the Ser, His and Asp active site residues. The underlined regions in ClpP3 and ClpR indicate the synthetic peptides used for specific antibody production. The boxed Ala residue in ClpP2 shows the putative amino-terminal processing site for the precursor protein.

fragment in the middle of the complete *clpPII* gene with a 1·8 kb chloramphenicol resistance cassette (Shapira *et al*., 1983). An *Eco*RI–*Xba*I fragment covering the 5′ end of *clpPII* and a *Hin*dIII–*Xba*I fragment covering the 3′ end were joined together with the *Xba*I-cut chloramphenicol resistance cassette. This construct was cloned into the *Eco*RI–*Hin*dIII site of pUC19 and the resultant plasmid was transformed into *E. coli* DH5α. Positive transformants were selected on media containing chloramphenicol and restriction endonuclease digests verified plasmids with the correct construct. Wild-type *Synechococcus* was transformed with the linearized inactivation plasmid (van der Plas *et al*., 1990). Putative transformants were selected on BG-11 plates supplemented with 5 μg chloramphenicol ml^{-1}. Correct insertion of the Δ*clpPII* construct and its complete segregation in selected transformants were confirmed by Southern blot analysis (Eriksson & Clarke, 1996).

Preparation of Clp-specific antibodies. Polyclonal antibodies specific for *Synechococcus* ClpP2 or ClpX were made against fusion proteins overexpressed in *E. coli* using the pMAL-c2 overexpression plasmid (New England Biolabs). The complete *clpPII* gene and the 3′ end downstream of the *clpX* ATP-binding domain were PCR-amplified from their genomic clone with the high-fidelity *pfu* DNA polymerase (Stratagene). PCR products were separately ligated in-frame to the 3′ end of the *malE* gene, encoding the maltose-binding protein (MBP), on the plasmid pMAL-c2, and then transformed in *E. coli* DH5α. Overexpression of pMAL/*clpPII* and pMAL/*clpX* under control of the *tac* promoter was induced by adding IPTG to actively growing cells, and the resulting MBP/ClpP2 or MBP/ClpX fusion proteins were purified as described previously (Riggs, 1990). For *Synechococcus* ClpP3 and ClpR, synthetic peptides were designed for regions specific for each isomer (ClpP3: MPIGVPSVPYRLPGS; ClpR: LESIQAVQAPYYGDV; Fig. 1), and conjugated at the C terminus to the carrier protein keyhole limpet haemocyanin. All purified proteins were injected into rabbits intramuscularly and subcutaneously to produce the specific antibodies (AgriSera AB).

Stress treatments. All stress treatments were performed with cultures grown under standard conditions and taken in early exponential growth phase at a Chl concentration of 2·5–3·5 μg ml^{-1}. For all stresses, standard conditions were maintained except for the specified stress factor. No changes in Clp protein content relative to Chl concentration occurs during standard, non-stressed growth of cultures over the time period used for the stress treatments. For heat shock, culture flasks were directly shifted from a 37 °C water-bath to one at 50 °C for 2 h, whereas for cold shock they were moved to a 25 °C water-bath for 24 h. For high light, cultures were directly shifted to 1000 μmol photons m^{-2} s^{-1} for 6 h, while the oxidative stress was done by adding 0·5 mM H_2O_2 (final concentration) directly to culture flasks. For each stress, cell samples were taken at the selected time points, pelleted by centrifugation and then frozen in liquid N_2 to await protein isolation.

Sample preparation and immunodetection. Total proteins were extracted from frozen cell pellets (Clarke *et al*., 1993). Samples containing equal Chl (0·4 μg) were separated on 4–12 % polyacrylamide Bis-Tris NuPAGE gels (Novex). Proteins were transferred to PVDF (0·45 μm) membranes (Millipore) or supported nitrocellulose (0·2 μm) (Bio-Rad). Each Clp protein was detected using specific polyclonal antibodies, with the antibody for *Synechococcus* ClpP1 described elsewhere (Clarke *et al*., 1998). All primary antibodies were detected with a horseradish peroxidase-conjugated, anti-rabbit secondary antibody made in donkey and visualized by enhanced chemiluminescence (Amersham Pharmacia).

RNA isolation. *Synechococcus* cultures grown to a Chl concentration of approximately 3·5 μg ml^{-1} were pelleted and resuspended in 1 ml DEPC-treated H_2O per 50 ml culture. Cells were then frozen in liquid N_2 and ground to a fine powder, to which 1 ml Trizol Reagent was added per 50 ml culture. Total RNA from *Synechococcus* was extracted using the Trizol Reagent method (Invitrogen). Isolated RNA samples were treated with DNase when used for RT-PCR analysis. RNA purity and concentration was determined spectrophotometrically.

Northern blotting, RT-PCR and 5′-RACE. Northern blot analyses were made with total RNA using the Northern Max-Gly/Blotting Kit and method (Ambion). For Northern blots, 19 μg total RNA was denatured by glyoxylation and separated on a 1 % agarose gel. Separated RNA was transferred to nylon membrane (BrightStar-Plus; Ambion) and cross-linked under UV light. Membranes were prehybridized with heated ULTRAhyb (Ambion) for at least 1 h at 42 °C, followed by addition of ^{32}P-labelled DNA probes. Hybridizations were

done overnight at 42 °C. DNA probes were specific for each of the different *Synechococcus clp* transcripts (as determined by Southern blotting) and prepared by PCR amplification from genomic clones. The gene probes corresponded to the following DNA regions relative to the start ATG: *clpPI*, −100 to +296; *clpPII*, +265 to +705; *clpPIII*, +80 to +545; *clpR*, +121 to +599. Following hybridization, membranes were washed at 42 °C once with low-stringency solution for 10 min, twice with high-stringency solution for 15 min (Northern Blotting Kit; Ambion) and then exposed to X-ray film.

RT-PCR reactions were performed with *Synechococcus* total RNA using the SuperScript One-step RT-PCR System kit (Life Technologies). Varying amounts of template RNA (10–100 ng) were used to determine the amount suitable for non-saturated amplifications. The following primers were used, along with their predicted product size: *clpPII/X*, 5′-TTGGTACCGGTAGTGGCTGGTATTA-3′ and 5′-CCTTCGAGTTGCGCCGTAGTAGATG-3′ (1098 bp); *clpR/P3*, 5′-TTGTTCTCGTCTGACGATGTGA-3′ and 5′-TCAGCAGACATGAAGTAGTCGCGA-3′ (1154 bp). For each set of RT-PCR reactions, an extra control reaction was included without reverse transcriptase, but with *Taq* DNA polymerase to detect potential DNA contamination. RT-PCR products were separated on 1% agarose gels and viewed with an AlphaImager and associated software (Alpha Innotech). The identity of each RT-PCR product was verified by DNA sequencing.

The 5′ end for *clpX* and *clpPIII* monocistronic transcripts was determined using the 5′-RACE kit and method (Invitrogen). DNase-treated total RNA (3 μg) was used for the first strand cDNA synthesis together with the gene-specific primers *clpX*, 5′-TGGTCTAGATATCGCTTGATGTCGTG-3′, and *clpPIII*, 5′-TCATAATCCGCGAATGAGGCAATG-3′. After cDNA purification, 10 μl was used in the TdT-tailing reaction from which 5 μl was used in the final nested PCR amplification using additional gene-specific primers: *clpX*, 5′-GGAATCTGCGACAGCGTTAGCGATC-3′; *clpPIII*, 5′-ATTGCATCGTGTCGTAGATCGCCAT-3′. PCR products were purified and then identified by DNA sequencing.

RESULTS

Cloning and sequencing of *Synechococcus clp* genes

Extra *clp* genes were cloned from *Synechococcus* using the PCR strategy of degenerate primers specific for conserved regions to amplify an internal portion of putative *clpX* and *clpPIII* genes from wild-type DNA. After sequence verification, these fragments were used as specific probes to isolate full-length clones from a *Synechococcus* genomic DNA library. Sequencing of the *clpX* clone revealed no extra ORFs over 500 bp downstream of the *clpX* termination, but upstream was the *clpPII* gene only 12 bp from the start ATG of *clpX*. The same scenario occurred for the *clpPIII* clone, with the *clpR* gene identified only 42 bp upstream of the *clpPIII* start codon, whilst no ORFs were found approximately 800 bp downstream of *clpPIII*. Additional sequencing upstream of *clpPII* and *clpR* revealed no further ORFs, suggesting *clpPII/X* and *clpR/PIII* are organized as bicistronic operons. The predicted ORFs for *clpPII*, *clpPIII*, *clpR* and *clpX* are 723, 600, 687 and 1353 bp, respectively. According to Southern blot analysis, only one copy of each *clp* gene occurs in the *Synechococcus* genome (data not shown).

Analyses of protein sequences

The predicted amino acid sequences for the new ClpP/R isozymes were compared in Fig. 1 with that of ClpP1 (Clarke *et al*., 1998). As for ClpP1, both ClpP2 and ClpP3 possess the three conserved amino acids that compose the catalytic triad (Ser-His-Asp) characteristic of serine-type proteases. Equally characteristic was the apparent absence of this catalytic triad in the ClpR protein (Clarke, 1999), the significance of which remains unknown. Of the three ClpP isomers, each is 75–80% identical to its homologous protein in the related strain *Synechocystis* sp. strain PCC 6803, and 57–70% to the other two. The three *Synechococcus* isozymes have a similar low level of conservation (42–50% identity) in comparison to ClpP forms in plants and non-photosynthetic eubacteria. Of the three, ClpP2 appears to be homologous to the single ClpP in Gram-negative eubacteria like *E. coli*, in terms of both sequence similarity and gene arrangement with *clpX*. *Synechococcus* ClpP2 also has an extended N terminus like the *E. coli* protein that is post-translationally removed to produce the active mature form (Maurizi *et al*., 1990a). Comparison with *E. coli* ClpP suggests a processing site in ClpP2 at an Ala residue in position 36 (Fig. 1).

Like the ClpP isomers, the *Synechococcus* ClpR is also closely related to its counterpart in *Synechocystis* (78% identity), but much less to the ClpP proteins in both *Synechococcus* and *Synechocystis* (41–47%). ClpR has two short extensions in the first half of the protein, which is conserved for ClpR in other cyanobacteria and the plant *Arabidopsis thaliana*. When overlaid with the known structure of *E. coli* ClpP, these stretches are situated in the 'head' region, the first being between β-sheet 2 and helix B, and the other inside helix C (Wang *et al*., 1997). Of the remaining protein, *Synechococcus* ClpX contained all the conserved amino acid domains characteristic of its class of Clp protein, in particular the signature single ATP-binding domain. Furthermore, the presence of the two Zn-finger, DNA-binding motifs in the N-terminal domain indicates the *Synechococcus* ClpX is homologous to other bacterial types rather than to eukaryotic ClpX proteins, which usually lack this domain (Halperin *et al*., 2001).

Preparation of specific antibodies

To characterize in more detail the new Clp proteins in *Synechococcus*, antibodies were made for each of them. Fusion proteins with MBP were used as antigens for the entire *Synechococcus* ClpP1 and ClpP2 proteins, and the C-terminal domain of ClpX, whereas specific synthetic peptides conjugated to keyhole limpet haemocyanin were used for ClpP3 and ClpR (Fig. 1). When tested against cell protein extracts from wild-type *Synechococcus*, each antibody detected a single polypeptide of

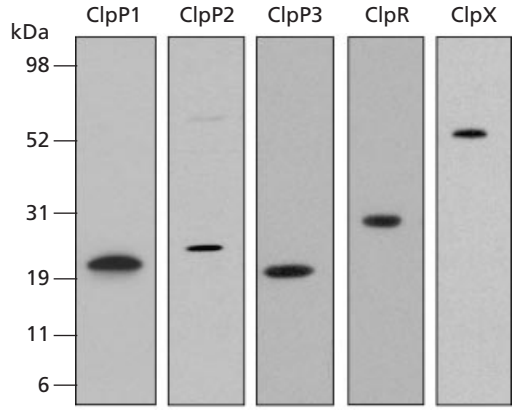

Fig. 2. Specificity of antibodies raised to *Synechococcus* ClpP1–3, ClpR and ClpX proteins. Total cell proteins were isolated from wild-type *Synechococcus* grown under standard culture conditions. Protein samples (0·4–0·5 μg Chl) were separated electrophoretically by SDS-PAGE and then analysed by immunoblotting. Identity of Clp protein recognized by each polyclonal antibody is indicated above the lane. Molecular mass standards are indicated on the left.

the expected size (Fig. 2). The estimated sizes are: ClpP1, 22 kDa; ClpP2, 23·5 kDa; ClpP3, 21 kDa; ClpR, 28 kDa; ClpX, 52 kDa.

Clp protein levels during stress

Since most Clp proteins in bacteria are induced by various stresses, especially heat, we examined if *Synechococcus* ClpP2, ClpP3, ClpR and ClpX were also similarly affected. Stresses known to severely impair *Synechococcus* phototrophic growth were chosen: heat, cold, high irradiance and oxidation (Fig. 3). Heat shock at 50 °C for 2 h produced no significant induction for any of the tested Clp proteins, similar to that observed for ClpP1 (Clarke *et al.*, 1998). A similar lack of Clp protein induction was also observed after the addition of 0·5 M H_2O_2, a severe oxidative stress. Indeed, the oxidative stress caused a significant decrease in ClpP2 content (approx. 25% of control level) after 30 min, followed by a partial recovery (approx. 50% of control) after 2 h (Fig. 3). A drop in ClpX protein was also observed during the oxidative stress, but only after 4 h and decreasing to approximately 60% of control levels at 6 h. During a cold shift (25 °C for 6 h) in which ClpP1 is strongly induced (Porankiewicz *et al.*, 1998), no change in ClpP2 or ClpX content occurred throughout, except for an increase in ClpX after 6 h. For ClpP3 and ClpR, a slight rise in protein content occurred after 2 h at 25 °C, but which then remained unchanged for the next 4 h. Like the cold treatment, high light exposure of 1000 μmol photons m^{-2} s^{-1} for 6 h caused strong induction of ClpP1 (Clarke *et al.*, 1998) and this same treatment also produced increases in ClpP2, ClpP3, ClpR and ClpX protein content, but to a lesser extent (Fig. 3).

Inactivation of *clp* genes

Earlier observations with a *clpPI* mutant showed that loss of ClpP1 produced pleiotropic changes in *Synechococcus*. Apart from a filamentous morphology (Clarke *et al.*, 1998), the Δ*clpPI* strain could not acclimate to cold or moderate UV-B irradiation (Porankiewicz *et al.*, 1998). To evaluate the roles of the new *clp* genes, we attempted to prepare gene-specific inactivation strains using the same deletion/insertion strategy that was successful for *clpPI* and other genes in *Synechococcus* (Clarke & Campbell, 1996; Eriksson & Clarke, 1996; Clarke *et al.*, 1998). Of the four genes, however, viable transformants were only obtained for *clpPII*, suggesting

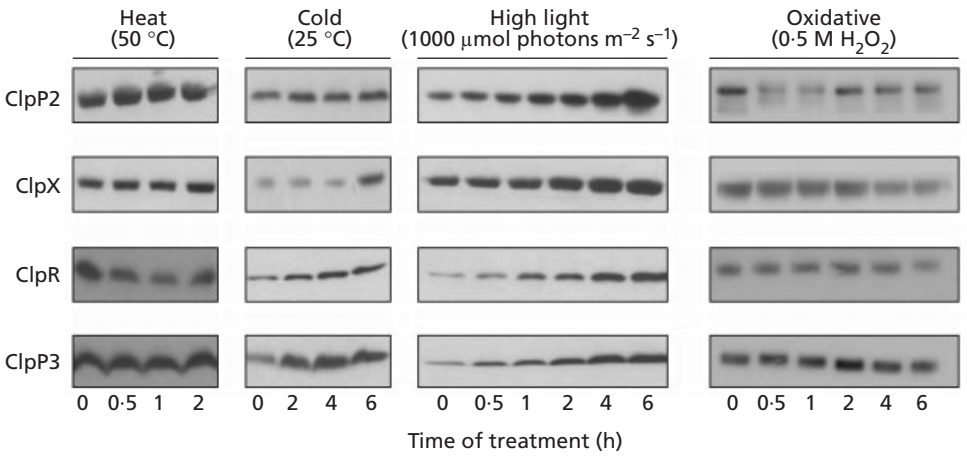

Fig. 3. Stress effects on *Synechococcus* Clp protein levels. Wild-type cells grown under standard culture conditions (Chl concentration of 2·5–3·0 μg ml^{-1}) were shifted to either 50 °C for 2 h (heat), 25 °C for 6 h (cold) or 1000 μmol photons m^{-2} s^{-1} for 6 h (high light), or were treated with 0·5 M H_2O_2 for 6 h (oxidative); all other growth parameters were kept constant. Cells were collected at the indicated times during each treatment. Protein samples (0·4 μg Chl) were separated electrophoretically and analysed by immunoblotting. The figure shows results representative of two to three replicate experiments.

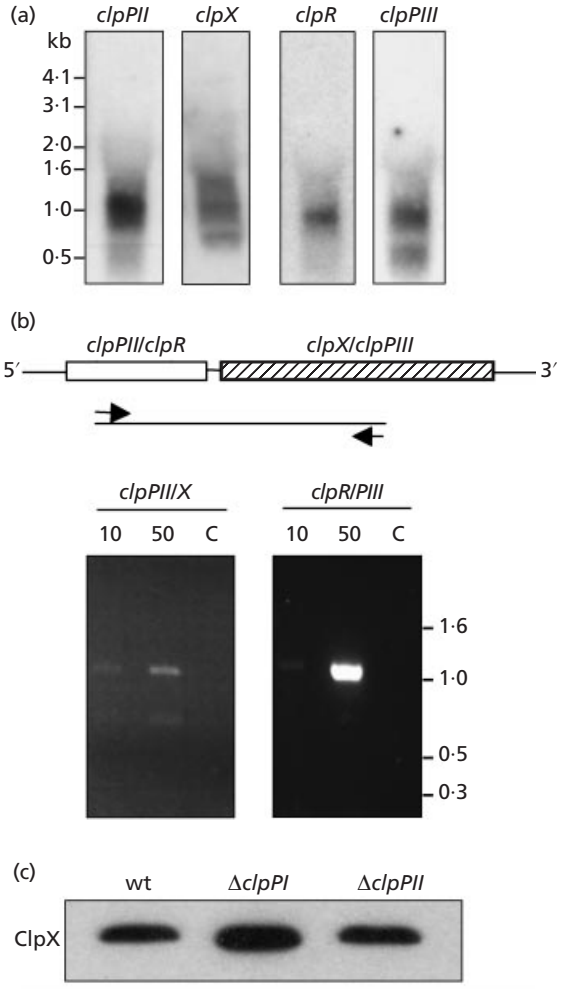

Fig. 4. Expression of *Synechococcus clpPII-III*, *clpR* and *clpX* genes. (a) Northern blot of total RNA extracted from wild-type in early exponential growth under standard conditions. Total RNA (19 μg) was separated electrophoretically and hybridized with ^{32}P-labelled DNA probes specific for each transcript (see Methods for probe details). Molecular size standards are indicated on the left. (b) Detection by RT-PCR of wild-type polycistronic mRNA for *clpPII/clpX* and *clpR/clpPIII*. Primers were selected that spanned the intervening gap between the *clpPII/clpX* or *clpR/clpPIII* genes, as depicted in the diagram. Template RNA (10 and 50 ng) was used for each *clp* gene pair in separate RT-PCR reactions, along with a control reaction (C) with 50 ng template RNA in which reverse transcriptase was replaced with *Taq* polymerase to detect possible DNA contamination. Molecular size markers are shown on the right. (c) ClpX content in wild-type (wt) *Synechococcus*, Δ*clpPI* and Δ*clpPII* strains grown under standard culture conditions. Protein samples were isolated from each strain at matching growth stages and separated by SDS-PAGE on the basis of equal Chl content (0·4 μg). ClpX protein was detected by immunoblotting using the specific polyclonal antibody described in Fig. 2.

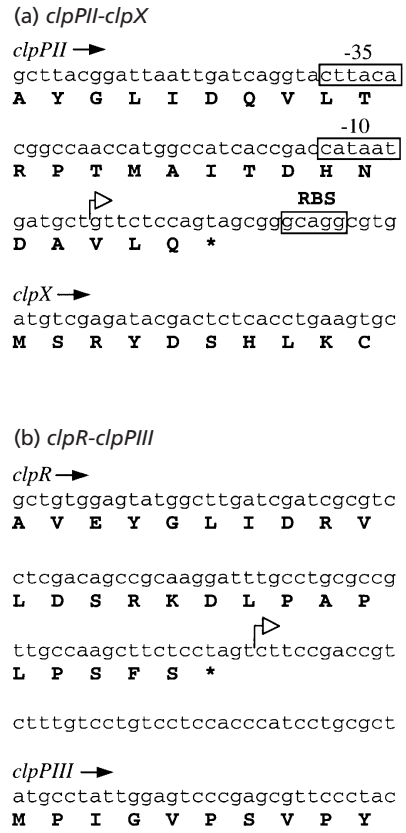

Fig. 5. Mapping the 5′ ends of the monocistronic transcripts for *clpX* (a) and *clpPIII* (b) genes. The 5′ end of each transcript was identified by 5′-RACE from total RNA isolated from wild-type *Synechococcus*. The nucleotide and deduced amino acid sequences are shown for the relevant region between *clpPII-clpX* and *clpR-clpPIII*. An open arrow indicates the 5′ end of each transcript, whereas putative RBS and −10/−35 promoter regions are boxed.

the ClpP3, ClpR and ClpX proteins are all essential for *Synechococcus* under standard growth conditions. Characterization of the Δ*clpPII* strain revealed no growth impairments relative to wild-type *Synechococcus* under standard culture conditions despite the loss of ClpP2 (see Fig. 6b). Neither did Δ*clpPII* exhibit the same phenotypic changes as Δ*clpPI*. There was no significant change in cell morphology and ClpP2 was unnecessary for acclimation to or growth at either high light or cold (data not shown).

Transcriptional regulation

Due to the short intervening distances between the *clpPII/X* and *clpR/PIII* genes (12 and 42 bases, respectively), we investigated their transcriptional regulation more closely. Genomic organization suggested that both gene pairs are arranged as bicistronic operons and are thus expressed as polycistronic messages. To test this, Northern blot analysis was performed using total RNA from wild-type *Synechococcus* in early exponential growth under standard conditions. As in Fig. 4(a), only monocistronic mRNAs were detected for both gene pairs, despite their apparent operon organization: the 0·9–1·0 kb transcripts for *clpPII*, *clpIII* and *clpR* and the less distinct transcript for *clpX* at 1·5 kb. Degradation of the main monocistronic mRNA was also evident for *clpX* and *clpPIII* by the shorter smeared

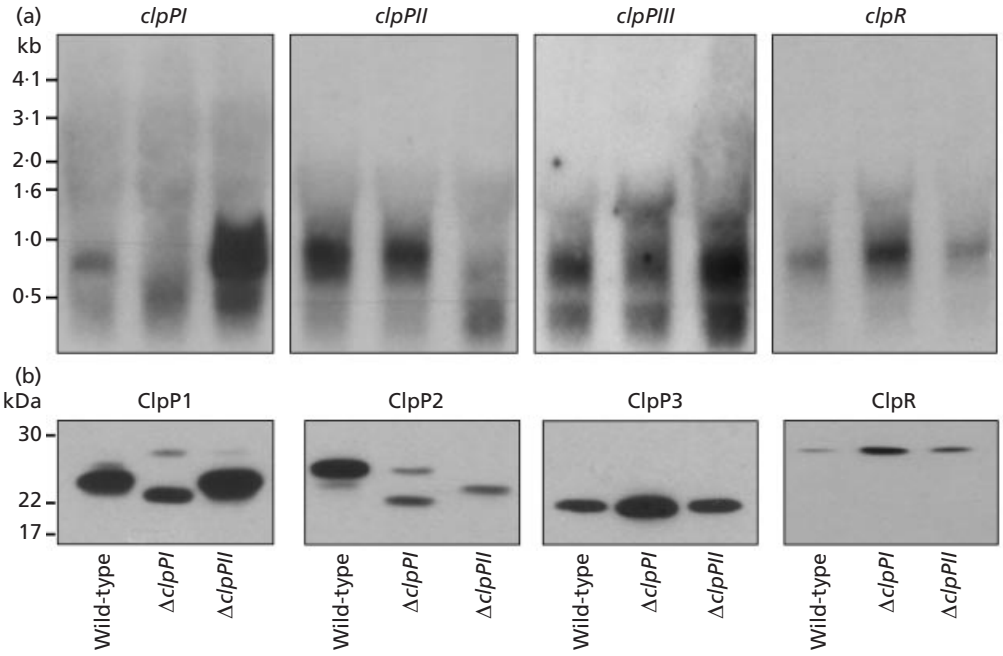

Fig. 6. Levels of mRNA (a) and protein (b) for *clpPI-III* and *clpR* in wild-type *Synechococcus*, Δ*clpPI* and Δ*clpPII* grown under standard conditions. (a) Northern blot of total RNA (19 μg) from each strain as described in Fig. 4. Size standards are indicated on the left. (b) Cell protein extracts from each strain were separated on linear 18% Tris/glycine gels (NOVEX) on the basis of equal Chl content (0·4 μg). The ClpP and ClpR proteins were detected by immunoblotting using the specific polyclonal antibodies described in Fig. 2. Molecular mass markers are shown on the left.

signals, suggesting both transcripts are less stable than those for their upstream partners.

Despite the main transcripts for *clpPII*, *clpIII*, *clpR* and *clpX* being monocistronic, the possibility remained of less abundant polycistronic mRNAs due to the weak detection of longer transcripts. For both gene pairs, faint smears were observed at the expected sizes for bicistronic transcripts: 2·5 kb for *clpPII/X* and 1·8 kb for *clpR/PIII* (Fig. 4a). Like for the monocistronic *clpX* and *clpPIII* messages, the degraded signal for the polycistronic transcripts suggests they are less stable than the monocistronic ones for *clpPII* and *clpR*. To confirm the existence of polycistronic transcripts, the more sensitive approach of RT-PCR was employed (Fig. 4b). By choosing a complementary primer annealing within the downstream gene of the operon and one annealing within the upstream gene, any resulting RT-PCR product would span the intervening gap and originate only from a polycistronic message. Primers were also selected whereby any resulting products would be longer than the monocistronic transcripts detected by Northern blotting. As shown in Fig. 4(b), products of the expected length were indeed obtained for *clpPII/X* and *clpR/PIII* (approx. 1·1 kb each), thereby verifying the existence of polycistronic messages.

Although polycistronic transcripts were detected for *clpPII/X* and *clpR/PIII* by RT-PCR, their low abundance as detected by Northern blot analysis suggests that the monocistronic messages for the four genes are the most abundant under standard growth conditions.

In the case of *clpPII/X*, we were able to examine this further by examining the relative ClpX content in the Δ*clpPII* strain. If the polycistronic mRNA was the main source of ClpX protein, then its disruption by the inactivation of *clpPII* should significantly decrease the amount of ClpX in the Δ*clpPII* strain. However, as seen in Fig. 4(c), no such polar decrease in ClpX content was observed in Δ*clpPII* compared to the wild-type, consistent with ClpX protein originating primarily from a monocistronic mRNA independent of *clpPII* gene expression. The absence of any ClpX loss in the Δ*clpPII* strain also precludes the unlikely possibility that the monocistronic transcripts for *clpPII* and *clpX* derive from rapidly processed polycistronic mRNAs, although this possibility cannot be excluded as yet for *clpR/PIII*.

Promoters for monocistronic *clpX* and *clpPIII* mRNA

The existence of monocistronic *clpX* and *clpPIII* messages infers that dedicated promoters exist for both genes. The short gap between *clpPII/X* (12 bp) and *clpR/PIII* (42 bp) equally implies that these independent promoters for *clpX* and *clpPIII* must be located close to or inside the 3′ region of their upstream partner. To locate these promoter regions, we first identified the 5′ end of the monocistronic *clpX* and *clpPIII* transcripts. By 5′-RACE analysis on total wild-type RNA, the 5′ end of the *clpX* mRNA was located inside the *clpPII* gene, 9 bases upstream from the *clpPII* stop codon (Fig. 5a). Putative constitutive −10 and −35 promoters for *clpX* gene expression were also identified, consistent with the

lack of stress induction for ClpX. For *clpPIII*, the transcript 5′ end was situated 1 bp downstream of the *clpR* stop codon (Fig. 5b). Although no obvious promoter motifs were found for *clpPIII* gene expression, they must be located inside the *clpR* gene like those for *clpX* in the *clpPII* gene.

Differential regulation of *clp* genes

Although multiple *clpP* forms, including the variant *clpR*, are now recognized in cyanobacteria and plants, little is known about their transcriptional and translational regulation. In particular, do changes in the amount of one Clp protein affect the level of one or more of the others? To analyse this, we compared the levels of mRNA and protein for the *clpP/R* genes in wild-type *Synechococcus* and the two viable mutants Δ*clpPI* and Δ*clpPII*. Levels of mRNA for each gene were detected by Northern blotting (Fig. 6a) using the gene-specific probes described in Fig. 4(a), while Clp protein contents were examined by immunoblotting (Fig. 6b) using the antibodies in Fig. 2. It should be noted that in the absence or low level of ClpP1 or ClpP2, as in the two mutants, the corresponding antibodies cross-react to the other ClpP isozymes since they were made to the entire ClpP1 or ClpP2 proteins.

Inactivation of the *clpPI* gene resulted in an increase of both transcript and protein levels for ClpP3 and ClpR (Fig. 6). In the case of ClpP3, it was the level of polycistronic transcript that rose (Fig. 6a). Although no change could be detected in the amount of *clpPII* mRNA, there was a significant decrease in ClpP2 protein content (Fig. 6). Concomitant to this loss of ClpP2 in Δ*clpPI* was a marked rise in ClpX protein content (approx. 75%, Fig. 4c), further indicating the regulatory separation between the *clpPII* and *clpX* genes. Contrary to the Δ*clpPI* strain, mutation of the *clpPII* gene caused much less dramatic alterations. Despite a significant increase in the amount of *clpPI* mRNA, the level of the ClpP1 protein increased to a much lesser extent (Fig. 6). Larger increases in *clpPIII* mRNA were detected compared to a smaller increase in the amount of *clpR* mRNA (Fig. 6a). However, the protein levels for ClpP3 and ClpR remained relatively unchanged (Fig. 6b).

DISCUSSION

In this study we have characterized four new *clp* genes in *Synechococcus*: *clpPII*, *clpPIII*, *clpR* and *clpX*. Together with *clpPI*, the discovery of the *clpPII* and *clpPIII* genes now confirms the presence of a multigene *clpP* family in this cyanobacterium. The arrangement of these genes in *Synechococcus* (i.e. monocistronic *clpPI* and bicistronic *clpPII-clpX* and *clpR-clpPIII*) is conserved in the genomes of *Synechocystis* sp. strain PCC 6803 (Kaneko *et al*., 1996) and other strains (*Synechococcus* WH8102, *Nostoc punctiforme* and *Prochlorococcus marinus*). Including the monocistronic *clpC*, *clpBI* and *clpBII* genes, cyanobacteria appear to have a common *clp* gene complement (eight in total), types and organization. Furthermore, multiple ClpP isomers and the ClpR variant also seem characteristic of all oxygenic photosynthetic organisms. Higher plants in particular have a great complexity of Clp proteins, with six distinct *clpP* and four *clpR* genes in the *Arabidopsis* genome (Adam *et al*., 2001). In contrast, archaea and lower eukaryotes like yeast (*Saccharomyces cerevisiae*) and *Drosophila* lack *clpP*, whilst mammals and most eubacteria have only one *clpP* gene and no *clpR* (reviewed by Porankiewicz *et al*., 1999). However, multiple *clpP* genes are not a feature exclusive to cyanobacteria and plants, since up to five *clpP* genes exist in different *Streptomyces* strains (Viala *et al*., 2000).

The three *Synechococcus* ClpP isozymes are typical of serine-type proteases, with the characteristic catalytic triad of Ser, His and Asp residues (Maurizi *et al*., 1990b). While the ClpP1 type appears specific for cyanobacteria, ClpP2 is homologous to the single ClpP in *E. coli*, in terms of both sequence similarity and gene arrangement with *clpX*. In contrast, ClpP3 has higher sequence similarity to the plastomic ClpP (ClpP1) in plants. Moreover, *Synechococcus* ClpP3 is essential for photosynthetic growth like the plant ClpP1 (Huang *et al*., 1994; Shikanai *et al*., 2001). Existence of a cyanobacterial homologue to ClpP1 in plants is consistent with the endosymbiotic theory for plastid evolution.

Clp proteins in most eubacteria are induced by one or more stresses and some are crucial for cellular acclimation to these less favourable conditions. ClpX and ClpP are typically induced by high temperatures (Kroh & Simon, 1990; Östersås *et al*., 1999) and other stresses like high salt, oxidation and glucose deprivation (Völker *et al*., 1994). In *B. subtilis*, ClpP also plays a vital role in stationary-phase adaptive responses like competence development, motility, degradative enzyme synthesis and sporulation (Msadek *et al*., 1998). In contrast, none of the studied *Synechococcus* Clp proteins responded to heat shock, similar to our previous observation for ClpC and ClpP1 (Clarke & Eriksson, 1996; Clarke *et al*., 1998). Cold stress also failed to elicit significant increases in ClpX, ClpP2, ClpP3 and ClpR protein, unlike that observed for ClpP1 (Porankiewicz *et al*., 1998), whereas all four Clp proteins were induced by high light intensities to different degrees, but again to a lesser extent than ClpP1 (Clarke *et al*., 1998). Instead, ClpX, ClpP3 and ClpR appear to be primarily constitutive proteins that perform roles essential for cell viability, as shown by the inability to inactivate each gene. Indispensable ClpX and ClpP proteins have also been observed in another eubacterium, *Caulobacter crescentus*, in which loss of these proteins blocks cell division (Jenal & Fuchs, 1998). In contrast, the functional importance of ClpP2 in *Synechococcus* remains unclear. Indeed, the lack of obvious phenotypic changes resulting from *clpPII* inactivation implies ClpP2 is redundant under the conditions tested in this study. Interestingly, a similar lack of significant functional importance was observed for the homologous ClpP in *E. coli* (Maurizi *et al*., 1990b).

In terms of transcriptional regulation, the single *clpP* gene in *E. coli* is one of the best studied. It is situated

upstream of *clpX* like the *Synechococcus clpPII* gene, but with a longer intervening distance (125 bases; Gottesman *et al*., 1993). In contrast to *clpPII*, however, the *E. coli clpP* gene is mainly co-transcribed with *clpX* as a single polycistronic mRNA. In *E. coli*, the *clpX* gene can also be expressed independently of *clpP* from a promoter proximal to the 3′ end of *clpP*, but the amount of ClpX produced from this monocistronic transcript is relatively low (Yoo *et al*., 1994). More transcriptional variation exists for *clpX* and *clpP* in Gram-positive bacteria. Both genes in *Salmonella enterica* are arranged like those in *E. coli* and are expressed only as a single polycistronic transcript (Yamamoto *et al*., 2001), whereas *clpP* and *clpX* in *C. crescentus* are separated by the *cicA* gene and transcribed monocistronically (Österås *et al*., 1999). In *B. subtilis*, *clpP* and *clpX* are located at different chromosomal loci and are also transcribed as monocistronic genes (Msadek *et al*., 1998). Yet another variation occurs in *Synechococcus* with both genes transcribed monocistronically despite being separated by only 12 bases. Indeed, the 5′ end of the monocistronic *clpX* mRNA is situated inside the 3′ region of the *clpP* gene, along with the putative promoters. To our knowledge, this is the first example of such a regulatory organization for these two *clp* genes, or indeed for any monocistronic genes in eubacteria.

As for *clpPII/X*, monocistronic transcripts predominate for the *Synechococcus clpR/PIII* genes, despite the bicistronic gene arrangement. However, the polycistronic message for *clpR/PIII* is relatively more abundant than that for *clpPII/X*, particularly in the Δ*clpPI* strain. Although the exact function of ClpR is unknown, results so far suggest that it interacts in some way with ClpP3. Besides the conserved gene arrangement in cyanobacteria, both *clpR* and *clpPIII* are essential for *Synechococcus* cell viability. Transcript and protein levels for ClpR and ClpP3 match closely in wild-type *Synechococcus* under various stress regimes, and increase equally in Δ*clpPI* in response to the loss of ClpP1. Moreover, ClpR homologues are localized in the stroma of plant chloroplasts, as is the ClpP isomer homologous to the cyanobacterial ClpP3. Although ClpR proteins are characterized by lacking the ClpP catalytic triad, whether it functions as a regulatory subunit or as a novel proteolytic one remains to be resolved.

The regulatory variations in *clpX* gene expression suggest an evolutionary selective pressure to uncouple ClpX protein synthesis from that of ClpP in certain eubacteria. In strains like *E. coli*, expression of *clpP* and *clpX* remains tightly co-ordinated and regulated from common promoters proximal to the upstream *clpP* gene, suggesting ClpX in these eubacteria functions primarily in a ClpXP protease. In cyanobacteria and many Gram-positive strains, however, the uncoupled expression of both *clp* genes infers that ClpX chaperone activity, independent of ClpP, may be increasingly crucial. ClpX can function as a chaperone apart from ClpP (Levchenko *et al*., 1995) and it is the importance of this function under different growth conditions that may underlie the development of independent *clpX* gene expression in certain eubacteria. The unchanged level of ClpX in the *Synechococcus* Δ*clpPII* strain is consistent with this proposal. Moreover, in Δ*clpPI* where ClpP2 content also drops significantly concomitant to ClpP1 loss, the amount of ClpX protein instead increased, again supporting a role for ClpX independent of ClpP2. Alternatively, in strains that have many ClpP forms like *Synechococcus*, ClpX may function as the regulatory partner of Clp proteolytic complexes with other ClpP isomers, thereby requiring *clpX* expression independent of the upstream *clpP* gene.

Genetic studies revealed interesting regulatory features for the multiple *Synechococcus clpP* genes at both the transcriptional and translational level (Fig. 6). While *clpPI* inactivation did not produce a significant change in the level of *clpPII* transcript, the ClpP2 protein was rendered highly unstable. Concomitant increases in both *clpPIII* and *clpR* mRNA and protein in the Δ*clpPI* strain suggested a possible compensatory response, although this would clearly be insufficient to substitute for ClpP1 function as shown by the pleiotrophic phenotype of Δ*clpPI*. Instead, the increased levels of ClpP3 and ClpR may be directly related to the degradation of ClpP2 in Δ*clpPI*. Absence of ClpP1 may somehow target ClpP2 for degradation by a Clp protease containing ClpP3 and/or ClpR. One possibility is that ClpP2 activity requires post-translational processing at the N terminus as does the homologous ClpP in *E. coli*, in which the first 14 aa are removed (Maurizi *et al*., 1990a). For *Synechococcus* ClpP2, this processing event may be facilitated by ClpP1 rather than being completely autoproteolytic as for *E. coli* ClpP. Accumulation of the immature, nonfunctional ClpP2 precursor would explain its instability and low constitutive level in the Δ*clpPI* strain. It would also explain why no reciprocal response occurred in Δ*clpPII*, in which the level of ClpP1 instead rose in response to the loss of ClpP2. Despite a large increase in the amount of *clpPI* transcript, there was only a slight increase in ClpP1 protein content in the *clpPII* mutant. Given that Δ*clpPII* showed no phenotypic changes, it is likely that the small increases in ClpP1 and ClpP3 are sufficient to compensate for the absence of ClpP2. Such compensatory responses by either ClpP1 or ClpP2, however, are clearly unable to substitute for ClpP3 and ClpR function given that both are apparently essential for *Synechococcus* viability.

Such differential regulation and functional importance of *clp* genes as shown in *Synechococcus* has also recently been observed in the high G+C Gram-positive bacterium *Streptomyces lividans* (de Crécy-Lagard *et al*., 1999; Viala *et al*., 2000). Interestingly, high G+C Gram-positive bacteria are now believed to be more closely related to Gram-negative bacteria, and in particular cyanobacteria, than to other Gram-positive bacteria (Gupta, 1998). *S. lividans* has five *clpP* genes, of which only the first four have so far been studied. Like the *clpP* homologues in *Synechococcus*, none of the four genes in *S. lividans* is induced by heat stress (de Crécy-Lagard *et al*., 1999; Viala *et al*., 2000). The *S. lividans clpPI* and

clpPII genes are located in tandem upstream of *clpX*, and both genes produce active ClpP proteins that require post-translational processing like the *E. coli* ClpP. In these regards, *S. lividans* ClpP1 and ClpP2 proteins are homologous to *Synechococcus* ClpP2. In contrast, however, inactivation of *clpPI/II* in *S. lividans* causes several phenotypic changes, including thermosensitivity at 37 °C and no aerial mycelium formation (de Crécy-Lagard *et al*., 1999), whereas the *Synechococcus* Δ*clpPII* strain exhibits no such obvious changes from the wild-type.

Similarities also exist between the *clpPIII-IV* genes in *S. lividans* and *clpR-PIII* in *Synechococcus* as shown in this study. In *S. lividans*, expression of the *clpPIII-IV* bicistronic operon is induced upon inactivation of the *clpPI-II* operon (Viala *et al*., 2000). Both transcript and protein levels for *Synechococcus* ClpP3 and ClpR are also elevated in the Δ*clpPII* strain, although more so in Δ*clpP*1. This up-regulation of *S. lividans clpPIII-IV*, however, is unable to fully compensate for the loss of ClpP1 and ClpP2, since the lack of aerial mycelium formation in the Δ*clpPI* strain persists (Viala *et al*., 2000), a finding reminiscent of the *Synechococcus* Δ*clpPI* strain with increased ClpP3 and ClpR proteins. This suggests that the ClpP isomers in both *S. lividans* and *Synechococcus* are not fully isofunctional. Despite such similarities, the *S. lividans clpPIII-IV* genes also differ from *Synechococcus clpR-PIII* in several regards. Whereas the *clpPIII-IV* genes in *S. lividans* are expressed polycistronically (Viala *et al*., 2000), the *clpR-PIII* genes in *Synechococcus* are expressed primarily as monocistronic transcripts. Inactivation of the two *Synechococcus* genes is also lethal, while a viable mutant of *clpPIII-IV* was obtained in *S. lividans* without obvious phenotypic changes (Viala *et al*., 2000). Furthermore, although the ClpP4 protein in *S. lividans* lacks the active site His residue in the expected position, another His residue lies just 6 aa upstream and may well function within the catalytic triad (Viala *et al*., 2000). In contrast, the *Synechococcus* ClpR lacks all three conserved amino acids of the catalytic triad and cannot be considered a true ClpP protein. The position of *clpR* upstream of *clpPIII* gene in *Synechococcus* also differs from the *clpPIV* position downstream of *clpPIII* in *S. lividans*. Expression of the *S. lividans clpPIII-IV* genes is also controlled by the activator protein PopR (Viala *et al*., 2000), whereas no homologue of this regulatory protein has yet been identified in cyanobacteria.

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