Role of spermidine in overwintering of cyanobacteria

Xiangzhi Zhu, Qiong Li, Chuntao Yin, Xiantao Fang, Xudong Xu

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei 430072, China

Received: February, 2015/Accepted:

Address correspondence to Xudong Xu, xux@ihb.ac.cn
ABSTRACT
Polyamines are found in all groups of cyanobacteria, but their role in environmental adaptation has been barely investigated. In Synechocystis 6803, inactivation of spermidine synthesis genes significantly reduced the survivability under chill(5°C)-light stress, and the survivability could be restored by addition of spermidine. To analyze effects of spermidine on gene expression at 5°C, lacZ was expressed from the promoter of carboxy(nor)spermidine decarboxylase gene (CASDC) in Synechocystis. Synechocystis 6803::P_{CASDC}-lacZ pretreated at 15°C showed a high level of LacZ activity for a long period of time at 5°C; without the pretreatment or with protein synthesis inhibited at 5°C, the enzyme activity gradually decreased. In a spermidine-minus mutant harboring P_{CASDC}-lacZ, lacZ showed an expression pattern as if protein synthesis were inhibited at 5°C, even though the stability of its mRNA increased. Four other genes, including rpoA that encodes the α subunit of RNA polymerase, showed similar expression patterns. The chill-light stress led to a rapid increase of protein carbonylation in Synechocystis. Afterwards, protein carbonylation quickly returned to background level in the wild type but continued to slowly increase in the spermidine-minus mutant. Our results indicate that spermidine promotes gene expression and replacement of damaged proteins in cyanobacteria under the chill-light stress in winter.

IMPORTANCE
Outbreak of cyanobacterial blooms in freshwater lakes is a worldwide environmental problem. In the annual cycle of bloom-forming cyanobacteria, overwintering is the least understood stage. Survival of Synechocystis 6803 under long-term chill(5°C)-light stress has been established as a model for molecular studies on overwintering of cyanobacteria. Here, we show that spermidine, the most common polyamine in cyanobacteria, promotes the survivability of Synechocystis under long term chill-light stress and that the physiological function is based on its effects on gene expression and recovery from protein
damage. This is the first report on the role of polyamines in survival of overwintering cyanobacteria. We also analyzed spermidine synthesis pathways in cyanobacteria on the basis of bioinformatic and experimental data.
Cyanobacteria are a large group of prokaryotes that carry out oxygenic photosynthesis. In freshwater eutrophic lakes, some cyanobacterial species may vigorously propagate and cause the outbreak of water blooms. Bloom-forming cyanobacteria overwinter in the sediment (1-3), and reinitiate growth in shallow areas in spring providing inocula for the pelagic region (4-6). Studies using \textit{Synechocystis} sp. PCC 6803 (\textit{Synechocystis} 6803) showed that preconditioning at a low temperature, such as 15°C, could greatly enhance its survivability under long-term chill (5°C)-light stress (7, 8). This finding suggests that cyanobacteria acquire the capability to overwinter in late autumn and early winter. Also, it allows the overwintering mechanism to be investigated under simulating conditions in laboratory.

Studies on how cyanobacteria adapt to low temperatures can be roughly divided into two categories: 1) cold acclimation of cells exposed to a suboptimal growth temperature, such as 22°C~24°C (9, 10), or 15°C (11); 2) survival under long-term (8-10 d) chill(5°C)-light stress as a simulation of overwintering (7, 8, 12). In previous reports, we showed that α-tocopherol, which is membrane-localized, is essential for the acquired chill-light tolerance (ACLT) of cyanobacteria (7), and that accumulation of RNA-binding protein 1 (Rbp1) leads to the formation of overwintering capability of cyanobacteria (8, 12). Nonetheless, information about the biology of overwintering cyanobacteria is still very limited. This is mainly due to cessation of growth and lack of photosynthetic oxygen evolution in mesophilic cyanobacteria (such as \textit{Synechocystis} 6803) under long-term chill-light stress.

Polyamines are polycationic hydrocarbon molecules with multiple amino groups. In prokaryotic cells, most polyamines are bound to phosphate groups of RNA, even though they can also bind to DNA, free nucleotides and phospholipids (13). In conjunction with Mg\textsuperscript{2+}, polyamines may cause conformational changes of certain mRNAs, leading to enhancement of protein synthesis; as scavengers of free radicals, they may reduce oxidative damage of macromolecules; specific binding of a polyamine molecule may also modulate the activity of a protein (13, 14). For these characteristics, polyamines are involved in variable cellular activities of prokaryotes, such as biofilm formation (15, 16), modification of outer membrane permeability (17,
and enhancement of acid resistance (19, 20). In cyanobacteria, there are certain clues that may relate polyamines to osmotic tolerance (21), but little is known about the role of polyamines in adaptation to freshwater environments. Because polyamines in plants are involved in cold responses and can enhance tolerance to cold and freezing temperatures (22), those in cyanobacteria may relate to overwintering.

The most common polyamines in bacteria include putrescine, spermidine, norspermidine, spermine, etc. (14, 23). Putrescine is formed through the decarboxylation of arginine or ornithine (14, 15, 24, 25). Arginine is decarboxylated into agmatine by arginine decarboxylase (ADC), and further converted into putrescine by agmatine ureohydrolase (AUH) or agmatine iminohydrolase (AIH) and N-carbamoylputrescine amidohydrolase (NCPAH). The decarboxylation of ornithine directly produces putrescine. There are two known pathways for synthesis of spermidine from putrescine (Fig.1). In the classical pathway, spermidine synthase (SPDS) transforms putrescine and decarboxylated S-adenosylmethionine (DC-SAM) into spermidine (14, 15). DC-SAM is generated from SAM by SAM decarboxylase (SAMDC). In the alternative pathway, carboxy(nor)spermidine dehydrogenase (CASDH) transforms putrescine and aspartic β-semialdehyde (ASA) into carboxyspermidine, which is then transformed into spermidine by carboxy(nor)spermidine decarboxylase (CASDC) (15, 26). In microbes with diaminobutyrate aminotransferase/decarboxylase (DABA AT/DC), from ASA and glutamate, diaminopropane (DAP) is produced via the intermediate DABA. ASA and DAP are transformed by CASDH into carboxynorspermidine, then by CASDC into norspermidine.

Spermidine and homospermidine (both triamines) are the two most common polyamines found in cyanobacteria (27). In most unicellular species that belong to Chroococcales, spermidine is the major polyamine; in most heterocyst-forming species that belong to Nostocales, homospermidine is the major polyamine; in non-heterocystous species that belong to Oscillatoriales and heterocyst-forming species that belong to Stigonematales, spermidine and/or homospermidine are the major polyamines. Genome sequence data indicate that both classical and alternative
pathways for spermidine synthesis can be found in cyanobacteria. Homospermidine synthase is not found, but deoxyhypusine synthase may transform putrecine into homospermidine, in certain groups of cyanobacteria (28). Like other bacteria, cyanobacteria can take up polyamines from the environment (29-32) and may secret polyamines (32).

In the study of overwintering mechanism of cyanobacteria, we employed microarray analysis to identify genes up-regulated in *Synechocystis* 6803 exposed to chill-light stress. *CASDC*, a gene involved in synthesis of spermidine, was up-regulated in response to the stress. Further analyses revealed roles of spermidine in survivability and transcription/translation activity of mesophilic cyanobacteria at 5°C in the light.

**MATERIALS AND METHODS**

**Strains, culture conditions and measurements of RACLRT.** *Synechocystis* 6803 was a gift from Zhao J of this institute. The wild type and its derivative strains were grown in BG11 as previously described (33). Measurements of the relative acquired chill-light tolerance (RACLRT) were performed according to Yang et al. (7). Polyamines except homospermidine were purchased from Sigma Chemicals. Homospermidine was synthesized in this lab and confirmed with nuclear magnetic resonance spectroscopy. Data (mean ± SD) were generated from results of 3 replicates.

**Construction of plasmids.** Molecular manipulations were performed according to standard methods or per manufacturers’ instructions. Clones of PCR products were confirmed by sequencing. Details of plasmid construction are described in Table S1. Plasmid pHB2193 was used to inactivate slr0873 (*CASDC* in *Synechocystis*) with a Km’ cassette excised from pRL446 (NCBI GenBank accession no. EU346690); plasmid pHB4239 was used to inactivate slr0049 (*CASDH* in *Synechocystis*) with the Km’ cassette; plasmid pH5450 was used to inactivate Synpcc7942_0628 (*SPDS* in *Synechococcus*) with the Km’ cassette; plasmid pH2972 was used to insert
7Ω-P_{prbcL-sll0873} into an integrative neutral platform of *Synechocystis* 6803 (34, 35) to complement the *sll0873::Kmr* mutation; plasmid pH4768 was used to insert Ω-P_{slr0049} into the integrative platform to complement the *slr0049::Kmr* mutation; plasmid pH4324 was used to insert Ω-P_{sll0873-lacZ} into the integrative platform of *Synechocystis* 6803. Ω is a spectinomycin/streptomyacin resistance cassette excised from pRL57 (36).

**Generation of cyanobacterial strains.** Transformation of *Synechocystis* 6803 was performed according to Williams (34). Complete segregation of mutants was confirmed by PCR. To complement a mutant, the wild type gene with a spectinomycin-resistance marker was inserted into the integrative platform in the genome. Transformation of *Synechococcus* 7942 was performed as Güler et al. (37) with modifications. Fifty milliliters of *Synechococcus* cells (4 day old, OD$_{730}$ ≈ 1.2) were collected by centrifugation (4,000 rpm, 10 min), washed with 50 ml of 10 mM NaCl and re-suspended in 1.5 ml of BG11. Plasmid DNA was added to cell suspension at 2 μg DNA/ml. After incubation overnight in the dark at 30°C on a rotating shaker, cells were spread on BG11 plates with 5 μg/ml kanamycin.

Cyanobacterial strains are described in Table 1, and primers are listed in Table S1.

**Microarray analysis.** *Synechocystis* 6803 grown at 30°C was preconditioned at 15°C for 6 days, cooled to 4°C in a water bath, and then incubated in a refrigerator at 4°C with illumination of 100 μE m$^{-2}$ s$^{-1}$ for 2 h. Total RNA was extracted using TRIZol reagent (Life Technologies), treated with RNase-free DNase I to eliminate contaminating chromosomal DNA and examined by agarose gel electrophoresis.

CyanoCHIP ver. 2.0 (Takara Biotechnology) was used for analyses of genome-wide transcriptional activities. Labeling of cDNA, hybridization, rinsing and scanning of microarrays and data analyses were performed by Takara Biotechnology at Dalian. Data (mean ± SD) were generated from 3 independent experiments, each with 2 repeats (3×2).

**Northern blot analysis.** Total RNA was extracted from *Synechocystis* 6803 preconditioned at 15°C and exposed to chill and light at different time points as indicated, separated by electrophoresis on agarose/formaldehyde gel and blotted onto
an Immobilon-Ny+ membrane (Millipore) by capillary transfer. Digoxigenin-labelled DNA probes were prepared by PCR using primer pairs sl0873n-1/-2 and rnpBn-1/-2. Hybridization and immunological detection were performed with a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche) following the manufacturer’s protocol.

**RACE, qRT-PCR and RNA stability.** Rapid amplification of cDNA ends (RACE) was performed according to Zhang et al. (38). The gene-specific primer for the first PCR was sl0873up-7, for the second PCR was sl0873up-2. PCR products were cloned into pMD18-T and sequenced. Quantitative reverse transcription polymerase chain reactions (qRT-PCR) were performed according to Gao and Xu (39). *rnpB* (RNase P subunit B) (40) was used as the internal control. RNA stability analyses were performed according to Wang and Xu (41) with modifications. Cells were pretreated at 15°C for 2 d and exposed to chill-light stress for 1 d, then rifampicin was added to inhibit mRNA synthesis. Total RNA was extracted at 0, 1 and 2 h. PCR primers are listed in Table S1.

**Assays of β-galactosidase.** β-galactosidase activity of *Synechocystis* 6803 cells was measured as described by Miller (42) with modifications. One point five milliliters of cells were spun down by centrifugation (12,000 rpm, 1 min) and resuspended in 1 ml of Z buffer and permeabilized by addition of 50 μl of 0.1% SDS and 50 μl of chloroform, then 200 μl of 13.28 mM ONPG (2-Nitrophenyl β-D-galactopyranoside) was added to initiate the reaction. After incubation at 30°C for 30 min, the reaction was terminated by addition of 500 μl of 1 M Na₂CO₃. Cells were removed from the reaction mixture by centrifugation (12,000 rpm, 2 min), and absorbance at 420 nm was measured for calculation of β-galactosidase activity (OD₄₂₀. min⁻¹ μl⁻¹ OD₇₃₀⁻¹).

**Western blot analysis.** *Synechocystis* cells grown at 30°C were preconditioned at 15°C and exposed to chill and light for different periods of time. Protein samples were prepared from *Synechocystis* cells by sonication in the presence of 1 mM PMSF (phenylmethylsulfonyl fluoride). Western blot analyses were performed as previously described (12) using rabbit antisera against Rbp1, EF-Ts and LacZ. The former two
antisera were generated by this laboratory, while the anti-LacZ antiserum was purchased from Creative Diagnostics. Twenty micrograms of proteins were loaded in each lane. Protein concentrations were determined by Bradford’s method (43).

**Measurements of polyamines.** Polyamines were extracted from *Synechocystis* 6803 with 5% cold HClO₄ and derivatized with benzoyl chloride as reported (21). The derivatization of polyamine standards (Sigma Chemicals) was performed similarly. The polyamine contents were analyzed by high performance liquid chromatography using a C-18 reverse phase column and a UV-Vis detector at 254 nm. The system was run using a 60% methanol over 25 min with a flow rate of 1 ml min⁻¹, and the oven temperature was 35°C.

**Measurement of protein carbonylation.** *Synechocystis* cells were broken by sonication on ice in presence of 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell debris was removed by centrifugation (12,000 rpm, 15 min) at 4°C. Protein Carbonylation was measured spectrophotometrically using OxiSelect™ Protein Carbonyl Spectrophotometric Assay Kit.

**RESULTS**

**Up-regulation of slr0873 (CASDC) in response to chill-light stress.** Pretreatment at 15°C can greatly enhance the chill-light survivability of *Synechocystis* 6803 (7). Previously, we had analyzed the response of 30°C-grown *Synechocystis* 6803 to 15°C using microarrays (12). In this study, we further performed a transcriptomic analysis of the response of 15°C-treated cells to chill-light stress. Many genes showed significant changes in mRNA level within 2 hours of exposure to chill-light, including 44 genes up-regulated (4°C/15°C ratio ≥ 2.0) and 39 genes down-regulated (4°C/15°C ratio ≤ 0.5) (Table S2).

By generating mutants of genes that are up-regulated upon exposure to chill-light (Table S2, denoted with a or b) and testing their chill-light survivability, we identified slr0083 (crhR) and slr0873 (CASDC) as two important genes for the survivability (data not shown). slr0083 that encodes an RNA helicase has been investigated for its
role in cold acclimation (temperature downshift from 34°C to 24°C) before (9). In our study, we focused on *sll0873*, a gene predicted to encode the carboxy(nor)spermidine decarboxylase, for its specific role in chill-light survivability.

Northern blot analysis showed that *sll0873* mRNA was gradually accumulated after transfer from 15°C to 5°C and reached the maximal level at 24 h (Fig. 2A). The mRNA level gradually decreased afterwards. Quantitative RT-PCR analysis confirmed this finding (Fig. 2B). The marked increase of mRNA abundance could be due to either activation of the promoter or enhancement of mRNA stability. Here, we analyzed the mRNA stability by measuring the rate of decay in the presence of rifampicin. In cells exposed to chill-light for 1 d, *sll0873* mRNA showed greatly enhanced stability than that in cells at 15°C (Fig. 2C). The promoter activity was analyzed later using the *lacZ* reporter.

**Requirement of spermidine for survival of *Synechocystis* 6803 under chill-light stress.** Jantaro *et al.* (21) reported that *Synechocystis* 6803 possesses spermidine, spermine and putrescine, with spermidine as the most abundant polyamine (21). However, spermidine was the only detectable polyamine in *Synechocystis* 6803 maintained in our laboratory (Fig. S1). Similarly, substrains of *Synechocystis* 6803 differ in tocopherol contents (7). Unlike the mRNA level of *CASDC*, the spermidine content decreased after transfer from 30°C to 15°C, then remained unchanged or increased slightly after further transfer to 5°C in the light (Fig. 3). In cells directly exposed to chill-light stress for 8 days, spermidine decreased to a much lower level than that in cells pretreated at 15°C.

To investigate the role of spermidine in chill-light survivability, we generated the *CASDC*-null mutant of *Synechocystis* 6803, namely, *sll0873::Km* (DRHB2193). *CASDC* can catalyze the synthesis of spermidine as well as norspermidine. However, no norspermidine was detected in *Synechocystis* 6803. Inactivation of *sll0873* abolished the synthesis of spermidine; after complementation, the synthesis of spermidine was resumed in the mutant (Fig. S1). The spermidine-minus mutant lost over 50% RACTL (relative acquired chill-light tolerance, calculated based on survivability of the mutant under the chill-light stress relative to the wild type treated...
in parallel; 7), while complementation with wild type sll0873 almost fully restored the RACLT (Fig. 4A).

It has been reported that Synechocystis 6803 can take up spermidine (31). We tested the effect of exogenous spermidine on the RACLT of sll0873::Kmr. Spermidine was added to the culture medium at 0.01 mM or 0.1 mM, and the CASDC-null mutant was grown in the medium with spermidine at 15°C for 6 days before transfer to the chill-light stress. The exogenous spermidine fully restored the RACLT of the mutant to the wild type level (Fig. 4A). However, 1 mM spermidine appeared to be toxic to Synechocystis cells. These results indicated that spermidine is required to promote the chill-light survivability of Synechocystis 6803.

To confirm the role of spermidine in chill-light survivability, we also generated the CASDH-null mutant, slr0049::Kmr (DRHB4239). Inactivation of this gene showed similar effects on synthesis of spermidine (Fig. S2) and chill-light survivability (Fig. 4B), and the RACLT was reduced to around 30%. Complementation with wild type slr0049 or addition of spermidine fully or partially restored the RACLT (Fig. 4B).

Even though spermidine is required for the chill-light survivability, inactivation of CASDH or CASDC showed no effect on the growth of Synechocystis 6803 in BG11 at 30°C and 15°C.

Using the CASDC mutant, we also tested whether spermidine, a triamine, can be replaced with other polyamines. We tested spermine (tetramine), homospermidine (triamine), cadaverine (diamine) and putrescine (diamine). Of these polyamines, at least putrescine has been shown to be taken up by Synechocystis 6803 (30), and the uptake of spermine has been shown in another cyanobacterium (29). Addition of 0.01 mM or 0.1 mM spermine / homospermidine restored the RACLT of CASDC mutant to the wild type level, while cadaverine and putrecine only slightly increased the RACLT (Fig. 5). Apparently, diamines can not serve as substitutes for spermidine in promoting chill-light survivability.

Role of spermidine in expression of P_{CASDC-lacZ} under chill-light stress.
Polyamines have been shown to affect gene expression in other bacteria (14). The requirement of spermidine for the chill-light survivability of Synechocystis 6803 may
relate to its role in gene expression under the chill-light stress. We attempted to employ the in vitro translation system (44) to test this hypothesis, but found it technically unfeasible due to precipitation of constituents at the low temperature. Alternatively, we employed lacZ as a reporter gene to test gene expression in vivo. CASDC was shown to be actively expressed at 5°C. RACE analysis indicated that transcription of this gene started at 324 bp (A at 1141984 bp of the chromosome) upstream of the start codon. On the basis of this information, we constructed the transcriptional fusion P_{CASDC}-lacZ and introduced this construct into the wild type and the sll0873::Km' mutant of Synechocystis 6803 via double-crossover integration into a neutral platform in the genome.

In the wild type strain carrying P_{CASDC}-lacZ, the expression of lacZ was consistent with the acquisition of chill-light tolerance. Without preconditioning at 15°C, the β-galactosidase activity increased in first 2 days after exposure to chill and light, then gradually decreased to a low level in next 6 days (Fig. 6A); with preconditioning, the enzyme activity was maintained at the high level within the same range of time under chill-light stress (Fig. 6B). Measurement of the relative mRNA level of lacZ with qRT-PCR showed similar results (Fig. 6C and 6D). When chloramphenicol was added to preconditioned cells at the beginning of chill-light stress, the β-galactosidase activity gradually decreased to the close-to-background level (Fig. 6B). It indicated that the high and stable β-galactosidase activity in such cells depended upon the activity of protein synthesis under the chill-light stress.

Using the lacZ reporter system, we examined the role of spermidine in gene expression under chill-light stress. After transfer of cells from 30°C to 15°C, Synechocystis 6803 sll0873::Km' with P_{CASDC}-lacZ, like the wild type with P_{CASDC}-lacZ, showed an increase of β-galactosidase activity (Fig. 7A). After exposure to chill-light stress, the enzyme activity in the spermidine-minus strain gradually decreased as if chloramphenicol were added; exogenous supplementation of spermidine restored the capability to maintain the enzyme activity under the stress (Fig. 7A).

Using Western blot analysis, we also detected LacZ at the protein level. Within 4
days under the chill-light stress, the two strains showed no difference in abundance of LacZ; on the 8th day, LacZ remained almost unchanged in the wild type with P_CASDC-lacZ but disappeared in the spermidine-minus strain (Fig. 7B).

The decrease of β-galactosidase activity and LacZ abundance in the mutant may reflect the role of spermidine in translation or transcription or both. Using qRT-PCR, we compared mRNA levels of lacZ in the two strains and found similar results as assaying β-galactosidase activity (Fig. 8A); lack of spermidine led to gradual decrease of the mRNA level. Because the decay of lacZ mRNA was slower in the spermidine-minus strain (Fig. 8B), spermidine was required for the transcription of P_CASDC-lacZ rather than the stability of lacZ mRNA.

Role of spermidine in expression of other genes under chill-light stress. To further investigate the role of spermidine in gene expression under chill-light stress, we compared the relative mRNA levels of 4 other genes, rbp1, tsf, rpoA and slr0338, in the wild type and the CASDC mutant of *Synechocystis* 6803 using qRT-PCR (Fig. 9). These genes encode RNA-binding protein 1 (12), elongation factor(EF)-Ts, RNA polymerase α-subunit and a potential oxidoreductase, respectively. In the wild type cells transferred from 30°C to 15°C then to 5°C, the mRNA levels of these genes showed stepwise increases and remained unchanged within 8 days of chill-light stress. In contrast, these mRNAs in the spermidine-minus mutant gradually decreased after exposure to chill and light.

Using rbp1 as an example, we tested the effect of spermidine on the stability of mRNAs and found a slight increase in the spermidine-minus mutant compared to the wild type (Fig. S3). Therefore, in *Synechocystis* cells exposed to chill-light stress, spermidine enhances transcription activity rather than mRNA stability.

Unlike LacZ, the abundance of Rbp1 and EF-Ts remained unchanged within 8 days of chill-light stress in strains with or without spermidine (Fig. S4). Apparently, in cyanobacterial cells exposed to chill-light stress, proteins differ from each other in stability.

Role of spermidine in recovery from protein oxidative damage. *Synechocystis* 6803 shows no growth and no photosynthetic O2 evolution activity after exposure to
the chill-light stress for over 24 h (data not shown). Turnover of macromolecules, such as replacement of damaged proteins with newly synthesized ones, is critical for maintaining the viability of cyanobacterial cells under such conditions. In Fig. 7, β-galactosidase activity decreased before the degradation of LacZ in the CASDC mutant with P_{CASDC-lacZ}. This could be due to protein oxidative damage under the chill-light stress. Spermidine may reduce protein oxidative damage by scavenging free radicals (14) or promote the recovery from oxidative damage by enhancing protein synthesis. We compared protein carbonylation in the wild type and the CASDC mutant of *Synechocystis* 6803. In the wild type transferred from 30°C to 15°C then to 5°C, protein carbonylation rapidly increased on the first day of exposure to 5°C but dropped back to the background level on the second day (Fig. 10). In the spermidine-minus mutant, protein carbonylation also rapidly increased on the first day at 5°C (to a slightly lower level in comparison to the wild type), but continued to slowly increase in next several days. Spermidine appeared to play an important role in recovery from oxidative damage under the chill-light stress.

**Requirement of spermidine for chill-light survivability of *Synechococcus* 7942.**

Many cyanobacteria possess SAMDC and spermine/spermidine synthase (SPDS) rather than CASDH and CASDC (Table 2). In other words, the classical pathway of polyamine synthesis is much more widely distributed in cyanobacteria. To confirm that spermidine is also required for the chill-light survivability of cyanobacteria with the classical pathway, we generated a *SPDS*-null mutant of *Synechococcus* sp. PCC 7942 (*Synechococcus* 7942), namely DRHB5450 (*SPDS::Kmr*). In this mutant, spermidine was no longer synthesized, but putrescine was detected at a low level (Fig. S5). Elimination of spermidine from *Synechococcus* 7942 reduced its RACLT to about 40%, and addition of 0.1 mM spermidine to the medium restored the RACLT to about 80% (Fig. 11).

**DISCUSSION**

Polyamines are involved in various cellular activities in heterotrophic bacteria, but...
their role in environmental adaptation of cyanobacteria has been barely investigated. In this study, we found that spermidine is required for the chill-light survivability of cyanobacteria and that the physiological function is based on its effects on gene expression and recovery from protein damage.

Before the finding of the alternative polyamine synthesis pathway in bacteria (15), spermidine synthesis in *Synechocystis* 6803 was thought to be via SAMDC, and spermine was reported to be detected in *Synechocystis* samples (21, 45). Our study, however, showed that polyamine synthesis in this cyanobacterium should be via the alternative pathway rather than the classical pathway, because inactivation of either *CASDH* or *CASDC* abolished the synthesis of spermidine and no gene has been predicted to encode SAMDC in the genome. Also, no spermine was detectable in *Synechocystis* 6803 under our conditions. This is supported by the lack of spermine synthase gene in the genome. Furthermore, in an investigation of polyamine distribution profiles, spermine was only found at very low levels in 7 of 63 cyanobacterial strains (Hosoya *et al.* [2005], with no information of axenicity of these strains).

According to the gene information shown in Table 2, many cyanobacteria can be predicted to synthesize spermidine via either classical or alternative pathway or both. *Synechocystis* 6803 and several cyanobacterial strains that possess the alternative pathway might have acquired *CASDH* and *CASDC* genes from heterotrophic bacteria by horizontal gene transfer. Without the two enzymes (diaminobutyrate aminotransferase, diaminobutyrate decarboxylase) for synthesis of diaminopropane (15), *Synechocystis* 6803 produces spermidine rather than norspermidine. In some cyanobacteria, such as *Anabaena* sp. and most other species of Nostocales, the major polyamine is homospermidine (27). Table 2 also lists 1 strain that lacks both AUH and AIH/NCPAH (*Leptolyngbya boryana*) and 1 strain that lacks all the enzymes involved in spermidine synthesis (*Cyanothece* ATCC51142). Possibly, such cyanobacterial species can acquire polyamines via import from environments or from coexisting bacteria.

The initial prompt for us to relate spermidine synthesis to chill-light survivability of
cyanobacteria was the up-regulation of \textit{CASDC} gene in response to chill and light. Preconditioning at 15°C enabled \textit{Synechocystis} cells to maintain a much higher level of spermidine after 8-d chill-light stress. Similar effects of preconditioning had been found on \(\alpha\)-tocopherol (7) and Rbp1 (12) in \textit{Synechocystis}. Downshift of temperature to 5°C greatly increased the stability of \textit{CASDC} mRNA, leading to accumulation of the mRNA. Unlike the mRNA abundance, the spermidine content decreased at 15°C to about half of that at 30°C and remained unchanged or slightly increased after transfer to 5°C. The decrease at 15°C could be due to slowed uptake of nitrate (46), because synthesis of spermidine is probably responsive to nitrogen status that affects the availability of arginine. At 5°C, spermidine synthesis might have significantly decreased if \textit{CASDC} were not up-regulated.

Our results clearly indicated that spermidine promoted the chill-light survivability. Inactivation of either \textit{CASDH} or \textit{CASDC} in \textit{Synechocystis} 6803 or \textit{SPDS} in \textit{Synechococcus} 7942 remarkably reduced the chill-light survivability, and supplementation with spermidine restored the survivability of mutants. The chain length of polyamine appeared to be important for such a physiological function, because the chill-light survivability of the \textit{Synechocystis} \textit{CASDC} mutant could be restored by spermine (tetramine), spermidine and homospermidine (triamines), but not putrescine and cadaverine (diamines). This result suggests that homospermidine, a major polyamine in some cyanobacterial groups, should also play a role in chill-light survivability.

To analyze the underlying molecular mechanism for the role of spermidine in promoting chill-light survivability, we introduced \textit{P\_CASDC-lacZ} into the wild type and the \textit{CASDC} mutant of \textit{Synechocystis} 6803. After preconditioning, the wild type with \textit{P\_CASDC-lacZ} showed a high level of \(\beta\)-galactosidase activity over 8 days under the chill-light stress. Inhibition of protein synthesis by chloramphenicol and elimination of spermidine synthesis by gene inactivation both led to a decrease of \(\beta\)-galactosidase activity to a very low level. On one hand, this showed the persistence of protein synthesis activity in \textit{Synechocystis} 6803 at 5°C over a relatively long period of time; on the other hand, this indicated the role of spermidine in gene expression. In parallel
to β-galactosidase activity, the abundance of lacZ mRNA decreased without compromised mRNA stability, and examination of the mRNA abundance for tsf, rhp1, rpoA and slr0338 showed similar results. The decrease of β-galactosidase activity is seemingly due to the decline of transcription in spermidine-minus cells. Equally possible, the effect of spermidine on transcription is attributed to its role in translation, because polyamines may enhance the synthesis of RNA polymerase at 5°C by binding to its mRNA.

Alternatively, polyamines can reduce oxidative stress by scavenging free radicals; therefore, lack of spermidine may intensify oxidative damage of LacZ and RNA polymerase under the chill-light stress. Oxidative modification of amino acid side chains to carbonyl derivatives results in protein carbonylation. Only a portion of protein molecules are carbonylated (0.05-0.4 carbonyl per 50 kDa protein) in cells (47), and the carbonylation appears to prefer some proteins over others (48-50). Oxidative damage could inhibit protein functions by modifying critical sites, destabilizing tertiary structure and promoting protein aggregation (51, 52). Due to the irreversible nature, carbonylation is widely used to indicate the degree of protein oxidation. On the first day at 5°C, the CASDC mutant showed a lower protein carbonylation level than the wild type, indicating that spermidine was actually not an important free radical scavenger in this case. After acclimation on the first day, free radicals presumably diminished in Synechocystis 6803. At this stage, protein carbonylation rapidly returned to the background level in the wild type but continued to slowly increase in the CASDC mutant (Fig. 10). This difference can be explained by lack of protein turnover in the mutant at 5°C; under the same conditions, damaged proteins in the wild type can be actively replaced with newly synthesized ones. The continually increased protein carbonylation would inactivate RNA polymerase and other enzymes in the mutant under chill-light stress. Consistently, in Synechocystis spermidine-minus background, the transcription activity (as seen with mRNA abundance in Figs. 8 and 9) and β-galactosidase activity (Fig. 7) continued to decrease within 8 days, and LacZ was even completely degraded between the 4th day and 8th day (Fig. 7).
On the basis of these analyses, we propose that spermidine is required for maintaining gene expression in cyanobacteria under chill-light stress. At 5°C, spermidine may promote translation by binding to mRNA, or promote transcription by binding to DNA, or both. In any case, gene expression under the chill-light stress promotes replacement of damaged proteins, so that cyanobacterial cells can maintain viability in the winter. Therefore, spermidine should play an important role in overwintering of cyanobacteria, even though it is not required for their growth at favorable temperatures.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (30825003) and the State Key Laboratory of Freshwater Ecology and Biotechnology at IHB, CAS (2014FBZ). We thank Dr. Anthony J. Michael of University of Texas Southwestern Medical Center for his critical reading of the manuscript.

REFERENCES


19


LEGENDS TO FIGURES

FIG 1 Pathways for synthesis of spermidine in prokaryotes. ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; AUH, agmatine ureohydrolase; CASDC, carboxyspermidine decarboxylase; CASDH, carboxyspermidine dehydrogenase; NCPAH, N-carbamoylputrescine amidohydrolase; SAMDC, S-adenosylmethionine decarboxylase; SPDS, spermidine synthase.

FIG 2 mRNA levels of CASDC in Synechocystis 6803 at different stages of acquisition of chill-light tolerance. (A) Northern blot analysis of expression of CASDC in cells exposed to chill-light stress. (B) qRT-PCR measurement of CASDC mRNA levels in cells grown at 30°C, treated at 15°C and exposed to chill(5°C)-light stress. (C) qRT-PCR analysis of CASDC mRNA decay at 15°C (solid square) or under chill-light stress (empty square).

FIG 3 Content of spermidine in Synechocystis 6803 at different temperatures. (A) Cells grown at 30°C were treated at 15°C for 2 days before exposure to chill-light stress. (B) cells grown at 30°C were directly exposed to chill-light stress.


FIG 5 Effects of spermine, cadaverine, putrecine and homospermidine on the RACLT of the CASDC mutant of Synechocystis 6803.

FIG 6 Expression of lacZ in Synechocystis 6803::P<sub>CASDC</sub>-lacZ under the chill-light stress with (B, D) or without (A, C) preconditioning. Expression of lacZ was shown with β-galactosidase activity (A, B) or qRT-PCR (C, D). Bars in (A) and (B): dark grey bars, Synechocystis 6803::P<sub>CASDC</sub>-lacZ; light grey bars, Synechocystis 6803::P<sub>CASDC</sub>-lacZ supplemented with chloramphenicol upon transfer to 5°C; empty bars, Synechocystis 6803 treated in parallel (background activity).

FIG 7 Assays of β-galactosidase activity and LacZ abundance showing the effect of spermidine on expression of P<sub>CASDC</sub>-lacZ in Synechocystis strains. (A) β-galactosidase activity in Synechocystis 6803::P<sub>CASDC</sub>-lacZ (dark grey), Synechocystis 6803
DRHB2193::P$_{\text{CASDC-lacZ}}$ without (light grey) or with (empty) exogenous spermidine.

(B) Western blot detection of LacZ in *Synechocystis* 6803::P$_{\text{CASDC-lacZ}}$ (I) and
*Synechocystis* 6803 DRHB2193::P$_{\text{CASDC-lacZ}}$ (II). DRHB2193, the CASDC mutant.

**FIG 8** Measurements of lacZ mRNA level showing the effect of spermidine on
expression of P$_{\text{CASDC-lacZ}}$ in *Synechocystis* strains. (A) qRT-PCR analysis of lacZ
mRNA levels in *Synechocystis* 6803::P$_{\text{CASDC-lacZ}}$ (dark grey) and *Synechocystis* 6803
DRHB2193::P$_{\text{CASDC-lacZ}}$ (light grey). (B) qRT-PCR analysis of lacZ mRNA decay in
*Synechocystis* 6803::P$_{\text{CASDC-lacZ}}$ (solid square) and *Synechocystis* 6803
DRHB2193::P$_{\text{CASDC-lacZ}}$ (empty square) under chill-light stress. DRHB2193, the
CASDC mutant.

**FIG 9** qRT-PCR measurement of mRNA levels showing the role of spermidine
synthesis in gene expression under chill-light stress. Dark grey bar, *Synechocystis*
6803; light grey bar, the CASDC mutant. *rhp1*, RNA-binding protein 1; *tsf*, EF-Ts;
*rpoA*, α subunit of RNA polymerase; *slr0338*, probable oxidoreductase.

**FIG 10** Comparison of protein carbonylation in the wild type and CASDC mutant of
*Synechocystis* 6803. Empty bar, wild type; grey bar, the CASDC mutant.

**FIG 11** Effect of spermidine on chill-light survivability of *Synechococcus* 7942.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Derivation, relevant characteristics, or sequences</th>
<th>Source or reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechocystis sp. PCC 6803 strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synechocystis 6803</td>
<td>Wild type, a glucose-tolerant strain</td>
<td>Zhao J, Peking University/Institute of Hydrobiology</td>
</tr>
<tr>
<td>DRHB2193</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;, <em>sll0873</em>::Km&lt;sup&gt;+&lt;/sup&gt; (<em>CASDC</em> insertion mutant), <em>Synechocystis</em> 6803 transformed with pHB2193</td>
<td>This study</td>
</tr>
<tr>
<td>DRHB2193/DRHB2972</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;/Sp&lt;sup&gt;+&lt;/sup&gt;, <em>sll0873</em>::Km&lt;sup&gt;+&lt;/sup&gt; complemented with wild-type <em>sll0873</em>, DRHB2193 transformed with pHB2972</td>
<td>This study</td>
</tr>
<tr>
<td>DRHB2193/DRHB4324</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;/Sp&lt;sup&gt;+&lt;/sup&gt;, Ω-<em>P</em>&lt;sub&gt;CASDC&lt;/sub&gt;<em>-lacZ</em> integrated into the neutral platform in the genome of <em>CASDC</em> mutant, DRHB2193 transformed with pHB4324</td>
<td>This study</td>
</tr>
<tr>
<td>DRHB4239</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;, <em>slr0049</em>::Km&lt;sup&gt;+&lt;/sup&gt; (<em>CASDH</em> insertion mutant), <em>Synechocystis</em> 6803 transformed with pHB4239</td>
<td>This study</td>
</tr>
<tr>
<td>DRHB4239/DRHB4768</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;/Sp&lt;sup&gt;+&lt;/sup&gt;, <em>slr0049</em>::Km&lt;sup&gt;+&lt;/sup&gt; complemented with wild-type <em>sll0049</em>, DRHB4239 transformed with pHB4768</td>
<td>This study</td>
</tr>
<tr>
<td>DRHB4324</td>
<td>Sp&lt;sup&gt;+&lt;/sup&gt;, Ω-<em>P</em>&lt;sub&gt;CASDC&lt;/sub&gt;<em>-lacZ</em> integrated into the neutral platform in the genome of wild type, <em>Synechocystis</em> 6803 transformed with pHB4324</td>
<td>This study</td>
</tr>
<tr>
<td>Synechococcus sp. PCC 7942 strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synechococcus 7942</td>
<td>Wild type</td>
<td>Freshwater Algae Culture Collection at IHB</td>
</tr>
<tr>
<td>DRHB5450</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;, Synpcc7942_0628::Km&lt;sup&gt;+&lt;/sup&gt; (<em>SPDS</em> insertion mutant), <em>Synechococcus</em> 7942 transformed with pHB5450</td>
<td>This study</td>
</tr>
<tr>
<td>Strains</td>
<td>ADC</td>
<td>AUH</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Acaryochloris marina MBIC11017</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anabaena PCC7120</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anabaena variabilis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ATCC29413</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthrospira platensis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chamaesiphon minutus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cyanothece ATCC51142</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cyanothece PCC7424</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cyanothece PCC7425</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cyanothece PCC8801</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Fischerella PCC 9605</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gloeobacter violaceus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PCC7421</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptolyngbya boryana</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mastigocladopsis repens</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Microcystis aeruginosa</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NIES-843</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nostoc punctiforme</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ATCC29133</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prochlorococcus marinus**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synechococcus WH8102</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Synechococcus elongatus PCC7942</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Synechococcus CC9311</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Synechocystis PCC 6803</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Synechocystis PCC 6714</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Scytonema hofmanni</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trichodesmium erythraeum IMS101</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Because spermidine rather than spermine is one of the major polyamines in cyanobacteria, the spermine/spermidine synthase gene is tentatively abbreviated as SPDS (spermidine synthase).

**Prochlorococcus marinus includes all 12 strains listed in Cyanobase (http://genome.microbedb.jp/cyanobase/).
FIG. 1

Arginine → Aspartic α-semialdehyde → Agmatine → Putrescine

S-adenosylmethionine (SAMDC) → Decarboxylated S-adenosylmethionine

Putrescine → Carboxyspermidine → Spermidine

ADC → Arginine

Agmatine → AIH → N-carbamoyl putrescine

Aspartic α-semialdehyde (CASDH) → N-carbamoyl putrescine (NCPAH)

N-carbamoyl putrescine (NCPAH) → S-adenosylmethionine (SAMDC)

Decarboxylated S-adenosylmethionine (SPDS) → Carboxyspermidine

Spermidine (CASDC) → Aspartic α-semialdehyde
Content of spermidine (mmol. ml$^{-1}$.OD$_{730}^{-1}$)

A

B

FIG. 3
Spermidine added to the mutant RACLT (%)

**A**

- Wild type
- CASDH mutant
- Spermidine added to DRHB4239
- CASDH mutant complemented

**B**

- Wild type
- CASDC mutant
- Spermidine added to the mutant
- CASDC mutant complemented

---

**FIG. 4**
<table>
<thead>
<tr>
<th></th>
<th>Spermidine</th>
<th>Spermine</th>
<th>Cadaverine</th>
<th>Putrescine</th>
<th>Homospermidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>0.01</td>
<td>0.01</td>
<td>0.1</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>CASDC</td>
<td>0.01</td>
<td>0.1</td>
<td>1</td>
<td>0.01</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**FIG. 5**
\[ \beta\text{-galactosidase activity (OD}_{420} \text{ min}^{-1} \cdot \mu\text{l}^{-1} \cdot \text{OD}_{730}^{-1}) \]

\begin{align*}
A & \quad 30^\circ C \quad 5^\circ C \\
B & \quad 30^\circ C \quad 15^\circ C \quad 5^\circ C \\
C & \quad 30^\circ C \quad 5^\circ C \\
D & \quad 30^\circ C \quad 15^\circ C \quad 5^\circ C
\end{align*}

Relative mRNA level

FIG. 6
FIG. 7

**A**

- β-galactosidase activity
- \( \text{OD}_{420} \cdot \text{min}^{-1} \cdot \text{µl}^{-1} \cdot \text{OD}_{730}^{-1} \)
- Temperatures: 30°C, 15°C, 5°C
- Days: 1, 2, 3, 4, 5, 6, 7, 8

**B**

- Temperatures: 30°C, 15°C, 5°C
- Days: 2, 1, 2, 4, 8
- Protein bands
  - I: LacZ
  - II: Control
FIG. 8
FIG. 9

Relative mRNA level

rbp1

tsf

rpoA

slr0338

30°C  15°C  5°C  2  1  2  4  8  d

30°C  15°C  5°C  2  1  2  4  8  d
Protein carbonyl (nmol.mg protein⁻¹)
FIG. 11

RACLT (%)

0 0.3 0.6 0.9 1.2

Wild type
SPDS mutant

Spermidine added to the mutant

0 0.01 0.1 1 mM