

## Induction of the Heat Shock Protein ClpB Affects Cold Acclimation in the Cyanobacterium *Synechococcus* sp. Strain PCC 7942

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**The heat shock protein ClpB is essential for acquired thermotolerance in cyanobacteria and eukaryotes and belongs to a diverse group of polypeptides which function as molecular chaperones. In this study we show that ClpB is also strongly induced during moderate cold stress in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942. A fivefold increase in ClpB (92 kDa) content occurred when cells were acclimated to 25°C over 24 h after being shifted from the optimal growth temperature of 37°C. A corresponding increase occurred for the smaller ClpB' (78 kDa), which arises from a second translational start within the *clpB* gene of prokaryotes. Shifts to more extreme cold (i.e., 20 and 15°C) progressively decreased the level of ClpB induction, presumably due to retardation of protein synthesis within this relatively cold-sensitive strain. Inactivation of *clpB* in *Synechococcus* sp. increased the extent of inhibition of photosynthesis upon the shift to 25°C and markedly reduced the mutant's ability to acclimate to the new temperature regime, with a threefold drop in growth rate. Furthermore, around 30% fewer  $\Delta clpB$  cells survived the shift to 25°C after 24 h compared to the wild type, and more of the mutant cells were also arrested during cell division at 25°C, remaining attached after septum formation. Development of a cold thermotolerance assay based on cell survival clearly demonstrated that wild-type cells could acquire substantial resistance to the nonpermissive temperature of 15°C by being pre-exposed to 25°C. The same level of cold thermotolerance, however, occurred in the  $\Delta clpB$  strain, indicating ClpB induction is not necessary for this form of thermal resistance in *Synechococcus* spp. Overall, our results demonstrate that the induction of ClpB contributes significantly to the acclimation process of cyanobacteria to permissive low temperatures.**

Induction of a specific subset of proteins is a universal response to different environmental stress factors among prokaryotic and eukaryotic organisms. The best characterized of these are proteins induced during high-temperature stress. Known as heat shock proteins (HSP), many of these induced polypeptides function as molecular chaperones or ATP-dependent proteases (25). Molecular chaperones directly influence protein structure and maturation and are involved in protein folding and assembly of oligomeric complexes. During heat stress, induced chaperones also function to stabilize protein structures, refold denatured polypeptides, and solubilize protein aggregates (25). Additional proteases are also synthesized to degrade those polypeptides irreversibly damaged during the stress period (8). In association, these two types of HSP are essential for cell survival at high temperatures.

Besides high temperature, certain HSP are also generally inducible by other types of stress such as oxidative stress and treatments with heavy metals (9, 41). At low temperatures, however, synthesis of most bacterial HSP is strongly repressed, and instead, specific cold shock proteins (CSP) are rapidly induced (13, 40). In contrast to HSP, little is known about the specific roles of CSP in bacteria, although they appear to be involved in a range of cellular functions such as transcription, translation, and DNA recombination (12, 14). The major CSP in *Escherichia coli*, for example, is CspA, a small 5-kDa protein that is exclusively synthesized at low temperature. It binds to single-stranded DNA and is thought to function as either a general transcriptional activator of the cold shock regulon in *E. coli* (18) or an RNA chaperone (14). Homologs to CspA have

now been identified in many different eubacteria (1, 28, 43). Of those proteins induced at low temperatures, however, few appear to function as molecular chaperones acting upon the structure of preexisting polypeptides, despite the increased potential for protein aggregation via intermolecular associations and the formation of disulfide linkages (10).

The Clp/HSP100 proteins constitute a new family of molecular chaperones which, as do other chaperone families, contains both constitutive and stress-inducible representatives. Two groups of Clp protein have been identified. The first consists of relatively large proteins with two distinct ATP-binding domains (ClpA to -D), while the second has smaller proteins with only one such domain (ClpM, -N, -X, and -Y) (34). ClpA from *E. coli* was the first of these proteins to be characterized and was originally shown to associate with an unrelated protein, ClpP, to form the ATP-dependent Clp or Ti protease (11, 16). As part of this protease complex, ClpA binds the polypeptide selected for degradation and initiates proteolysis by apparently unfolding the polypeptide and thereby exposing it to the proteolytic action of ClpP (17, 39). ClpA also has chaperone activity independent of ClpP in vitro, substituting for the Hsp70 chaperones in the activation of RepA (42).

Another well-characterized member of the Clp/HSP100 family is ClpB. It is distinguishable from other large Clp proteins by its relatively long intervening region between the two ATP-binding domains (37). Two distinct ClpB proteins exist in prokaryotes and eukaryotes, and in each case both forms are HSP. Separate nuclear *clpB* genes in eukaryotes code for cytosolic-nuclear (100 to 110 kDa) and mitochondrial (78-kDa) proteins (19, 31), whereas a single gene in eubacteria codes for two different-sized proteins (78 and 94 kDa) via a second translational initiation site within the *clpB* transcript (7, 23).

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The role of the smaller prokaryotic ClpB protein (ClpB') remains unknown, although a regulatory function when complexed to the larger form (94 kDa) has been suggested (23).

Both the cytosolic and mitochondrial ClpB forms in yeast function as molecular chaperones. The cytosolic protein (Hsp104) promotes the resolubilization of unfolded proteins that have aggregated during severe heat stress (27), while the smaller mitochondrial ClpB (Hsp78) prevents protein denaturation at high temperatures (35). Loss of the cytosolic ClpB in *Saccharomyces cerevisiae* has little effect on cell survival during a sudden and severe heat shock, but it does prevent the development of thermotolerance (31), which is the acquired resistance to a normally lethal high temperature by being preconditioned to a nonlethal, permissive high temperature. In contrast, the ClpB homolog in *E. coli* is apparently not necessary for acquired thermotolerance but does influence cell survival during direct shifts to severe high temperatures (38). This feature of the *E. coli* ClpB is not characteristic of all eubacterial proteins, however, since the ClpB homolog in the cyanobacterium *Synechococcus* sp. strain PCC 7942 is essential for sustained thermotolerance, similar to the yeast Hsp104 protein (7).

In addition to high temperatures, ClpB is also inducible by other types of physiological change. The yeast Hsp104 is induced during early stages of sporulation and upon the transition from exponential to stationary growth phase (31), as well as after exposure to ethanol and arsenite (32). In *E. coli*, ClpB synthesis is also stimulated by ethanol, in addition to cadmium treatment and periods of nutrient starvation (38). In order to better understand the role of ClpB in cyanobacteria, we examined whether this known HSP is also important during cold stress in *Synechococcus* sp. strain PCC 7942, a thermophilic cyanobacterium particularly sensitive to low temperatures (36). We show that ClpB is induced by moderate chilling and, by comparing the wild type with a *clpB* deletion strain ( $\Delta clpB$ ), we demonstrate that its loss significantly retards the acclimation process to this low-temperature growth regime. We also analyze the ability of this cyanobacterial strain to develop sustained thermotolerance to severe cold and whether ClpB is involved in this process.

#### MATERIALS AND METHODS

**Culture conditions.** Cell cultures were grown in BG-11 inorganic medium (30) buffered with 10 mM 3-(*N*-morpholino) propanosulfonic acid (pH 7.5) and bubbled with 5% CO<sub>2</sub> in air (1 ml s<sup>-1</sup>) at 37 or 25°C with continuous, even illumination of 50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (2). Batch cultures were inoculated from liquid precultures to a concentration of 0.5  $\mu\text{g}$  of chlorophyll (Chl) ml<sup>-1</sup>. All experiments were performed with exponentially growing cells at a Chl concentration of 2.3 to 3  $\mu\text{g ml}^{-1}$ . Chl content was calculated from whole-cell spectra according to the method described by Myers et al. (21). The  $\Delta clpB$  strain was maintained on solid BG-11 plates and in liquid precultures with 5  $\mu\text{g}$  of the antibiotic kanamycin ml<sup>-1</sup> added to maintain selection. Batch cultures for all experiments, however, were grown without kanamycin to exclude the possibility of antibiotic-induced changes in the phenotype.

**Cold shock experiments.** The culture flask was moved directly from 37°C to a water bath-shaker at 25, 20, or 15°C. The photon irradiance was maintained at 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and the culture was continuously bubbled with 5% CO<sub>2</sub> in air. At each time point a certain volume was taken for photosynthetic and absorbance measurements, while another was pelleted and frozen in liquid nitrogen to await protein isolation. For the cold thermotolerance assay, cultures were pretreated at 25°C for 2 or 4 h and then transferred to a 50-ml glass tube placed in a 15°C water bath. Cultures were bubbled with 5% CO<sub>2</sub> in air, with a photon irradiance maintained at 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Cell survival determinations were performed as previously described (7) with 30  $\mu\text{l}$  of culture taken at the specified time points and serially diluted in fresh, sterile BG-11 medium from 1:1 to 1:10,000. A 3- $\mu\text{l}$  aliquot from each dilution was then spotted onto a predried BG-11 plate, and cells were grown for several days under low light (ca. 5  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at 33°C. Cell survival was determined from the number of colonies counted with a light microscope and multiplied by the appropriate dilution factor. Variations in cell number for each culture were normalized by dividing the calculated value by  $A_{750}$ . Three to four replicates for each experiment were carried out. Micros-

copy was also used to estimate the number of attached cells prior to division by using a hemocytometer chamber for three to four replicate cultures grown at either 37 or 25°C for 24 h.

**Photosynthetic measurements.** Chl *a* fluorescence parameters and oxygen evolution were measured simultaneously at 37 and 25°C with a pulse amplitude modulated fluorometer (Walz, Efeltrich, Germany) and a Clark-type oxygen electrode (Hansatech, King's Lynn, United Kingdom), respectively, as previously described in detail (2, 4). The degree of inhibition of photosystem II and photosynthetic electron transport was monitored by determining the parameters of maximal photochemical efficiency ( $F_v/F_m$ ) and oxygen evolution ( $\mu\text{mol of O}_2 \text{ mg of Chl}^{-1} \text{ h}^{-1}$ ), respectively.

**Production of Clp-specific antibodies.** Fusion proteins containing unique regions of *Synechococcus* sp. strain PCC 7942 ClpB or ClpC proteins were overexpressed in *E. coli* by using the inducible pMAL-c2 overexpression plasmid (New England Biolabs, Beverly, Mass.). For ClpB, the 490-bp 3' region downstream of the second ATP-binding domain of *clpB* (7) was PCR amplified with the high-accuracy *pfu* DNA polymerase (Stratagene) by using the primers *clpB1* (5' - AGC GAA TTC GGT TCG CAA TAC ATT CTC G - 3') and *clpB2* (5' - GAT TCT AGA CTT TTA CTT CGC TCA AAT T - 3'). The primers included *EcoRI* and *XbaI* restriction sites, respectively, to facilitate directional cloning. For ClpC, the primers *clpC1* (5' - AGG GAA TTC ATG TTT GAA CGC TTT ACC G - 3') and *clpC2* (5' - GAG TCT AGA TTA GAC TTG GGT CCG TAC TTT T - 3'), which also included *EcoRI* or *XbaI* sites, were used to amplify the 430-bp 5' domain upstream of the first ATP-binding domain of *clpC* (5). The amplified *clpB* and *clpC* fragments were purified, restricted, and then ligated in frame to the 3' end of the *malE* gene, coding for the maltose-binding protein on the plasmid pMAL-c2. The resulting plasmids were transformed into *E. coli* DH5 $\alpha$ . Overexpression of the pMAL/*clp* plasmids under the control of the *tac* promoter was induced by the addition of isopropyl- $\beta$ -D-thiogalactopyranoside to actively growing cells, and the resulting maltose-binding protein-Clp fusion proteins were purified according to a method described previously (29). The purified fusion proteins were then injected into rabbits intramuscularly and subcutaneously to produce ClpB- and ClpC-specific polyclonal antibodies (Agriseria AB, Vännäs, Sweden).

**Protein electrophoresis and immunodetection.** Total cellular proteins were extracted and samples containing equal amounts of Chl (0.75  $\mu\text{g}$ ) were separated electrophoretically on 7.5 or 11% lithium dodecyl sulfate polyacrylamide gels and subsequently transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, Mass.). Immunoblot analysis of the filter was performed according to the method of Clarke and Critchley (3). In addition to ClpB and ClpC, GroEL was also detected with polyclonal antibodies (Stress-Gen, Victoria, Canada) raised against the *Synechococcus* sp. strain PCC 7942 protein. All antibody reactions were detected with an enhanced chemiluminescence kit (Amersham). Immunoblots were performed on samples from at least three independent replicate experiments. Density scanning of X-ray films was carried out with an Image desktop scanner and software (Pharmacia LKB, Uppsala, Sweden).

#### RESULTS

**Induction of ClpB at cold temperatures.** Potential induction of ClpB protein at low temperatures was investigated in *Synechococcus* sp. strain PCC 7942 by shifting wild-type cultures from 37 to 25, 20, or 15°C. The full-length ClpB (94 kDa) and truncated ClpB' (78 kDa) proteins were detected with polyclonal antibodies directed to the C-terminal domain common to both proteins. During exponential growth at 37°C, the two ClpB proteins were almost undetectable. Upon shifting to 25°C, ClpB and ClpB' content steadily increased to up to five to six times the 37°C control level after 8 h. This level remained high once the cells had acclimated to the lower temperature after 24 h (Fig. 1A). Shifts to more severe, nonacclimative cold temperatures resulted in lower induction of the ClpB proteins, with a less than fivefold increase in ClpB content after 24 h at 20°C (Fig. 1B) and no discernible increase throughout the 15°C treatment (data not shown).

**Growth at 37 and 25°C.** Due to the relatively large induction of ClpB proteins at 25°C, the ability of a  $\Delta clpB$  strain to acclimate to this low temperature was compared to that of the wild type over a 24-h period. Under our culture conditions at 37°C, the average exponential doubling time of the wild type ( $7.5 \pm 0.4$  h,  $n = 3$ ) was slightly shorter than that of the  $\Delta clpB$  strain ( $8.6 \pm 0.7$  h,  $n = 10$ ). Cultures were shifted to 25°C once they had reached a Chl concentration of 2.5 to 2.7  $\mu\text{g ml}^{-1}$ . Growth of both strains ceased upon the cold shift and only

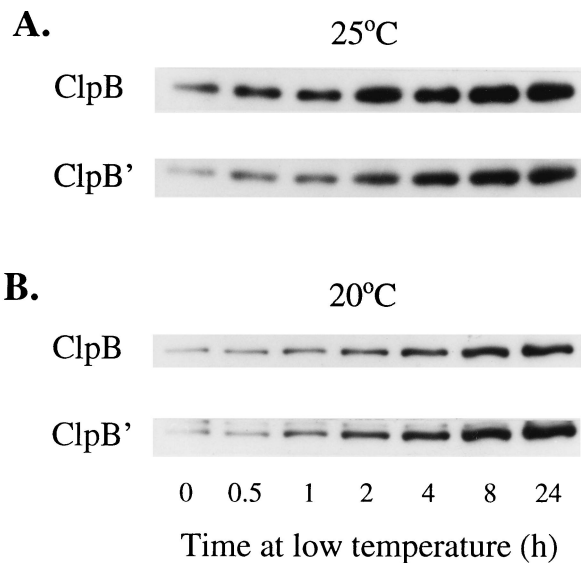


FIG. 1. Levels of ClpB and ClpB' during cold acclimation. Wild-type cells were shifted from 37 to 25 (A) or 20°C (B) for 24 h. Cellular proteins were isolated and then separated by polyacrylamide gel electrophoresis on the basis of equal Chl content. ClpB and ClpB' were detected with polyclonal antibodies raised against the C-terminal domain common to both proteins. Results are representative of three replicates.

slowly resumed after 4 h (Fig. 2). By 24 h, wild-type cultures had acclimated to the low temperature with an average generation time of  $15.0 \pm 0.9$  h ( $n = 3$ ). In contrast, the generation time of the  $\Delta clpB$  strain was over three times longer than that of the wild type (i.e.,  $54.4 \pm 7.5$  h,  $n = 10$ ).

**Photosynthetic activity at 25°C.** In addition to growth, the photosynthetic activities of the wild-type and  $\Delta clpB$  strains were analyzed during the 24-h cold shift. Photosynthetic activities, as determined by the rates of oxygen evolution, were comparable for both strains at the optimal growth temperature (i.e., 150 to 160  $\mu\text{mol}$  of  $\text{O}_2$   $\text{mg}$  of  $\text{Chl}^{-1}$   $\text{h}^{-1}$ ). When shifted to 25°C, the two strains suffered a dramatic loss in photosynthetic activity (ca. 50%) after 2 h (Fig. 3A). Afterwards, the wild type steadily recovered photosynthetic activity during acclimation

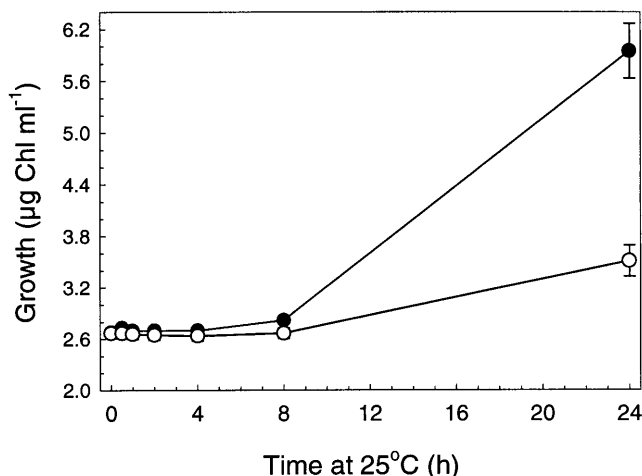


FIG. 2. Growth characteristics at 25°C. Growth rates of wild-type (●) and  $\Delta clpB$  (○) strains after being shifted at time zero from 37 to 25°C for 24 h. Values represent averages  $\pm$  standard errors for 3 to 10 replicates.

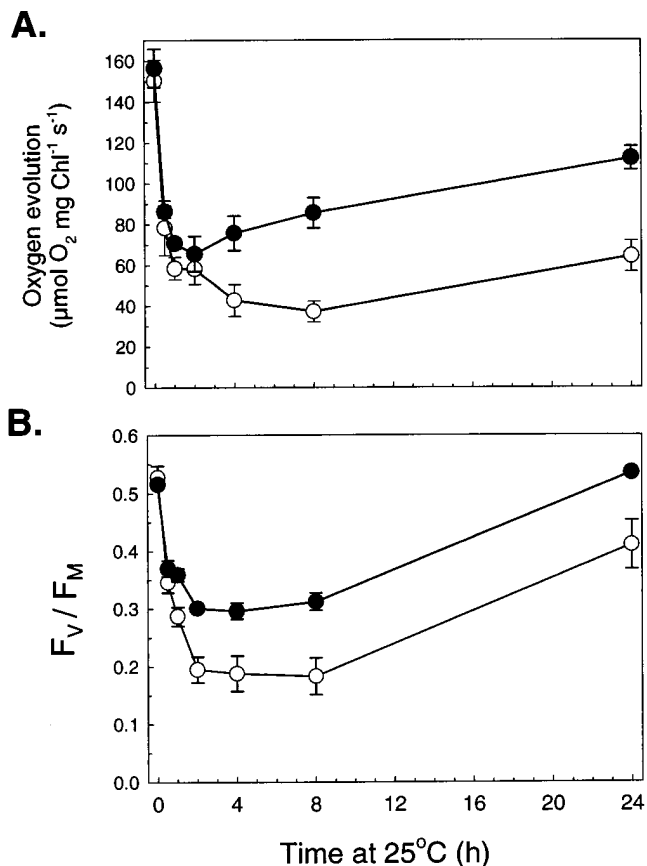


FIG. 3. Changes in photosynthesis during cold acclimation. Wild-type (●) and  $\Delta clpB$  (○) strains were shifted from 37 to 25°C for 24 h. Gross oxygen evolution (A) and photochemical efficiency of photosystem II reaction centers ( $F_v/F_m$ ) (B) are shown. Values represent averages  $\pm$  standard errors for three replicates.

to the new growth temperature, returning to 70% of the 37°C control level after 24 h at 25°C. The extent of this drop in photosynthetic electron transport is consistent with that previously observed in wild-type *Synechococcus* spp. acclimated to 25°C (2). In contrast, the  $\Delta clpB$  strain continued to lose activity after the initial drop, with only 25% activity remaining after 8 h at 25°C. By 24 h, the  $\Delta clpB$  strain had only marginally recovered photosynthetic activity, attaining 43% of the control value (Fig. 3A). The increased sensitivity of the  $\Delta clpB$  strain to 25°C was also shown in the photochemical efficiency of photosystem II reaction centers (i.e.,  $F_v/F_m$ ) (Fig. 3B), with a more severe inhibition upon the cold shift and slower subsequent recovery of  $\Delta clpB$  relative to those of the wild type.

**Cell survival and division at 25°C.** In addition to photosynthesis, cell survival and morphology of the wild-type and  $\Delta clpB$  strains were monitored during the cold treatment (Fig. 4). Cell number determinations showed that around 80% of wild-type and  $\Delta clpB$  cells survived the shift from 37 to 25°C after the first 2 h (Fig. 4A). The number of viable wild-type cells later stabilized, remaining at 80% of the 37°C control level after 24 h at 25°C. In contrast, the number of viable  $\Delta clpB$  cells continued to drop steadily throughout the cold treatment, with less than 60% of that of the control level remaining after 24 h. Microscopic examination of both strains revealed a somewhat higher number of  $\Delta clpB$  cells still connected after septum formation during exponential growth at 37°C (Fig. 4B). Most of these

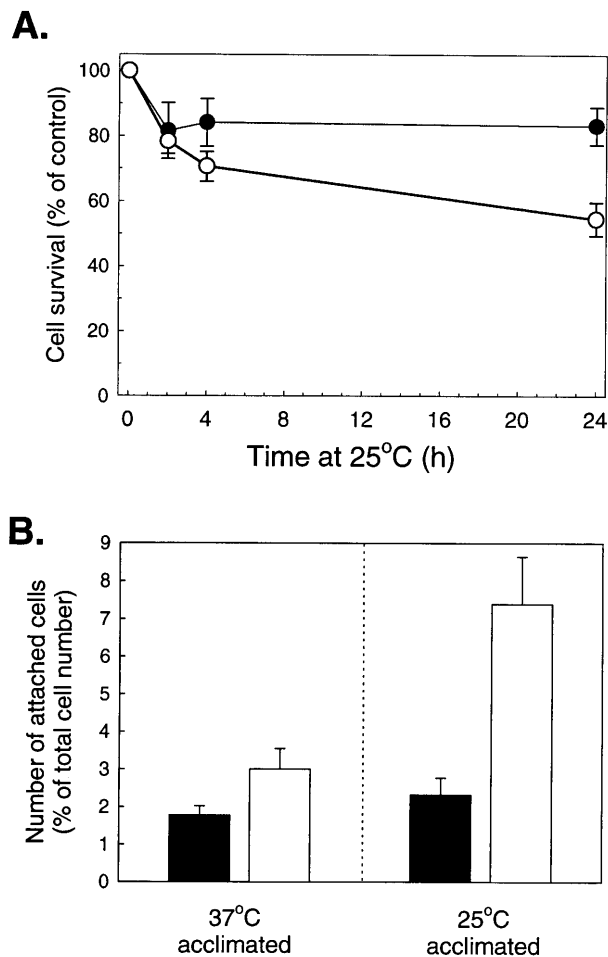


FIG. 4. Cell survival and morphology during cold acclimation. (A) Wild-type (●) and  $\Delta clpB$  (○) strains were shifted from 37 to 25°C for 24 h. At each indicated time, cell aliquots were taken and serially diluted in BG-11 medium. The numbers of viable wild-type and  $\Delta clpB$  cells were plotted as percentages of the value for the 37°C control (100%). (B) Numbers of wild-type (solid bars) and  $\Delta clpB$  (open bars) cells remaining attached following septum formation in cultures acclimated to either 25 or 37°C. All values represent averages  $\pm$  standard errors for three replicates.

attached cells had also entered elongation phase, which normally occurs only after separation. This phenotype was extenuated at 25°C, with the percentage of attached cells after division in the mutant increasing over twofold after 24 h (Fig. 4B). The corresponding wild-type cells, however, showed no significant rise in the level of attached cells following the cold shift.

**Acquisition of cold thermotolerance.** Synthesis of ClpB is necessary for acquired thermotolerance, or the ability to survive an otherwise lethal high temperature by being pretreated at a permissive high temperature, in yeast and cyanobacteria (7, 31). In this study we examined whether *Synechococcus* sp. strain PCC 7942 could also develop resistance to a normally severe cold temperature and if so whether this form of thermotolerance required the induction of ClpB. In developing this cold thermotolerance assay, the first step was to determine a severe treatment for the wild-type and  $\Delta clpB$  cultures. As shown in Fig. 5A, shifting both strains from 37 to 15°C caused a dramatic decrease in cell viability, with only 2% of wild-type and  $\Delta clpB$  cells surviving after 8 h. Since most of this loss in cell

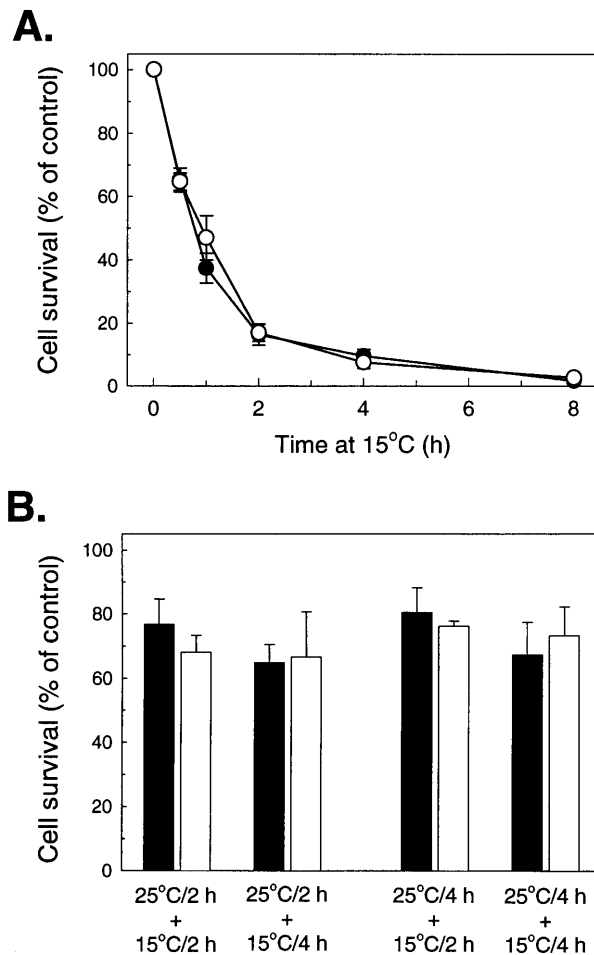


FIG. 5. Acquisition of cold thermotolerance. (A) Determination of severe, nonpermissive treatment for cold thermotolerance assay. Wild-type (●) and  $\Delta clpB$  (○) strains were shifted from 37 to 15°C for 8 h, and the number of viable cells at each time point was measured as described in the legend for Fig. 4. (B) Wild-type (solid bars) and  $\Delta clpB$  (open bars) strains grown at 37°C were pretreated at 25°C for 2 or 4 h and then exposed to 15°C for 2 or 4 h. The numbers of viable cells were calculated as described in the legend for Fig. 4. All values represent averages  $\pm$  standard errors for three replicates.

viability occurred after 2 h at 15°C, both 2 and 4 h at this temperature were used for the severe cold treatment.

The next step was to determine a pretreatment suitable to both establish cold thermotolerance in *Synechococcus* sp. strain PCC 7942 and induce the synthesis of ClpB. Since a shift to 25°C produced a relatively high level of ClpB (Fig. 1), we tested 2 and 4 h at this temperature as a preconditioning treatment. As shown in Fig. 5B, the wild-type strain was able to develop a substantial level of thermotolerance to the 15°C stress after first being incubated at 25°C. Of the 80% of wild-type cells that survived the 2- and 4-h pretreatments at 25°C (Fig. 4A), almost all remained viable when exposed to 15°C for 2 h, with only a further 15% drop in cell viability after 4 h at 15°C (Fig. 5B); this is in comparison to a less than 20% survival rate after cells were directly shifted from 37 to 15°C for the same time period (Fig. 5A). In comparison, the  $\Delta clpB$  strain developed an equivalent degree of thermotolerance to the 15°C treatment as the wild-type after being preconditioned at 25°C (Fig. 5B), indicating that ClpB induction is not essential for acquired cold thermotolerance in *Synechococcus* spp.

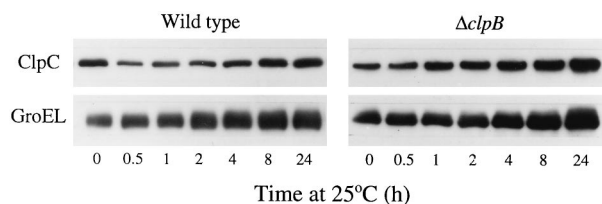


FIG. 6. ClpC and GroEL content during cold acclimation. Wild-type and  $\Delta clpB$  strains were shifted from 37 to 25°C for 24 h, and proteins were isolated at the indicated time points. Proteins were separated by polyacrylamide gel electrophoresis, and the ClpC and GroEL proteins were detected immunologically with specific polyclonal antibodies. Results are representative of three replicates.

**Cold induction of ClpC and GroEL.** Due to the apparent unimportance of ClpB synthesis for acquired cold thermotolerance, we investigated whether raised levels of other chaperones occurred in the  $\Delta clpB$  mutant that may have compensated for the absence of ClpB. Using specific antibodies we examined first the level of the ClpC protein, another member of the Clp/HSP100 family. In contrast to ClpB, ClpC is constitutively expressed in *Synechococcus* sp. strain PCC 7942 (5) and is equally abundant in both wild-type and  $\Delta clpB$  cultures grown at 37°C. During the 25°C treatment, the amount of ClpC slightly increased in the wild type after 24 h (i.e.,  $1.5 \pm 0.4$  times the control value,  $n = 3$ ), whereas it increased threefold in the  $\Delta clpB$  strain (i.e.,  $2.9 \pm 0.3$  times the control value,  $n = 3$ ) (Fig. 6). In comparison, the content of another molecular chaperone, GroEL, doubled during the low-temperature shift in both strains after 24 h (Fig. 6).

## DISCUSSION

Induction of ClpB/HSP100 proteins has been observed in a variety of stress regimes (7, 24, 31, 32), but this study is the first to show increased synthesis of ClpB during cold stress. Induction of HSP under conditions other than heat shock is common for many different organisms, and in the yeast *S. cerevisiae*, the ClpB homolog (Hsp104) is induced during certain developmental and growth transitions and upon exposure to inhibitory levels of cadmium, arsenate, and ethanol (31, 32). The yeast Hsp104 also appears to be important for long-term storage of spores at low temperatures, with spore viability lower in the  $\Delta clpB$  strain compared to that of the wild type after several months of cold storage (32). In contrast, induction of HSP during cold stress is unusual, particularly in prokaryotes where HSP synthesis is normally strongly repressed in favor of the induction of specific CSP (13, 40). Conversely, CSP production is actively down-regulated during heat shock (40). The differential regulation of these proteins has long suggested distinct functions for HSP and CSP. Many CSP help stabilize transcriptional and translational processes at low temperatures and are involved in DNA recombination (12, 14), whereas most HSP act upon preexisting protein structures as molecular chaperones and proteases, stabilizing and refolding denatured polypeptides and promoting degradation of damaged proteins (26). Despite the apparent functional differences between HSP and CSP, our data clearly demonstrate the importance of ClpB induction for low-temperature acclimation in *Synechococcus* sp. strain PCC 7942. Loss of ClpB prolongs the lag phase caused by the cold shift prior to resumption of growth. It also reduces the growth rate at 25°C threefold. The growth phenotype is reflected metabolically in the  $\Delta clpB$  strain by a more severe loss in photosynthesis upon the cold shift, followed by a slower recovery. Furthermore, the loss of ClpB impairs cell

separation after septum formation during the division process. These features all point to a significantly reduced capacity to acclimate to cold in the *Synechococcus* sp.  $\Delta clpB$  strain. The sixfold increase in ClpB content in *Synechococcus* sp. strain PCC 7942 during cold acclimation was similar to that observed during moderate heat shock upon a shift to 50°C (7). This suggests that activation of ClpB synthesis in cyanobacteria is responding to stress factors common to both transient heat shock and prolonged cold stress.

In yeast, ClpB functions as a molecular chaperone by dissolving protein aggregates that increasingly form during severe high temperatures (27), and it is this role that presumably confers thermotolerance. In fact, ClpB alone is sufficient for the development of thermotolerance in yeast (20), suggesting that its role in protein resolubilization cannot be adequately performed by other chaperones. Since ClpB is also critical for high-temperature thermotolerance in *Synechococcus* spp. (7), it is very likely that it performs a similar role to that of the yeast homolog. If so, then the increase in ClpB content during chilling would indicate that ClpB is involved in protein aggregate disruption during cold acclimation. It is known that extensive protein denaturation and aggregation of metabolically important enzymes can occur at low temperatures (10) and that the failure to resolubilize these nonfunctional aggregates may well retard acclimation to this new temperature regime.

Reduced protein translation is a common effect of chilling in bacteria and is thought to be the primary cause for the lag phase immediately following a cold shift (12, 14), such as that observed in this study with *Synechococcus* sp. strain PCC 7942. In fact, certain CSP in *E. coli* exclusively associate with ribosomes following a cold shift and are presumably involved in preserving partial protein synthesis (15). One such protein, CsdA, exhibits helix-destabilizing activity and thereby facilitates translation via unwinding of stable secondary structures in mRNAs (15). This activity is thought to complement the RNA chaperone role of CspA which prevents the reannealing of transcripts once unwound (15). Given that protein synthesis continues to some extent during permissive cold stress, the induction of ClpB and its importance for cold acclimation in *Synechococcus* sp. PCC 7942 is less surprising. Under conditions of continued protein synthesis, protein renaturation and aggregate resolubilization facilitated by ClpB may well be vital for the initial steps of cold acclimation, relieving the requirement for extensive protein degradation and resynthesis. This may be especially important due to the significant reduction in photosynthetic activity, and presumably in available metabolic energy, after a cold shift as observed at 25°C in this study (ca. 40%).

Although ClpB induction plays a critical role in cold acclimation in *Synechococcus* sp. strain PCC 7942, it is not involved in acquired thermotolerance to severe, nonacclimative cold, in contrast to its vital contribution to high-temperature thermotolerance. *Synechococcus* sp. cells suffer rapid and almost complete loss in cell viability when shifted from the normal growth temperature of 37°C to 55°C for 10 min (7). If exposed at the moderate heat shock temperature of 50°C for 90 min, wild-type *Synechococcus* sp. develops substantial resistance to a subsequent 55°C treatment, with over 50% of cells surviving. In contrast, the same  $\Delta clpB$  strain used in this study failed to develop the same level of thermotolerance, with over four times fewer cells surviving the 55°C treatment (7). This study is the first to clearly demonstrate that cyanobacteria can also readily develop an equivalent degree of resistance to extreme cold shock by the preconditioning of cells to permissive low temperatures. The fact that ClpB activity is unnecessary for cold thermotolerance, however, suggests that the potential ac-

cumulation of inactive protein aggregates does not affect the acquisition of this form of thermal resistance. Instead, it is likely other mechanisms such as increases in solute concentration (6) and the induction of other specific CSP confer cold thermotolerance in *Synechococcus* spp., although CSP homologous to those in other eubacteria such as CspA have yet to be identified in cyanobacteria. Alternatively, changes in membrane saturation are known to influence long-term acclimation of cyanobacteria to extreme cold (22, 33) and may be involved in acquired cold thermotolerance, although recent evidence suggests such membrane alterations occur too slowly to facilitate this thermal resistance (27a).

At high temperatures, ClpB appears to be the sole or principal chaperone involved in resolubilization of protein aggregates (20). Even elevated levels of related chaperones, as in the case of ClpC in *Synechococcus* sp. PCC 7942, fail to adequately compensate for the loss of ClpB in the development of high-temperature thermotolerance (7). In this study, raised levels of ClpC in the  $\Delta clpB$  strain also failed to fully compensate for the inactivation of ClpB synthesis, reemphasizing the apparent functional difference between these two related Clp proteins as previously suggested (7). It is possible, however, that the eventual slow return to active growth in the mutant may be related to some extent to the additional ClpC protein. The slight rise in ClpC content in the cold-acclimated wild type may be related to its putative roles as both a chaperone and regulator of ATP-dependent proteolysis by ClpP. We have observed a dramatic increase in ClpP content during a corresponding 25°C shift in *Synechococcus* sp. strain PCC 7942 (27b), suggesting that increased proteolytic activity could also be important for cold acclimation. The extra ClpC protein in the cold-shifted  $\Delta clpB$  cells may therefore be related to the loss of ClpB resolubilization activity and the presumable accumulation of protein aggregates, requiring a higher proteolytic activity within the cell.

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#### REFERENCES

- Av-Gay, Y., Y. Aharonowitz, and G. Cohen. 1992. *Streptomyces* contains a 7.0 kDa cold shock like protein. *Nucleic Acids Res.* **20**:5478–5479.
- Campbell, D., G. Zhou, P. Gustafsson, G. Öquist, and A. K. Clarke. 1995. Electron transport regulates exchange of two forms of photosystem II D1 protein in the cyanobacterium *Synechococcus*. *EMBO J.* **14**:5457–5466.
- Clarke, A. K., and C. Critchley. 1992. The identification of a heat shock protein complex in chloroplasts of barley leaves. *Plant Physiol. (Bethesda)* **100**:2081–2089.
- Clarke, A. K., D. Campbell, P. Gustafsson, and G. Öquist. 1995. Dynamic responses of photosystem II and phycobilisomes to changing light in the cyanobacterium *Synechococcus* sp. PCC 7942. *Planta* **197**:553–562.
- Clarke, A. K., and M.-J. Eriksson. 1996. The cyanobacterium *Synechococcus* sp. PCC 7942 possesses a close homologue to the chloroplast ClpC protein of higher plants. *Plant Mol. Biol.* **31**:721–730.
- Deshnium, P., Z. Gombos, Y. Nishiyama, and N. Murata. 1997. The action in vivo of glycine betaine in enhancement of tolerance of *Synechococcus* sp. strain PCC 7942 to low temperature. *J. Bacteriol.* **179**:339–344.
- Eriksson, M.-J., and A. K. Clarke. 1996. The heat shock protein ClpB mediates the development of thermotolerance in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J. Bacteriol.* **178**:4839–4846.
- Gottesman, S., and M. R. Maurizi. 1992. Regulation by proteolysis: energy-dependent proteases and their targets. *Microbiol. Rev.* **56**:592–621.
- Graumann, P., K. Schröder, R. Schmid, and M. M. Maraha. 1996. Cold shock stress-induced proteins in *Bacillus subtilis*. *J. Bacteriol.* **178**:4611–4619.
- Guy, C. L. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41**:187–223.
- Hwang, B. J., W. J. Park, C. H. Chung, and A. L. Goldberg. 1987. *Escherichia coli* contains a soluble ATP-dependent protease (Ti) distinct from protease La. *Proc. Natl. Acad. Sci. USA* **84**:5550–5554.
- Jones, P. G., R. A. Van Bogelen, and F. C. Neidhardt. 1987. Induction of proteins in response to low temperature in *Escherichia coli*. *J. Bacteriol.* **169**:2092–2095.
- Jones, P. G., M. Cashel, G. Glaser, and F. C. Neidhardt. 1992. Function of a relaxed-like state following temperature downshifts in *Escherichia coli*. *J. Bacteriol.* **174**:3909–3914.
- Jones, P. G., and M. Inouye. 1994. The cold-shock response—a hot topic. *Mol. Microbiol.* **11**:811–818.
- Jones, P. G., M. Mitta, Y. Kim, W. Jiang, and M. Inouye. 1996. Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **93**:76–80.
- Katayama-Fujimura, Y. S., S. Gottesman, and M. R. Maurizi. 1987. A multiple-component, ATP-dependent protease from *Escherichia coli*. *J. Biol. Chem.* **262**:4477–4485.
- Kessel, M., M. R. Maurizi, B. Kim, E. Kocsis, B. L. Trus, S. K. Singh, and A. C. Steven. 1995. Homology in structural organization between *E. coli* ClpAP protease and the eukaryotic 26S proteasome. *J. Mol. Biol.* **250**:587–594.
- La Teana, A., A. Brandi, M. Falconi, R. Spurion, C. Pon, and C. O. Gualerzi. 1991. Identification of a cold shock transcriptional enhancer of the *Escherichia coli* gene encoding nucleoid protein H-NS. *Proc. Natl. Acad. Sci. USA* **88**:10907–10911.
- Leonhardt, S. A., K. Fearon, P. N. Danese, and T. L. Mason. 1993. Hsp78 encodes a yeast mitochondrial heat shock protein in the Clp family of ATP-dependent proteases. *Mol. Cell. Biol.* **13**:6304–6313.
- Lindquist, S., and G. Kim. 1996. Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. *Proc. Natl. Acad. Sci. USA* **93**:5301–5306.
- Myers, J., J. R. Graham, and R. T. Wang. 1980. Light harvesting in *Anacystis nidulans* studied in pigment mutants. *Plant Physiol. (Bethesda)* **66**:1144–1149.
- Nishida, I., and N. Murata. 1996. Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**:541–568.
- Park, S. K., K. I. Kim, K. M. Woo, J. H. Seol, K. Tanaka, A. Ichihara, D. B. Ha, and C. H. Chung. 1993. Site-directed mutagenesis of the dual translational initiation sites of the *clpB* gene in *Escherichia coli* and characterization of its gene product. *J. Biol. Chem.* **268**:20170–20174.
- Parsell, D. A., Y. Sanchez, J. D. Stitzel, and S. Lindquist. 1991. Hsp104 is a highly conserved protein with two essential nucleotide-binding sites. *Nature* **353**:270–273.
- Parsell, D. A., and S. Lindquist. 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27**:437–496.
- Parsell, D. A., J. Taulien, and S. Lindquist. 1993. The role of heat-shock proteins in thermotolerance. *Philos. Trans. R. Soc. Lond. Ser. B* **339**:279–286.
- Parsell, D. A., A. S. Kowal, M. A. Singer, and S. Lindquist. 1994. Protein disaggregation mediated by heat-shock protein Hsp104. *Nature* **372**:475–478.
- Porankiewicz, J. et al. Submitted for publication.
- Porankiewicz, J., and A. K. Clarke. Unpublished data.
- Ray, M. K., T. Sitaramamma, S. Ghandhi, and S. Shivaji. 1994. Occurrence and expression of *cspA*, a cold shock gene, in Antarctic psychrotrophic bacteria. *FEMS Microbiol. Lett.* **116**:55–60.
- Riggs, P. 1990. Expression and purification of maltose-binding protein fusions. In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. Greene Publishing Associates Inc, New York, N.Y.
- Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **111**:1–61.
- Sanchez, Y., and S. L. Lindquist. 1990. HSP104 required for induced thermotolerance. *Science* **248**:1112–1115.
- Sanchez, Y., J. Taulien, K. A. Borkovich, and S. L. Lindquist. 1992. HSP104 is required for tolerance to many forms of stress. *EMBO J.* **11**:2357–2364.
- Sato, N., and N. Murata. 1981. Studies on the temperature shift-induced desaturation of fatty acids in monogalactosyl diacylglycerol in the blue-green alga (cyanobacterium), *Anabaena variabilis*. *Plant Cell Physiol.* **22**:1043–1050.
- Schirmer, E. C., J. R. Glover, M. A. Singer, and S. Lindquist. 1996. HSP100/Clp proteins: a common mechanism explains diverse functions. *Trends Biol. Sci.* **21**:289–296.
- Schmitt, M., W. Neupert, and T. Langer. 1995. Hsp78, a Clp homologue within mitochondria, can substitute for chaperone functions of mt-hsp70. *EMBO J.* **14**:3434–3444.
- Siva, V., K. Rao, J. J. Brand, and J. Myers. 1977. Cold shock syndrome in *Anacystis nidulans*. *Plant Physiol. (Bethesda)* **59**:965–969.
- Squires, C., and C. L. Squires. 1992. The Clp proteins: proteolysis regulators or molecular chaperones? *J. Bacteriol.* **174**:1081–1085.
- Squires, C. L., S. Petersen, S. Ross, and C. Squires. 1991. ClpB is the *Escherichia coli* heat shock protein F84.1. *J. Bacteriol.* **173**:4254–4262.

39. **Thompson, M. W., and M. R. Maurizi.** 1994. Activity and specificity of *Escherichia coli* ClpAP protease in cleaving model peptide substrates. *J. Biol. Chem.* **269**:18201–18208.
40. **Van Bogelen, R., and F. C. Neidhardt.** 1990. Ribosomes as sensors of heat and cold shock in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **87**:5589–5593.
41. **Völker, U., S. Engelmann, B. Maul, S. Riethdorf, A. Völker, R. Schmid, H. Mach, and M. Hecker.** 1994. Analysis of the induction of general stress proteins of *Bacillus subtilis*. *Microbiology* **140**:741–752.
42. **Wickner, S., S. Gottesman, D. Skowrya, J. Hoskins, K. McKenney, and M. R. Maurizi.** 1994. A molecular chaperone, ClpA, functions like DnaK and DnaJ. *Proc. Natl. Acad. Sci. USA* **91**:12218–12222.
43. **Willimsky, G., H. Bang, G. Fischer, and M. A. Marahiel.** 1992. Characterization of *cspB*, a *Bacillus subtilis* inducible cold shock gene affecting cell viability at low temperatures. *J. Bacteriol.* **174**:6326–6335.