ClpB in a Cyanobacterium: Predicted Structure, Phylogenetic Relationships, and Regulation by Light and Temperature

MARTINA CELERIN,‡*, ANDREA A. GILPIN,† NICHOLAS J. SCHISLER,‡ ALEXANDER G. IVANOV,§ EWA MISKIEWICZ, MARIANNA KROL, AND DAVID E. LAUDENBACH

Department of Plant Sciences, University of Western Ontario, London, Ontario, Canada N6A 5B7

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The Clp (caseinolytic protease) system was first identified as a heat shock-inducible, multicomponent, ATP-dependent protease complex able to hydrolyze casein (24, 28; for a review, see reference 35). Subsequent studies showed that the Clp system can hydrolyze numerous other proteins and peptides in both aggregated and nonaggregated forms (31). The Clp system members include three nonhomologous gene families: clpAB, ClpC, ClpX, and ClpY. Small quantities of a 4.5-kb p-clpB transcript and 110-kDa cytosolic p-ClpB protein were detected in cells grown under optimal conditions; however, increases in the quantities of the transcript and protein were observed in cells grown under excess light and low temperature conditions. Finally, we analyzed ClpA, ClpB, and ClpC sequences from 27 organisms in order to predict phylogenetic relationships among the homologs. We have used this information, along with an identity alignment, to redefine the Clp subfamilies.

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liquid cultures were maintained at 15°C with either 150 or 300 μmol of photons m⁻² s⁻¹. Periodic dilution with fresh growth medium ensured that cells were harvested in their exponential growth phase.

Growth was measured as an increase in chlorophyll a concentration. Aliquots of cell-free glass microsomes (934-AL; Whatman, Maidstone, United Kingdom) and transferred to 2-ml screw-cap tubes containing 0.1-mm zirconium oxide beads and 1.5 mL of 90% acetone. Cells were broken with a Mini-Beadbeater (BioSpec Products, Bartlesville, Okla.) for 60 s and subsequently centrifuged at 12,000 × g for 5 min. The amount of chlorophyll a in the supernatant was determined by the equations of Jeffrey and Humphrey (26). The specific constant rate (μ), which represents the increase in biomass per unit time, was calculated by the equation \( \Delta Chl \sim \mu t \), where \( \Delta Chl \) is the biomass concentration (μg Chl per mL) and \( t \) is time. The doubling time, \( t_D \), was determined by the equation \( t_D = 0.693/\mu \).

Analysis of pigment composition. Pigments were extracted with 100% acetone at 4°C under dim light. The supernatant was filtered through a 0.22-μm pore-size syringe filter, and samples were stored at −80°C. Pigments were separated and quantified by high-performance liquid chromatography (HPLC) as described by Ivanov et al. (25), with some modifications. The system consisted of a Beckman System Gold programmable solvent module 126, diode array detector module 168 (Beckman Instruments, San Ramon, Calif.), and C8-spherisorb ODS-1 reverse-phase column (5-μm particle size; inside diameter, 25 by 0.46 cm) with an Upchurch Perisorb A guard column (both columns from Chromatographic Specialties, Inc., Concord, Ontario, Canada). Samples were injected with a Beckman 200S pump system equipped with a 200S loop. Pigments were eluted isocratically for 6 min with a solvent system (acetonitrile–methanol–0.1 M Tris-acetate, pH 8.0) [72:83:5; vol/vol/vol], followed by a 2-min linear gradient to 100% methanol–hexane [75:25 [vol/vol]], which continued isocratically for 4 min. Total run time was 12 min. Flow rate was 2 mL/min. Absorbance was detected at 440 nm, and peak areas were integrated by Beckman System Gold software.

Retention times and response factors of chlorophyll a and b-carotene were determined by injection of known amounts of pure standards purchased from Sigma (St. Louis, Mo.). The retention times for chlorophyll a and b-carotene, which was tentatively identified as myoxanthophyll, were determined by using pigments purified by thin-layer chromatography (13). To determine the concentration of the putative myoxanthophyll, the response factor was calculated by a specific extinction coefficient of 2,160 at 478 nm (10).

PCR amplification and Southern blots. Two degenerate 20-mer oligonucleotides (oligo-I and oligo-II) were used to amplify DNA from the genome of P. boryanum, as described elsewhere (9). At the end of the PCR product were made blunt (T4 polymerase; Pharmacia, Mississauga, Ontario, Canada) and were made blunt (T4 polymerase; Pharmacia, Mississauga, Ontario, Canada) and were ligated into pUC18 plasmids that had been linearized with SmalI. Single-stranded constructs were isolated for sequence analysis, and double-stranded inserts were purified (QIAprep 8 Plasmid kits; Qiagen, Santa Clarita, Calif.) for use as probes. The PCR was radiolabeled with the random primer method with T7 polymerase (QuickPrime kit; Pharmacia) and [³²P]dCTP (NEN; >3,000 Ci mmol⁻¹). Southern blots were performed as described elsewhere (9).

Cloning of the cPb gene. Genomic DNA fragments ranging from 8.7 to 8.9 kb (generated by digestion of HindIII) were ligated into pBR322 from an agarose gel and subsequently ligated and ligated into pUC18 plasmids that had been linearized with HindIII and dephosphorylated. The heterologous plasmid constructs were electroporated into Escherichia coli DH10B competent cells. Cells harboring a plasmid construct were selected by plating on LB agar-Luria plates supplemented with ampicillin (LBA; 50 μg ml⁻¹), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 30 μg ml⁻¹), and isopropyl-β-D-thiogalactopyranoside (IPTG; 50 μg ml⁻¹) (53). Colonies were picked in sets of 200 and amplified (1 ml of Terrific Broth medium [53] and 50 μg of ampicillin ml⁻¹). Plasmids were isolated by the boiling miniprep method (53) and screened on Southern blots for their ability to hybridize with a radiolabeled probe, the cloned Sun8A1I fragment of the PCR product. Clones were verified by the converse experiments: inserts of positive clones were radiolabeled and used to screen blots of P. boryanum genomic DNA, which was digested with each of the restriction enzymes BglII, HindIII, and EcoRI. A restriction map of one of the positive clones, mPCX8, was created by single- and double enzyme digests. A Southern blot of the digests was probed with the cloned Sun8A1I fragment of the PCR product, and the smallest positive band, a 1.7-kb ClaI-AccI fragment, was subcloned into pUC18. In addition, the 1.4-kb ClaI-ClaI DNA fragment, proximal to the 1.7-kb ClaI-AccI DNA fragment in a restriction map of the 8.8-kb HindIII clone, was subcloned into pUC18. Inserts were released from vectors with EcoRI and HindIII, radiolabeled by random priming, and used as probes in Southern blots of total P. boryanum genomic DNA.

Sequencing. DNA sequencing with both single-stranded M13- and plasmid-based vectors was performed by the dye-terminator method using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif.) and using the program BLAST (12) to identify similar sequences. Cellular localization was predicted with the program PSORT, version 6.3 (42).

Alignments of Cpb amino acid sequences were carried out with the ClustalW program, version 1.7.29, 65). The program Sequence Similarity Predictor (ftp://ftp.bio.indiana.edu) was used to visualize alignments (16). Maximum likelihood analyses were performed with the program Puzzle, version 3.1 (63), with the quartet-puzzling tree search algorithm, the neighbor-joining (52) tree parameter estimation, the Dayhoff model of substitution (11), and standard rate model of divergence. Maximum likelihood analyses used frequencies of puzzling steps in the quartet-puzzling tree search as a measure of confidence which is comparable to the number of bootstrap replicates (63). The program NJPlot (Manolo Gouy, University of Lyon, Lyon, France) was used to visualize phylogeny. The plastid Cpb sequence from Plasto- midae falciparum was used as the outgroup in all cases.

Percent identities (pairwise comparisons) of amino acids between ClpB homologs and Cpb space regioners were calculated with Genetics Computer Group (GCG) Gap (12). In addition, the predicted amino acid sequences of CpfA, Cpb, and CpfC were analyzed by using ClustalW (37) and pairs (5) programs designed to augur secondary structures. The Coils2 program was used with a specific extinction coefficient of 2,160 at 478 nm (10).

RESULTS

Growth rates and culture morphology. Under optimal growth conditions (29°C/150 μmol of photons m⁻² s⁻¹), cells...
of *P. boryanum* have a doubling time of 8.4 ± 0.06 h. Conversely, cells of *P. boryanum* grown under ELLT conditions (15°C/150 mmol of photons m⁻² s⁻¹) have a doubling time of 55.74 ± 0.73 h.

Macroscopically, cultures of *P. boryanum* grown under optimal light and temperature conditions exhibited the blue-green hue typical of cyanobacteria (Fig. 1A). In contrast, cultures of *P. boryanum* grown under ELLT conditions showed a marked change in color (Fig. 1B); distinctively, the culture was tinged with an orange-red appearance. Analysis of the HPLC-separated cell pigments (Table 1) showed that cells grown under ELLT conditions (Fig. 1B) had altered pigment compositions compared to cells grown under optimal conditions (Fig. 1A). The quantity of the carotenoids (Table 1) increased differentially; myxoxanthophyll and zeaxanthin showed approximately five- and twofold increases, respectively, whereas the amount of β-carotene showed no appreciable increase. Carotenoids have the ability to dissipate excess energy generated under excess light conditions and thereby provide protection to the photosensitive cell components. This phenomenon has been well-studied in plants (59), and, as expected, we have found that the cellular quantities of these pigments also increase in *P. boryanum*. Additional environmental stresses, such as low temperature, exacerbate the decreased phototimization. Britten (7) and others have shown that, in plants, the amount of carotenoids increases differentially; myxoxanthophyll and zeaxanthin showed approximately five- and twofold increases, respectively, whereas the amount of β-carotene showed no appreciable increase. Carotenoids have the ability to dissipate excess energy generated under excess light conditions and thereby provide protection to the photosensitive cell components. This phenomenon has been well-studied in plants (59), and, as expected, we have found that the cellular quantities of these pigments also increase in *P. boryanum*. Additional environmental stresses, such as low temperature, exacerbate the decreased phototimization.

![FIG. 1. Cultures of *P. boryanum* grown under optimal (A) and ELLT (B) conditions.](Image)

**FIG. 2.** Southern blot analysis of the *clpB* gene in *P. boryanum*. In all panels, genomic DNA was digested with *Bgl* (B), *EcoR* (E), and *Hind* (H). The blot was probed with the *Sau* fragment of the PCR product (A), the 8.8- and 8.8-kb *HindIII* genomic DNA fragment (B), and the 1.7- and 1.4-kb *Clal*-Clal subfragments (C and D, respectively). The hybridization pattern of *clpB* is indicated by the size markers 4.9 (*Bgl*), 4.1 (*EcoR*), and 8.8 (*HindII*) kb. (The stronger bands, i.e., 8.1 (*Bgl*), 5.6 (*EcoR*), and 4.5 (*HindIII*) kb, are the hybridization pattern for *p-KLC* [9].)

**TABLE 1.** Carotenoid composition of *P. boryanum* cells grown at 29 and 15°C

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Result under the following growth conditions ( \mu \text{mol m}^{-2} \text{s}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optimal (29°C/150)</td>
</tr>
<tr>
<td>Myxoxanthophyll</td>
<td>0.101 ± 0.015</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>0.035 ± 0.007</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.249 ± 0.009</td>
</tr>
</tbody>
</table>

*a* Results are micromolar concentrations per micromolar chlorophyll. Mean values ± standard errors of the means were calculated from three to four independent experiments with three separate measurements in each experiment.
and 622 to 629, as well as two chaperonin ClpAB signature motifs at aa 307 to 319 and 648 to 666.

The DNA sequence of the ORF was compared to all sequences in the GenBank database (NCBI, Bethesda, Md.) with the BLAST program (12). Overwhelming identity to the ClpB family of ATP-dependent proteins was observed, although similarities to the ClpC and ClpX families were also noted. Figure 3 shows a representation of percent sequence identity across the multiple sequence alignment profile (alignment not shown) of the predicted P. boryanum ClpB to the other members of the ClpABC family. Several regions showed high sequence similarity, especially near and including the nucleotide binding sites. The N1 and N2 regions, the regions of highest similarity, are indicated. ClpB from P. boryanum was typical of other cyanobacterial and bacterial ClpB sequences.

Another feature of note in p-ClpB is the presence of a putative phosphorylation site for casein kinase II (CK2 [49] [S-X-X-E-X-X-E]) adjacent to Serine-420. The putative phosphorylation site, which is adjacent to the N1 domain, is a highly conserved feature of all ClpB sequences (Fig. 4) but is absent from all ClpA, ClpC, ClpX, and ClpY sequences examined. Surprisingly, in other unrelated proteins that have a site for phosphorylation adjacent to a predicted coiled-coil domain, the phosphorylation status is involved in regulating the three-dimensional (3D) conformation of the domain (21); we suspect that stabilization or regulation of the predicted
coiled-coil structures in ClpB (discussed below) may occur by phosphorylation of the conserved serines.

Additional differences among the Clp sequences that are noteworthy include the following: (i) the clpB sequences from Mycoplasma are unusual in that they lack a leader region; (ii) ClpB from \textit{S. cerevisiae} mitochondria has a truncated leader region, the amino acid sequence preceding the N1 domain; (iii) plants appear to have an extended trailer region; and (iv) \textit{Plasmodium berghei} contains a longer spacer region that upon further analysis is predicted to form three coiled coils separated by 20 instead of 3 aa (data not shown), as is the case in all other ClpB spacers (described below).

Coiled-coil region predicted in the spacer of other ClpB genes. The spacer region, a stretch of amino acids that separates the N1 and N2 regions, has been used to delineate relationships among the Clp homologs (19, 61). Squires and Squires (61) assign Clp proteins into subfamilies based on the size of the spacer region. ClpA family members have spacer regions composed of 5 aa, ClpC members have 62 to 69 aa, and ClpB members have the longest spacers (123 to 131 aa). The spacer region comprises 13.5% by number of amino acids (14.6% by weight) of the total predicted polypeptide in p-ClpB. The programs Coils2 (37) and paircoils (5) predicted that the coiled-coil is composed of coiled coils (Fig. 5 and http://mold.bio.indiana.edu/mcelerin/index.htm). Also, Coils2 was able to resolve the domain into four discrete helical regions. Each predicted helical region contains four sets of heptad repeats, a-b-c-d-e-f-g, where a and d are often hydrophobic residues, and b, c, e, f, and g are hydrophilic and form the solvent-exposed part of the helical surface (37). The sets of four are separated by three residues that are not part of a heptad repeat and thus are not part of the helical structure. Presumably, the three interhelical residues cause local instability of the coiled coil that could result in bends or hinges in the 3D structure, similar to those for the skip residues described elsewhere (45) for myosin. Of the nine residues that comprise the three sets of 3-aa interhelical hinges, seven are defined by Bhaskaran and Ponnuswamy (6) as highly or exceptionally flexible amino acids based on their computed flexibility indices. Additionally, the calculated total bond length of the 3 aa (10.1 Å) would permit a 90° turn in the direction of the helix in a manner similar to that of the 4 aa involved in the turn of helix-turn-helix motifs.

Because of the novel prediction that coiled coils are present in the spacer region of p-ClpB, we pursued an analysis of available ClpB sequences from the NCBI database and, using Coils2 (37), we have found that all ClpB spacer regions are predicted to form coiled-coil domains (http://mold.bio.indiana.edu/mcelerin/index.htm). Table 2 shows that although the overall identity among pairwise comparisons of the ClpB sequences from diverse organisms is high (mean, 51.1%; standard deviation [SD], 7.9; n = 78; shaded areas), the percent identity of pairwise comparisons within the predicted coiled-coil region is surprisingly low (mean, 36.7%; SD = 11.3; n = 78; white areas), given the amount of conservation at the secondary structural level. In all but two pairwise comparisons (Glycine max with Arabidopsis thaliana and Leishmania major with \textit{Trypanosoma brucei}), the spacer region shows less percent identity between sequences than the overall sequence percent identity. This indicates that the predicted 3D structure of the domain, but not the sequence per se, is conserved.

Phylogenetic analysis of ClpABC. Members of the ClpAB CXY family in eubacteria and in eukaryotes, including plants, fungi, and protists, have been identified. The putative relationship of p-ClpB to other ClpA, ClpB, and ClpC sequences that contain both the N1 and N2 domains is shown in Fig. 3. Since ClpX and ClpY lack the leader region, spacer, and first nucleotide-binding domain characteristic of ClpABC sequences, they were excluded from phylogenetic analysis.

Figure 6 shows the deduced phylogenies of ClpABC as calculated from maximum likelihood analyses of amino acid sequences. Distance and maximum parsimony trees are available at http://mold.bio.indiana.edu/mcelerin/index.htm. With the exception of the \textit{Plasmodium} sequence, ClpB sequences are well-resolved into bacterial and eukaryotic clades, with \textit{P. bo-ryanum} ClpB appearing most closely related to \textit{Synochococcus} sp. and other cyanobacterial ClpB polypeptides.

The plant ClpA polypeptides appear to be more closely related to ClpC than to ClpA sequences (Fig. 3). ClpA, in general, is not particularly well-resolved, possibly due to the small number of taxa available for analysis (although it appears distinct from ClpB and ClpC by distance-based methods, albeit with low bootstrap support). Since (i) the ClpC sequences in the phylogenetic tree are predicted to be monophyletic and (ii) analysis of the amino acid alignment (described above) suggests that the spacer region, which is used to delineate Clp homologs, is absent from the ClpA plant sequences, we suggest that ClpA plant polypeptides are, in fact, members of the ClpC subfamily.
Expression of p-clpB. A p-clpB transcript of approximately 4.5 kb was identified by Northern blot analysis of total RNA from *P. boryanum* cells grown under optimal (29°C/50 μmol of photons m⁻² s⁻¹) conditions (Fig. 7A, lane 1b, large arrowhead). Total RNA collected from cells grown under ELLT (15°C/300 μmol of photons m⁻² s⁻¹) conditions also contained the 4.5-kb p-clpB transcript (Fig. 7A, lane 2b); however, the quantity of transcript was fivefold more abundant in the ELLT-grown cells. In addition to the predominant 4.5-kb transcript, two larger transcripts were detected from cells grown under ELLT conditions (Fig. 7A, small arrowheads). These may represent stages of processing of a polycistronic message that contains the p-clpB transcript. Additionally, a large quantity of RNA is detected through the length of the lane (Fig. 7A, lane 2b). Based on the integrity of the rRNA bands (Fig. 7A, lane 2a), we presume that the total RNA is not degraded; the smear of RNA may be reflective of the instability of the p-clpB transcript.

We addressed the question of localization of p-ClpB using immunodetection. Differential sedimentation was used to separate total soluble proteins from cell wall components, plasma, and thylakoid membranes. Figure 7BII (lane 2) shows that a single protein band of approximately 110 kDa was detected in the total soluble protein fraction from cells grown under optimal conditions. The apparent molecular mass of the protein is slightly higher than the predicted molecular mass of the p-ClpB polypeptide (100,513 Da), possibly a result of poor resolution of large proteins by SDS-PAGE. The 110-kDa band was not detected in any other cell fraction (data not shown), a finding in agreement with the results of the analysis by PSORT (42), which predicted, based on sequence features related to protein sorting signals, that p-ClpB would localize to the cytoplasmic fraction of the cell components. Furthermore, a single band of the same molecular mass but of greater intensity, which indicated that an increased quantity of the protein was present, was detected only in the cytosolic fraction of cells grown under ELLT conditions (Fig. 7BIII, lane 1). This is consistent with results of the Northern blot analysis and supports the idea that p-clpB encodes a single-copy gene that is transcribed and translated to make a single protein product. The results also indicate that a certain quantity of transcript and protein is present in cells growing under optimal conditions but that the quantities of both increase during growth under stressful conditions such as ELLT. It is interesting to note that although the results of the Northern blot suggest that there is a fivefold induction of the p-clpB transcript after ELLT treatment, there is not the same increase in the amount of protein present after ELLT treatment. Thus, the data suggest that ELLT growth conditions may induce more transcription of clpB but not more synthesis of the protein.

**DISCUSSION**

We have cloned and sequenced the p-clpB gene from *P. boryanum*. Based on available sequence data, p-ClpB has 77.3% identity with its most closely related subfamily member, ClpB from *Synechococcus* sp.; it has 44.2% identity with the most distantly related ClpB sequence (hsp104; *S. cerevisiae*, cytosolic). The predicted protein has a number of features which enable us to suggest that ClpB from *P. boryanum* is a paralog of ClpA, ClpC, ClpX, and ClpY and an ortholog of ClpB. All ClpABCXY family members, which can show variations in size (61), possess at least one ATP-binding motif (66) and one or both conserved N1 and N2 domains. Like other ClpB subfamily members, p-ClpB contains (i) both the N1 and the N2 domains, each of which contain an ATP-binding motif (66); (ii) leader and trailer sequence; (iii) a central spacer region, which we have predicted forms a coiled-coil domain; and (iv) a newly identified, highly conserved putative site for phosphorylation adjacent to the predicted coiled-coil domain.

**TABLE 2.** Pairwise comparison of the percent identities of the spacer region (white areas) and the entire amino acid sequences of ClpBs (shaded areas)

<table>
<thead>
<tr>
<th>Species</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. boryanum</em></td>
<td>77.3</td>
</tr>
<tr>
<td><em>Synechococcus</em> sp.</td>
<td>71.2</td>
</tr>
<tr>
<td><em>Corynebacterium glutamicum</em></td>
<td>45.9</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>51.4</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>40.8</td>
</tr>
<tr>
<td><em>Bacteroides nodosus</em></td>
<td>36.9</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>34.2</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>33.3</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> (mt)</td>
<td>28.3</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> (cytosolic)</td>
<td>27.5</td>
</tr>
<tr>
<td><em>Leishmania major</em></td>
<td>28.8</td>
</tr>
<tr>
<td><em>Trypanosoma brucei</em></td>
<td>28.2</td>
</tr>
<tr>
<td><em>Plasmodium berghei</em></td>
<td>34</td>
</tr>
</tbody>
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**5178 CELERIN ET AL. J. BACTERIOL.**

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The phylogenetic analysis in the present study supports previous suggestions that the ClpA, ClpB, and ClpC subfamilies are three separate yet evolutionarily related groups of polypeptides. However, our analysis of the sequence data shows that previously used nomenclature may not express accurately the evolutionary history of the proteins. We have redefined the subfamily groupings in what seems to be a more credible reflection of their amino acid sequence identities.

We suspect that the conserved recognition region for phosphorylation may be involved in regulating conformation of ClpB. In some coiled-coil proteins, such as lamins, the phosphorylation states of the serine residues that are immediately adjacent to the coiled-coil domains are pivotal in regulating the conformation (21). Although ClpB is known to form homotetramers, we suspect that the coiled-coil domain is involved in heteropolymerization. ClpA and ClpB are similar over many regions (reviewed in references 57 and 61), with the notable exception of the predicted coiled-coil domain. Moreover, homopolymerization occurs in both ClpA and ClpB; by inference, the coiled-coil domain is unlikely to be involved in this process.

Based on the difference between ClpB and either the ClpA or the ClpC protein, namely, the presence or absence of the
Predicted coiled-coil region, it is tantalizing to speculate that the lack of this domain may permit protein-protein interactions with ClpP. It would be interesting to determine if, by removing the coiled-coil domain in ClpB, one could construct a truncated ClpB that may be able to interact with ClpP.

It is well-established that heat shock proteins (hsp) are in fact stress proteins. In vivo, the occurrence of heat shock is rare; heat stress is usually a gradual shift to an extreme condition. Therefore, other stresses such as exposure to heavy metals, excess salt, low temperature, and, in photosensitive organisms, excess light are more prevalent. Differential expression of stress proteins is contingent on the type of stress (see reference 35 and the organism (48), but the hsp70 that tend to be induced most consistently include hsp70-DnaK, hsp60-GroEL, and the hsp100-Clp complex members (3). ClpBs have central roles in ameliorating environmental stresses in addition to heat shock. Sanchez et al. (55) showed that the ClpB homolog of S. cerevisiae (hsp104) is responsible for tolerance not only of heat but also of ethanol, arsenite, and long-term exposure to cold. Clarke and coworkers (14, 50) have demonstrated that in Synechococcus, ClpB is responsible for sustained thermotolerance at high temperatures and contributes to acclimation at moderately low (25°C) temperatures. However, Porankiewicz and Clarke (50) also report that the level of ClpB found in cells decreases with immoderately cold (15°C) conditions. It is important to note that in the latter study, all cells were grown at 50 μmol of photons m⁻² s⁻¹; thus, the effect of increased light intensity in combination with low temperatures was not examined.

Although all aerobic organisms must contend with reactive oxygen species, the by-products of respiration that result from the incomplete reduction of molecular oxygen, photosynthetic organisms have the added vicissitude of addressing a second potential source of oxidative damage. The excitation of chlorophyll results in the singlet excited state, which eventually can lead to the production of singlet oxygen (51). Prolonged exposure of photosynthetic organisms to a combination of light intensities and temperature such that the excitation energy exceeds the capacity for dissipation can result in the production of additional singlet oxygen. Oxidative damage can be manifested as lipid peroxidation, protein oxidation, and DNA damage, all of which potentially hamper normal cellular activities. Numerous proteins, including stress proteins, must function to compensate for these severe environmental conditions in order to permit normal cellular metabolism and growth.

Under optimal growth conditions, either very low levels of the clpB transcript are detected (P. boryanum [present study] and Leishmania major [22]) or no clpB is observed (S. cerevisiae [54], Synechococcus sp. [14], and Glycine max [33]). Remarkably, the quantity of clpB transcript present in P. boryanum increases in cells exposed to excessive light (as exacerbated by low temperatures [present study]) and sulfur limitation (8a). At least in P. boryanum, both of these two stresses can induce SODs (8) and, thus, presumably cause oxidative damage (directly or indirectly). Porankiewicz and Clarke (50) showed that Synechococcus sp. cells grown at 15°C (50 μmol of photons m⁻² s⁻¹) showed no induction of ClpB. They suggested that the lack of induction may be due to the retardation of protein synthesis during growth under immoderately cold conditions. In this light, the comparatively small (fivefold) induction observed in P. boryanum cells grown at 15°C with 300 μmol of photons m⁻² s⁻¹ is even more striking. Clearly, under these adverse conditions, cells must be expending a considerable amount of energy to produce this protein, and thus the end product, ClpB, must have a critical role in cell survival. Alternatively, the differences in induction of ClpB may simply be due to the fact that although both are cyanobacteria, P. boryanum and Synechococcus sp. are two different species, and thus each may employ disparate strategies for coping with stresses.

ClpB is a stress-induced protein, but it is not a protease. Parsell et al. (47) suggested that ClpB may function by controlling the aggregation or denaturation of vital cellular structures rather than by operating as a proteolytic regulator. Others (48, 61, 71) have suggested that ClpB may function as a molecular chaperone, like hsp70-DnaK or hsp60-GroEL. Consequently, ClpB may either prevent the formation of protein aggregates, catalyze ATP-dependent refolding, or reassemble unfolded proteins in stressed cells. Squires and Squires (61) also suggested that members of the ClpB family might be involved in controlling some enzymatic activities other than proteolysis. Nonetheless, a clear answer to the question “What does ClpB do?” remains elusive.

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