The Ssl2245-Sll1130 Toxin-Antitoxin System Mediates Heat-induced Programmed Cell Death in Synechocystis sp. PCC6803*‡§¶

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Two putative heat-responsive genes, ssl2245 and sll1130, constitute an operon that also has characteristics of a toxin-antitoxin system, thus joining several enigmatic features. Closely related orthologs of Ssl2245 and Sll1130 exist in widely different bacteria, which thrive under environments with large fluctuations in temperature and salinity, among which some are thermo-epithelial biofilm-forming cyanobacteria. Transcriptome analyses revealed that the clustered regularly interspaced short palindromic repeats (CRISPR) genes as well as several hypothetical genes were commonly up-regulated in Δssl2245 and Δsll1130 mutants. Genes coding for heat shock proteins and pilins were also induced in Δsll1130. We observed that the majority of cells in a Δsll1130 mutant strain remained unicellular and viable after prolonged incubation at high temperature (50 °C). In contrast, the wild type formed large cell clumps of dead and live cells, indicating the attempt to form biofilms under harsh conditions. Furthermore, we observed that Sll1130 is a heat-stable ribonuclease whose activity was inhibited by Ssl2245 at optimal temperatures but not at high temperatures. In addition, we demonstrated that Ssl2245 is physically associated with Sll1130 by electrostatic interactions, thereby inhibiting its activity at optimal growth temperature. This association is lost upon exposure to heat, leaving Sll1130 to exhibit its ribonuclease activity. Thus, the activation of Sll1130 leads to the degradation of cellular RNA and thereby heat-induced programmed cell death that in turn supports the formation of a more resistant biofilm for the surviving cells. We suggest to designate Ssl2245 and Sll1130 as MazE and MazF, respectively.

Microorganisms are constantly exposed to various kinds of biotic and abiotic environmental challenges that can threaten their very survival. Therefore, various acclimative mechanisms have evolved to cope with such unfavorable conditions. These mechanisms make them tolerant to the stress, letting them survive until the return of favorable conditions. One such response is the ability to alternate from unicellular to multicellular organization like microbial colonies, aggregates, and biofilms (1, 2). In such microbial colonies, a subpopulation of cells can be genetically determined to undergo programmed cell death (PCD).4 Thus, a bacterial population, upon being subjected to stress, utilizes PCD to sacrifice a portion of their population for survival of the remaining cells (3). Thus, based on the various strategies reviewed so far, it can be hypothesized that by utilizing the essential nutrients from the cells that are programmed to die, the surviving cells (persisters) are sustained until the return of favorable conditions after which they can resume reproduction and replenish their population (4–6). Autocatalytic PCD has been reported in certain cyanobacteria with possible implications for the export of carbon and nitrogen (7, 8), and metacaspases appear to be mechanistically involved in PCD in the marine cyanobacterium Trichodesmium (9) of which many molecular details are unknown.

Bacterial PCD is frequently mediated by specialized genetic systems known as the toxin-antitoxin (TA) modules (10). There are at least six major types of TA systems from which type II TA systems are the best characterized (11). The TA gene pair consists of a stable toxin and a labile antitoxin. The toxic properties of the toxin are masked by the antitoxin either by binding to it or by inhibiting its translation (12). In Escherichia coli, some of the well characterized TA systems are mazEF (13, 14), relBE (15, 16), chpAB (17), and yefM-yoeB (18). These systems are found on plasmid as well as chromosomal loci but were first identified in E. coli on low copy number plasmids (19). Several TA systems have been reported to trigger PCD under various conditions like amino acid starvation, exposure to antibiotics, plasmid segregation during cell division, phage infection, and high temperature (13, 15, 19–22).

The abbreviations used are: PCD, programmed cell death; TA, toxin-antitoxin; CRISPR, clustered regularly interspaced short palindromic repeats; ITC, isothermal titration calorimetry; qRT-PCR, quantitative RT-PCR; AFM, atomic force microscopy; DSC, differential scanning calorimetry; Sp, spectinomycin gene cassette.

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‡ This article contains supplemental Figs. S1–S4 and Tables S1 and S2.

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A well known example is the CcdAB TA system that triggers PCD during bacterial cell division. The purpose of PCD in this case is to ensure plasmid partitioning during cell division (22). When the daughter cell fails to receive the plasmid harboring the TA operon, it is selectively killed by the toxin as the labile antitoxin, unable to replenish itself, is degraded faster. The toxin is released from the TA complex and targets DNA gyrase, triggering cell death (19, 22). Another TA system, \textit{relBE}, is involved in PCD during amino acid starvation. The RelB antitoxin levels fall due to a decrease in translation and Lon-dependent proteolysis, freeing the RelE toxin to inhibit translation, thereby arresting growth until the return of favorable conditions (15). Upon exposure to antibiotics, the MazEF-induced PCD is activated due to a decrease in the amount of the antitoxin MazE (and its degradation by cellular proteases), leading to an increase in levels of the free MazF toxin (5, 6, 10). Under lethal concentrations of antibiotics, a small portion of the cells can even manage to survive until they experience favorable conditions (23).

MazEF also prevents the spread of infective phages to healthy individuals by initiating PCD in the infected cell. The lysogenic phage contains a gene that encodes a repressor that lets them replicate in the host cells without entering the lytic cycle (24). Inactivation of \textit{mazEF} led to higher heat tolerance in \textit{E. coli}, suggesting a possible role in the heat stress response. However, the mechanism of heat-induced cell death has not been reported (13). MazEF TA systems are known for their autoregulation by conditional cooperativity (14), and TA systems in general may function as global regulators (25–27). A role in the regulation of several hypothetical, pilin, and heat shock genes has been reported for the MazF-type transcriptional modulator \textit{Sll1130} in the cyanobacterium \textit{Synechocystis} (28).

\textbf{Results}

**Phylogenetic Analysis**—Sll2245 and Sll1130 are encoded by two overlapping genes in the genome of \textit{Synechocystis}. Sll2245 is an unknown protein with similarity to AbrB-like transcriptional regulators. The second gene, \textit{sll1130}, codes for a putative PemK-like MazF transcriptional modulator \textit{Sll1130} in the cyanobacterium \textit{Synechocystis} (28). In this study, we identified Sll2245-Sll1130 as a pair of putative heat-responsive TA-like transcriptional regulators encoded by a dicistronic operon. We report the mechanism of heat-induced PCD mediated by this TA system as a survival strategy under high temperature.

\textbf{FIGURE 1.} Phylogenetic relationship of Ssl2245 and Sll1130 with their closely related orthologs. The complete amino acids sequences of Ssl2245 and Sll1130 were concatenated and aligned with their putative orthologs using ClustalW (45), yielding a multiple sequence alignment of 208 positions. All cyanobacterial proteins are highlighted in blue, and the here investigated \textit{Synechocystis} proteins, Ssl2245 and Sll1130, are in bold font. The archetypical \textit{E. coli} K-12 MazEF (\textit{black boldface}) and four proteins more closely related to it were also included. Phylogenetic relationships were inferred by Minimum Evolution (48) as implemented in MEGA 7.0 (49) using the pairwise deletion option. The optimal tree with the sum of branch length = 7.90605607 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches when \textit{>60}. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are given in the number of amino acid substitutions per site as indicated by the scale bar representing 0.2 substitutions per amino acid. The compared protein sequences were retrieved from the KEGG and NCBI databases.
Supplemental
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8305 isolated from a heliothermal saline pool (31) and 50 and 70.3% identity with those from Microcystis aeruginosa sp. PCC 9432, respectively (32). Because homologs of the ssl2245-sll1130 operon appear well conserved among thermophilic bacteria, it appeared likely that the two proteins might be antagonistic to each other and play a key role in heat stress response. Ssl2245 and Sll1130 Are Conserved Proteins Encoded by a Dicistronic Operon—The open reading frames (ORFs) ssl2245 and sll1130 have a four-nucleotide overlap where the stop codon of ssl2245 overlaps with the start codon of sll1130. To validate whether both belong to a single operon controlled by the same promoter, cDNA made from total RNA by reverse transcription was used as template for PCR. Amplification using the primers ssl2245-F and sll1130-R specifically annealing to ssl2245 and sll1130, respectively, gave a product of ~600 bp, which corresponds to the size of the entire operon. No amplification was obtained without the cDNA (non-template control). This result confirms co-transcription and dicistronic organization of ssl2245 and sll1130 (Fig. 2A), originating from a single transcriptional start site found in the genome-wide mapping of the primary transcriptome at position 1049139 on the reverse strand of the chromosome (33). Overlap between the stop codon of the first and the start codon of the second gene has been described in many other dicistronic TA systems (34). Such overlaps probably are required for stringent regulation of genes involved in common physiological or cellular roles. We found this as a common feature among the homologs of ssl2245-sll1130 with overlaps ranging from 4 to 26 bp (Fig. 2B). The thermo-epilithic biofilm-forming cyanobacterium Stanieria cyanosphaera has a 26-bp overlap between sta7437 and sta7437. In addition, we also observed that introduction of an extra copy of this operon into the wild type leads to a higher thermotolerance in Synechocystis. In our previous work, the DNA microarray used for gene expression profiling of Δsll1130 contained only probes for chromosomal genes; therefore no differential expression was observed in plasmid genes (28). In the current study, a new custom made chip was designed that included probes covering genes located in the genome as well as all seven Synechocystis plasmids. We analyzed the changes in gene expression in both Δssl2245 and Δsll1130. Supplemental Table S1 shows the list of genes that were significantly either

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up- or down-regulated in the Δssl2245 and Δsll1130 mutants in comparison with wild type cells. Genes whose mean induction was greater than 2.0 at least in one of the two mutants are listed as up-regulated, and those with an induction ratio below 0.3 in the Δssl2245 and Δsll1130 mutants are listed as down-regulated (supplemental Fig. S2). We observed up-regulation of several genes located on the large plasmids pSYSA, pSYSX, and pSYSM in Δssl2245 as well as Δsll1130 (supplemental Table S1). Most of the up-regulated genes on plasmid pSYSA encoded parts of the clustered regularly interspaced short palindromic repeats (CRISPR) system. CRISPR systems provide adaptive immunity against phage invasion. Plasmid pSYSA harbors three separate CRISPR-Cas systems called CRISPR1, CRISPR2, and CRISPR3 (35). These were classified as subtype I-D, subtype III-D, and subtype III-B according to Makarova et al. (36). The genes up-regulated in Δssl2245 and Δsll1130 belong to CRISPR2 (genes slr7062–slr7067) and CRISPR3 (genes slr7085–sll7090), whereas expression of the CRISPR1 cassette remained unaffected. Plasmid pSYSA also harbors several other TA systems (37). However, expression of these TA systems was not affected, similar to the unchanged expression of CRISPR1 genes. Thus, the higher expression of the pSYSA-located CRISPR2 and CRISPR3 genes in the two mutants is not a consequence of a higher pSYSA plasmid copy number but points to a specific regulatory effect. The up-regulation of CRISPR-associated genes in Δssl2245 and Δsll1130 suggests that they might function as master regulators of these plasmid-located viral defense genes. Indeed, repression of CRISPR arrays by a global transcriptional repressor, the heat-stable nucleoid-structuring (H-NS) protein, has been described in some E. coli strains (38). Alternatively, Ssl2245 and Sll1130 could control a process that leads indirectly to their activation, e.g. as part of a lifestyle change. Certain TA systems were reported previously to be involved in virus defense (24). Thus, the Ssl2245-Sll1130 system could act in a multifunctional way like E. coli MazEF, which exhibits multiple stress responses (13). The genes of the slr1788-slr1789 dicistronic operon, heat-responsive frpC, and pilin genes that were previously confirmed to be up-regulated in the Δsll1130 mutant were also commonly up-regulated (28).

qRT-PCR Analysis Confirms DNA Microarray Expression Changes—We chose the first gene of selected plasmid-localized operons, which were differentially expressed due to mutation in both ssl2245 and sll1130 for qRT-PCR validation of their expression changes as observed in the DNA microarray analysis.
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(Supplemental Table S1, ORF numbers in bold letters). These genes were sll7064, sll7067, sll7085, sll7090, slr7091, sll5097, and sll6010. In addition, we chose two chromosome-located genes, slr0909 and sll1396 (Supplemental Table S2). The gene expression changes observed by both DNA microarray and qRT-PCR analyses were consistent (Fig. 4) and indeed suggested the direct or indirect involvement of Ssl2245 and Sll1130 in the regulation of expression of CRISPR2 and CRISPR3 as well as of certain chromosomal genes.

Enhanced Formation of Pili in Δsll1130 —As we observed up-regulation of pilin genes due to mutation in Δsll1130, we compared the pilus formation in wild type and Δsll1130 cells using atomic force microscopy (AFM). Wild type and Δsll1130 cells deposited onto the mica were around 1–1.5 μm in diameter. The surface of the wild type cells appeared to have very few appendages or pili (Fig. 5A). In contrast, the Δsll1130 cells were distinctly surrounded with multiple threadlike appendages (Fig. 5B), suggesting a possible role of Sll1130 in regulating pilin expression and pilus formation in Synechocystis.

Ssl2245 and Sll1130 Interact with Each Other and Form a Stable Complex —Because Ssl2245 and Sll1130 showed similarity to TA systems of other bacteria, we addressed their possible interaction. A bacterial two-hybrid system involving two plasmids, pT25 and pT18, harboring the ORFs sll1130 and ssl2245, respectively, was utilized (39). Simultaneously, the interaction of thioredoxin B (Slr1139) with Ssl2245 as well as Sll1130 was tested because Ssl2245 was reported as a possible target of this thioredoxin (40). As expected, the DHP1 cells harboring empty plasmids, pT25 and pT18 (as a negative control) without any DNA inserts, did not change their color upon incubation of cells at 30 °C even after 48 h (Fig. 6A, a). The DHP1 cells harboring pT25-RhlB and pPNP-T18 (as a positive control) turned a dark pink color upon plating onto MacConkey agar (41) (Fig. 6A, b). We observed a dark pink color formation on plating the DHP1 cells co-transformed with pT25-Sll1130 and pSsl2245-T18, demonstrating the strong interaction between Ssl2245 and Sll1130 (Fig. 6A, c). Color change was not observed with the cells harboring pT25-Slr1139 and pSsl2245-T18 or pT25-Slr1139 and pSll1130-T18, suggesting that Slr1139 interacts with neither Ssl2245 nor Sll1130 (Fig. 6A, d and e). To verify the possible physical association between Ssl2245 and Sll1130, they were expressed together in such a way that both proteins were expressed from the same plasmid upon induction, and only one protein contained the His6 tag. Thus, only Ssl2245 was expressed with an N-terminal His6 tag (His-Ssl2245). SDS-PAGE analysis of the eluates revealed the presence of Sll1130 along with the His-tagged Ssl2245 despite lacking a purification tag (Fig. 6B, lanes 5–10). The mass spectrometric analysis showed the masses of Ssl2245 and Sll1130 to be 9.20 and 12.925 kDa, respectively. These results indicated a stable and strong physical interaction between Ssl2245 and Sll1130. Their physical association could be mainly due to electrostatic interactions as Ssl2245 is acidic with a pI of 4.2 and Sll1130 is basic with a calculated pI of 8.8.

Thermal Stability of Ssl2245 and Sll1130 Proteins —To characterize the thermal stability of Ssl2245 and Sll1130, we,...
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performed high sensitivity differential scanning calorimetry (DSC). Typical DSC thermograms of Ssl2245 and Sll1130 are shown in Fig. 7, A and B. From these thermograms, the obtained temperatures corresponding to the unfolding transition of Ssl2245 and Sll1130 proteins were 60.6 and 62.8 °C, respectively.

Temperature-dependent Association of Ssl2245 and Sll1130—Thermodynamic parameters characterizing the interaction between Ssl2245 and Sll1130 were determined by isothermal titration calorimetry (ITC) at various temperatures. Fig. 7, C and D, show typical ITC profiles for the binding of Ssl2245 to Sll1130 at 25 and 50 °C, respectively. These titration profiles indicated that injection of small aliquots of Ssl2245 into Sll1130 gives large exothermic heat of binding, which decreased in magnitude with subsequent injections, showing saturation behavior. The data obtained at all temperatures could be best fitted by a non-linear least square approach to the “one set of sites” binding model, which yielded the association constant ($K_a$), stoichiometry of binding ($n$), and the thermodynamic parameters, enthalpy of binding ($\Delta H$) and entropy of binding ($\Delta S$). These values are listed in Table 1. At 25 °C, the stoichiometry, $n$, was found to be 0.46, indicating that each molecule of Ssl2245 binds to two molecules of Sll1130; i.e. the two proteins form a complex with a 1:2 (Ssl2245:Sll1130) stoichiometry. Table 1 clearly indicates that although the binding constant $K_a$ did not change significantly between 25 and 50 °C, the stoichiometry of interaction decreased quite dramatically from 0.46 at 25 °C to 0.16 at 50 °C. Because the thermal unfolding temperature of both proteins is clearly well above 50 °C, it is unlikely that the steep decrease in the stoichiometry is due to the significant thermal inactivation of one of the proteins. Instead, it is likely that at the elevated temperature one of the two proteins undergoes some conformational changes but not complete unfolding due to which its interaction with the second protein is altered. To investigate this in more detail, we performed circular dichroism (CD) spectroscopic studies. CD spectra of Sll1130 and Ssl2245 recorded at various temperatures between 25 and 60 °C are shown in Fig. 8. The far-UV CD spectra of Sll1130 and Ssl2245 in Fig. 8, A and C, respectively, show that the secondary structure of the two proteins remains essentially unaltered between 25 and 60 °C, which is consistent with the results of DSC studies presented above. Interestingly, although the near-UV CD spectra of Ssl2245 were largely unchanged in the temperature range of 25 and 60 °C, the molar ellipticity in the corresponding spectra of Sll1130 exhibited a significant decrease at and above 40 °C. These results are consistent with the formation of a molten globule-like structure by Sll1130 at temperatures above 40 °C (42, 43). The apparent decrease in stoichiometry then suggests that only a fraction of Sll1130 (which is still in the native form) complexes with Ssl2245, whereas the fraction that is in the molten globule-like structure does not bind to it. Because Sll1130 exhibits ribonuclease activity up to 60 °C (as shown below), these observations suggest that the molten globule-like form is functionally active and acts as an RNase.

Sll1130 Is a Ribonuclease, and Its Function Is Masked by Ssl2245—In vitro synthesized partial 16S rRNA and slr1788/slr1789 mRNA substrates were incubated with purified Sll1130, purified Ssl2245, or both. Addition of Sll1130 alone resulted in the cleavage of the RNA, whereas the incubation with Ssl2245 alone had no effect on the RNA integrity. The simultaneous addition of both Sll1130 and Ssl2245 also had no effect on the RNA integrity (Fig. 9, A and B). Significantly high decay of both RNA substrates by Sll1130 was inhibited by Ssl2245. Thus, Sll1130 in its free form can degrade RNA, and this function seems to be lost while it is associated with Ssl2245. Because a decreased association of Sll1130 and Ssl2245 at high temperature using ITC analysis was demonstrated, we examined the ribonuclease activity of Sll1130 alone and in the presence of Ssl2245 at 37, 50, and 60 °C on the in vitro synthesized slr1788/slr1789 mRNA. Irrespective of the temperature of incubation, Sll1130 degraded the mRNA...
substrates efficiently, whereas Ssl2245 alone had no effect on integrity of mRNA at all tested temperatures. Degradation of RNA was observed at both 50 and 60 °C when the RNA was incubated together with Ssl2245 and Sll1130. Conformational change in Sll1130 within the Ssl2245-Sll1130 complex probably released the free molten globule-like form of Sll1130, leading to the observed mRNA cleavage (Fig. 9, C, D, and E). Consistent with this result, we found that mRNA isolated from Δsll1130 cells was relatively more stable than that from wild type cells during prolonged incubation of cells at high temperature (data not shown).

**Discussion**

*Synechocystis* contains multiple putative TA systems, but only a very few of them have been experimentally addressed thus far. The *Synechocystis* TA system initially identified was the chromosomally located *relNE* (ssr1114-slr0664) that is proteolytically regulated by ATP-dependent proteases (44). At least seven TA systems are located on the plasmid pSYSA of which only *sll7003-sll7004* has been biochemically character-
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Sll1130 possesses endoribonuclease activity that is counteracted by Ssl2245. In vitro synthesized partial Synechocystis 16S rRNA fragment (A) and slr1788-slr1789 mRNA (B) were incubated for 30 min with Sll1130 (T), Ssl2245 (AT), or both Sll1130 and Ssl2245 (T+AT), respectively. The addition of Sll1130 resulted in degradation of the RNA, which was prevented by concurrent addition of Sll1130 and Ssl2245 together. The effect of temperature on endoribonuclease activity of Sll1130 in the presence or absence of Ssl2245 was determined. In vitro synthesized slr1788-slr1789 mRNA was incubated with Sll1130, Ssl2245, or both Sll1130 and Ssl2245 at 37 (C), 50 (D), and 60 °C (E), respectively. Sll1130 degrades RNA at high temperature even in the presence of Ssl2245. NC, negative control in which RNA was incubated for 30 min in the elution buffer without any protein.

FIGURE 8. Circular dichroism spectra of Sll1130 and Ssl2245 recorded at various temperatures. Far- and near-UV CD spectra, respectively, of Sll1130 (A and B) and far- and near-UV CD spectra, respectively, of Ssl2245 (C and D) are shown. Spectra recorded at various temperatures are identified by different colors as indicated in the figure. deg, degrees.

The DNA microarray- and qRT-PCR-based gene expression profiling of mutants revealed the role of this TA system in the regulation of expression of plasmid genes (Fig. 4 and supplemental Table S1). The gene expression changes observed in both cases were found to be similar. Especially, we observed inducible expression of CRISPR-associated genes, which are pivotal for defense (35). There is a report demonstrating roles of TA systems in viral defense in E. coli (24). Our results based on the AFM imaging showed a large number of distinct pili in the Δsll1130 mutant, but the wild type cells were found to have a comparatively smoother cell membrane (Fig. 5). Hence, the Sll2245-Sll1130 TA system could be multifunctional, analogous to e.g. E. coli MazE-MazF. The detailed mechanism of regulation of plasmid genes and their role in formation of pili need to be further elucidated.

Some TA systems induce PCD under various stress conditions whereby a portion of the bacterial population is sacrificed, aiding the survival of the rest of the cells (persisters) (21). In some cases, the bacterial populations form biofilms upon induction of PCD in which the persisters reduce their metabolic activities and survive on the nutrients obtained from the surrounding dead cells until the return of favorable conditions. High temperature is one such harsh condition that can induce biofilm formation in cyanobacteria (2). Interestingly, the more closely related orthologs of these two proteins were identified in Dactylococcopsis salina from a heliothermal saline pool; Microcystis aeruginosa; Stanieria cyanosphaera, a thermo-epilithic biofilm-forming cyanobacteria; and several other colonforming cyanobacteria. M. aeruginosa shows colony size distribution in its population as an adaptive strategy to disturbances.

FIGURE 9. Sll1130 possesses endoribonuclease activity that is counteracted by Ssl2245. In vitro synthesized partial Synechocystis 16S rRNA fragment (A) and slr1788-slr1789 mRNA (B) were incubated for 30 min with Sll1130 (T), Ssl2245 (AT), or both Sll1130 and Ssl2245 (T+AT), respectively. The addition of Sll1130 resulted in degradation of the RNA, which was prevented by concurrent addition of Sll1130 and Ssl2245 together. The effect of temperature on endoribonuclease activity of Sll1130 in the presence or absence of Ssl2245 was determined. In vitro synthesized slr1788-slr1789 mRNA was incubated with Sll1130, Ssl2245, or both Sll1130 and Ssl2245 at 37 (C), 50 (D), and 60 °C (E), respectively. Sll1130 degrades RNA at high temperature even in the presence of Ssl2245. NC, negative control in which RNA was incubated for 30 min in the elution buffer without any protein.
in its surroundings and in which PCD-related orthocaspase and TA genes are physically linked (46). These examples strongly support the role of this TA system in PCD under heat and the switch between a free living planktonic unicellular lifestyle and a colonial/biofilm sessile lifestyle (29–32). We noticed clumps of the wild type cells upon incubation at high temperature, hence resembling biofilm formation, and the population seemed to undergo PCD, sacrificing a portion of the cells and letting the rest of the cells survive through the stress (Fig. 3).

For the toxin and antitoxin proteins to induce PCD, they have to separate from each other to free the toxin protein to function and kill the cells. Therefore, under heat stress, we expected the dissociation of these two proteins from each other such that the toxin Sll1130 would be free to function. This hypothesis was tested using ITC, which demonstrated that the stoichiometry of interaction decreased between Ssl2245 and Sll1130 at high temperatures (Fig. 7). Because at such high temperature most proteins are unstable, we verified the stability of the two proteins by DSC, which confirmed the thermal stability of these proteins to be quite high with unfolding temperatures above 60 °C (Fig. 7), which was well above the temperature where the decrease in association of these proteins was observed. CD spectroscopic studies revealed that Sll1130 exists predominantly in a molten globule-like structure above 40 °C, whereas the structure of Ssl2245 is essentially unaltered up to 60 °C.

One of the mechanisms by which the toxin of the TA system induces cell death is by endoribonuclease activity. Sll1130 is indeed a stable ribonuclease that degrades RNA (Fig. 9). At optimal growth temperature, Ssl2245 inhibits the ribonuclease activity of Sll1130. When cells experience high temperature, Sll1130 undergoes conformational changes, leading to the formation of the molten globule-like structure, which does not interact with Ssl2245. The free form of Sll1130 (not associated with Ssl2245) degrades cellular RNA, thus inducing PCD (Fig. 10B). Thus, we report for the first time a putative TA system involving heat-responsive antitoxin (MazE) and toxin (MazF) encoded by the ORFs ssl2245 and sll1130, respectively, which mediate heat-induced PCD in *Synechocystis*, when cells are exposed to high temperature. In conclusion, based on the experimental evidence obtained, we have designated these two proteins, Ssl2245 and Sll1130, as MazE and MazF, respectively.

**Experimental Procedures**

**Bacterial Strains and Culture Conditions—Synechocystis,** a glucose-tolerant strain that was originally obtained from Dr. J. G. K. Williams (Dupont de Nemours, Wilmington, DE) served as the wild type. Wild type cells were grown photoautotrophically at 34 °C in a specialized cyanobacterial BG11 medium buffered with 20 mM HEPES/NaOH (pH 7.5) under continuous illumination at 70 μmol of photons/m²/s as described previously (28). The Δsll1130 and Δssl2245 cultures in which the sll1130 and ssl2245 genes were disrupted by inserting a kanamycin resistance gene cassette and Ω-spectinomycin gene cassette (SpR) were grown under the same conditions as described above with the exception that the culture medium...
contained kanamycin at 25 μg/ml and spectinomycin at 20 μg/ml, respectively, in the precultures. Density of the cells in the culture was measured by optical density at 730 nm using a spectrophotometer (Thermo Scientific Nanodrop 2000c). For heat treatment, wild type culture was grown to mid-exponential phase (an absorbance of ~0.6 at 730 nm) at 34 °C and then shifted to a water bath maintained at 50 °C with continuous illumination (70 μmol of photons/m²/s). For the viability test, wild type and Δssl1130 mutant cultures were grown at 34 °C to mid-exponential phase (~0.6 OD at 730 nm) and then incubated at 50 °C for a course of time in a water bath. Cells were harvested before and during heat treatment for the viability test.

**Phylogenetic Analysis of Ssl2245 and Sll1130**—Orthologs of Ssl2245 and Sll1130 were downloaded from the Kyoto Encyclopedia of Genes and Genomes (KEGG) and NCBI databases. The amino acid sequences of Sssl2245 and Sll1130 or of the respective two homologs from other bacteria were concatenated and aligned using ClustalW (47). Phylogenetic relationships were inferred by Minimum Evolution (48) as implemented in MEGA 7.0 (49). Bootstrap values were obtained through 1,000 repetitions.

**Targeted Mutagenesis of ssl2245 (Δssl2245)**—We generated a Δssl2245 mutant by insertional inactivation of the ssl2245 gene. A DNA fragment containing the ssl2245 and sll1130 ORFs was amplified by PCR using primers ssl2245-F (5′-ATG TCT ATC AAT GCT TAC AAA CTA GCT ACG-3′) and sll1130-R (5′-GCG AAG CTT ACC GAG TTT AAA AAC ATG GGG-3′). The resulting 615-bp fragment was ligated to a linear T vector (GeNei™ INSTANT Cloning kit, catalog number 107416, Bangalore Genie Pvt. Ltd., Bangalore, India) to generate pT2245-1130. Then ssl2245 was inactivated by EcoRI digestion and blunting by T4 DNA polymerase followed by ligation with the Ω-spectinomycin cassette, yielding pTssl2245::SpR (5). This construct was used to transform wild type Synechocystis. Developing mutant colonies were re-plated on BG11 with increasing concentrations of spectinomycin and finally maintained on BG11 plates with 20 μg/ml final concentration of spectinomycin. Genomic DNA of the Δssl2245 mutant cells grown for several rounds in BG11 medium was prepared and the extent of ssl2245 replacement by ssl2245::SpR was checked by PCR using primers ssl2245-F and sll1130-R. The mutant thus generated was named Δssl2245. Segregation analysis of this mutant by comparing the size of amplified PCR products showed that ssl2245 gene copies were replaced by the disrupted version ssl2245::SpR after several generations of antibiotic selection pressure. When the genomic DNA of wild type cells was used as template, a PCR product of 615 bp corresponding to the uninterrupted ssl2245-sll1130 genes was amplified (supplemental Fig. S3 in supplemental material). In contrast, when the genomic DNA of Δssl2245 cells was used as template with the same set of primers, a 2698 bp DNA fragment was obtained, corresponding to the wild type fragment (615 bp) including the inserted Ω-spectinomycin gene cassette (2083 bp) (supplemental Fig. S3).

**Preparation of cDNA for DNA Microarray Analysis**—Fifty milliliters each of wild type, Δssl1130, and Δssl2245 cells grown at 70 μmol of photons/m²/s were killed instantaneously by the addition of 50 ml of ice-cold 5% phenol in ethanol (w/v), and then total RNA was extracted as described previously in Prakash et al. (50). The RNA was treated with DNase I (catalog number EN0521, Thermo Fisher Scientific Inc.) to remove any contaminating DNA. The cDNA was prepared from 10 μg of total RNA and labeled using FairPlay III microarray labeling kit (catalog number 252012-5, Agilent Technologies Inc., La Jolla, CA) according to the manufacturer’s instructions. For labeling cDNA, monoreactive Cy3 and Cy5 fluorescent dyes (catalog numbers PA23001 and PA25001, GE Healthcare) were used.

**DNA Microarray Analysis**—Genome-wide analysis of transcript levels was performed with custom made DNA microarray chips (Synechocystis microarray chip with 8 × 15,000 format, Agilent Technologies Inc.) that covered 3611 genes including the genes of all native plasmids of Synechocystis. Dye-coupled cDNA was purified using microspin columns and hybridized to the DNA microarray chip according to the manufacturer’s recommendations (gene expression hybridization kit, catalog number 5188-5281, Agilent Technologies Inc.). A total of 45 μl of the hybridization mixture with Cy3- and Cy5-labeled DNA was allowed to hybridize at 65 °C for 16 h in a hybridization chamber. The chip was scanned using an Agilent Technologies Inc. microarray chip scanner (G2505B microarray scanner). Feature extraction was done using Agilent Technologies Inc. feature extraction (FE) software version 9.5.1 according to the manufacturer’s protocol. The signal from each gene on the microarray was normalized by reference to the total intensity of signals from all genes, and then the change in the level of the transcript of each gene relative to the total amount of mRNA was calculated.

**Quantitative Real Time PCR Analysis**—RNA isolated from wild type, Δssl2245, and Δssl1130 cells was used for cDNA synthesis with the Affinity Script cDNA synthesis kit following the manufacturer’s protocol (catalog number 600559, Agilent Technologies Inc.). qRT-PCR was carried out using the Power SYBR Green Master Mix kit (catalog number 4368708, Applied Biosystems). Each reaction was carried out in a 25-μl volume containing 12.5 μl of SYBR Green Master Mix, a 0.2 μM concentration of each of the forward and reverse primers, and 5 μl of diluted cDNA (35 ng). All reactions were run in duplicates using a qRT-PCR instrument (Mx3005P, Agilent Technologies Inc.). The instrument was programmed for 95 °C for 10 min, 40 cycles of 30 s at 95 °C, 60 s at 60 °C, and 60 s at 72 °C. For each reaction, the melting curve was analyzed to check the specific amplification of the target gene by corresponding primers. Expression levels were normalized using the gap1 gene as an internal reference. Primers used for qRT-PCR are listed in supplemental Table S2.

**Overexpression and Co-elution of Ssl2245 and Sll1130**—A DNA fragment covering the ssl2245 and sll1130 ORFs was amplified from Synechocystis genomic DNA by PCR using the primer set His2245-1130-F (5′-GGG AGG ATC TCT ATC AAT GCT TAC AAA CTA GCT ACG-3′) and His2245-1130-R (5′-GGG AGG ATC TCT ATC AAT GCT TAC AAA CTA GCT ACG-3′). The amplified fragment was digested with NdeI and HindIII and then ligated to pET28a(+)-vector (catalog number 69864-3, Novagen), which had also been digested with the same enzymes, to generate the HisSsl2245-Sll1130-pET28a construct. The N-terminal His₆-tagged Ssl2245 and untagged...
Sll1130 proteins were transformed and expressed in the BL21(DE3) pLYsS E. coli host strain and purified using nickel affinity gel (His60 Ni Superflow Resin, catalog number 635660, Clontech). Expression of these proteins was induced by addition of a 1 mM final concentration of isopropyl 1-thio-β-d-galactopyranoside. Bacterial cells were collected 3 h after induction by centrifugation at 10,000 rpm for 10 min at 4 °C. Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 1 mM PMSE, lysed by French press thrice at a pressure of 1,000 p.s.i., and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was filtered through a 0.45-μm filter to remove unlysed cells and then loaded onto the nickel affinity column. The column was washed twice with 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 40 mM imidazole, and sequentially the His-tagged Sll1130 was eluted with 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 100, 200, 300, and 400 mM imidazole, respectively. The purity of each fraction was examined by SDS gel electrophoresis. The fractions that gave a single band at the expected region on the gel were combined and dialyzed against 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl.

Viability Test—Synechocystis wild type and Δsll1130 cells were stained with ViaGram™ Red™ Bacterial Gram Stain and Viability kit according to the manufacturer’s instructions (catalog number V-7023, Molecular Probes, Invitrogen). Sixty microliters of water was added to dilute 3 μl of the SYTOX Green stain. To 50 μl of the cell suspension, 2.5 μl of the diluted SYTOX Green stain was added and incubated for 15 min at room temperature (28 °C). Thereafter, 10 μl of the stained cell suspension was examined with an inverted fluorescence microscope (model IX71, Olympus, Tokyo, Japan).

Sample Preparation for AFM—Synechocystis wild type and Δsll1130 cells were cultivated in liquid BG11 until an optical density at 730 nm of around 1.0 was reached. Twenty microliters of this bacterial suspension was placed onto freshly cleaved mica surface and dried in airflow for 30 min for adsorption. The samples were then imaged with a scanning probe microscope (SPA400, Seiko, Japan) using the tapping mode at a scan rate of 1–2 Hz and a resonance frequency of 110–150 kHz.

Bacterial Two-hybrid System—Constructs carrying sll1130 and slr1139 were cloned into plasmid pT25 following PstI and BamHI digestion. Constructs carrying ssl2245 and sll1130 were cloned into plasmid pT18 following XhoI and HindIII digestion. The plasmid combinations pT25-Sll1130 and pSsl2245-T18, pT25-Slr1139 and pSsl2245-T18, pT25-Slr1139 and pSll1130-T18, and positive control pT25-RhlB and pPnP-T18, respectively, were co-transformed into DH5α to screen for protein-protein interaction as described and plated onto MacConkey agar plates (39). Color changes on the MacConkey agar due to the interactions between the proteins were monitored.

Expression and Purification of Ssl2245 and Sll1130 Proteins for Isothermal Titration Calorimetry, Ribonuclease Assays, and CD Spectroscopic Measurements—Individual pET28a constructs carrying the His-tagged ssl2245 and sll1130, respectively, were generated. DNA fragment covering the ssl2245 and sll1130 ORFs were amplified from Synechocystis genomic DNA by PCR using the specific primer sets Ssl2245-Exp-FP (5′-CGG CAT ATG TCT TAT CAA TGA TTA CAA ACT AGC TAC G-3′) and Sll2245-Exp-RP (5′-GGC AAG CTT TCA TAG GTG TCG GTA TGC CAG AAT TAT CAG C-3′) and Sll1130-Exp-FP (5′-GCA GGC ATA TGA ATA CAA TTT ACG AAC-3′) and Sll1130-Exp-RP (5′-CGT CGA ATT CCT AAG CAA CGT TAA AAA CAT GG-3′), respectively. The amplified ssl2245 fragment was ligated with Ndel and HindIII, and sll1130 fragment was ligated with Ndel and EcoRI, respectively, and then ligated to pET28a(+) vector, which had also been digested with the same enzymes, to generate the HisSsl2245-pET28a and HisSll1130-pET28a constructs, respectively. These constructs were transformed and expressed in the BL21(DE3) pLYsS E. coli host strain and purified using nickel affinity gel (His60 Ni Superflow Resin). Expression of these proteins was induced by addition of a 1 mM final concentration of isopropyl 1-thio-β-d-galactopyranoside. Bacterial cells were collected 3 h after induction by centrifugation at 10,000 rpm for 10 min at 4 °C. Cell pellets were resuspended in lysis buffer, lysed by French press thrice at a pressure of 1,000 p.s.i., and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was filtered through a 0.45-μm filter to remove unlysed cells and then loaded onto the nickel affinity column. The column was washed twice with 20 mM Tris-HCl (pH 6.5), 500 mM NaCl, and 40 mM imidazole, and sequentially the His-tagged Sll1130 was eluted with 20 mM Tris-HCl (pH 6.5), 500 mM NaCl, and 100, 200, 300, and 400 mM imidazole, respectively. The fractions that gave a single band at the expected region on the gel were combined and dialyzed against 20 mM Tris-HCl (pH 6.5) and 150 mM NaCl. The purified and dialyzed proteins were then concentrated using Amicon Ultra centrifugal filters with a 3-kDa cutoff. The purity of both the proteins was examined by SDS gel electrophoresis (supplemental Fig. S4). These proteins were further used for ITC experiments, ribonuclease assays, and CD spectroscopic measurements.

Isothermal Titration Calorimetric Studies—Thermodynamics governing the interaction between Ssl2245 and Sll1130 proteins was investigated by ITC measurements using a MicroCal VP-ITC instrument (MicroCal LLC, Northampton, MA) (51). The purified protein solutions were first degassed under vacuum before use in the ITC experiments. Typically, 15–25 consecutive injections of 5-μl aliquots of Ssl2245 at a concentration of 500–900 μM were added with the help of a rotator stirrer-syringe into the 1.445-ml-volume calorimeter cell filled with Sll1130 protein at a final concentration of 70–100 μM. To minimize the contribution of heat of dilution to the measured heat change, the protein solutions were prepared in the same buffer. Injections were made at intervals of 5 min for all titrations. To ensure proper mixing after each injection, a constant stirring speed of 300 rpm was maintained throughout the experiment. Control experiments were performed by injecting Ssl2245 into the buffer solution in an identical manner, and the resulting heat changes were subtracted from the measured heats of binding. Because the first injection is often inaccurate, the first data point was deleted before the remaining data were analyzed. The data obtained from these calorimetric titrations were analyzed using the one set of sites binding model available in the Origin ITC data analysis software (51, 52) supplied by the instrument manufacturer.
Differential Scanning Calorimetric Studies—The thermal stability of Ssl2245 and Sll1130 proteins was investigated by DSC studies using a MicroCal VP-DSC microcalorimeter (MicroCal LLC) equipped with fixed reference and sample cells (0.545 ml each) (53). Protein solutions were dialyzed extensively against large volumes of 20 mM Tris buffer (pH 6.5) containing 150 mM NaCl, thoroughly degassed under vacuum for 5 min at room temperature, and then carefully loaded in the calorimeter cells. DSC thermograms were recorded at a scan rate of 60 °C/h. A background scan collected with buffer in both cells was subtracted from each scan. The temperature dependence of the molar heat capacity of the protein was further analyzed using the Origin DSC analysis software supplied by the manufacturer.

Circular Dichroism Spectroscopy—Circular dichroism measurements were carried out in a Jasco-J1500 CD spectrometer at a scan rate of 50 nm/min with a 1-mm data pitch and 2-s response using a 2-mm bandwidth. Temperature was varied by means of a Peltier thermostat supplied by the manufacturer. Spectra in the far-UV region (260–190 nm) were recorded with samples taken in a 1.0-mm quartz cell, whereas for measurements in the near-UV region (320–250 nm) a 10-mm-path length cell was used. For measurements in the far-UV region, the concentrations of Sll1130 and Ssl2245 used were 10 and 8.5 μM, respectively, whereas the corresponding concentrations used for measurements in the near-UV region were 66 and 285 μM, respectively.

In Vitro Transcription and Ribonuclease Activity of the Sll1130—In vitro transcription for all RNAs was performed with the MEGAscript or MAXIscript kit (Invitrogen). PCR products containing a T7 RNA polymerase promoter sequence were used as templates, and transcription was carried out according to the manufacturer’s instructions including the optional DNase treatment and phenol/chloroform extraction. Purified Sll1130 and Ssl2245 (100–250 ng of each) were incubated with 50–400 ng of target in vitro transcripts at 30 or 37 °C for 30–120 min in 25 mM Tris–HCl (pH 7.5), 60 mM KCl, 100 mM NH₄Cl, 5 mM MgCl₂, and 0.1 mM DTT, respectively. Duplex RNA formation was achieved as described previously (54) with the following modification. The reaction mixture was complemented with 25 mM Tris–HCl (pH 7.5), 60 mM KCl, 5 mM MgCl₂, 100 mM NH₄Cl, and 0.1 mM DTT. Purified Sll1130, Ssl224, and controls were also incubated with target transcripts at 37, 50, and 60 °C to test temperature dependence on the toxic activity-masking property of the Ssl2245. Reactions were stopped by adding 1 volume of RNA loading buffer (New England Biolabs or Fermentas). Samples were heated for 5 min at 95 °C prior to electrophoretic separation on 6–10% 7 M urea polyacrylamide gels (6 mA, 1.5–2.5 h).

Author Contributions—A. S. performed the majority of the experiments and wrote the paper. P. S. K. generated the mutant strains and performed DNA microarray experiment with Δsll1130. S. K. performed ribonuclease assays. D. S. and A. S. performed ITC and DSC experiments together. M. J. S. analyzed the ITC, DSC, and CD spectroscopic results and edited the paper. S. L.-C. and W. R. H. advised on experiments and edited the paper. J. S. S. P. conceived the idea for the project and wrote the paper with A. S.

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