Role of Spermidine in Overwintering of Cyanobacteria

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ABSTRACT

Polyamines are found in all groups of cyanobacteria, but their role in environmental adaptation has been barely investigated. In *Synechocystis* sp. strain PCC 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 the 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*. The protein carbonylation then quickly returned to the 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* sp. strain PCC 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 *Synechocystis* sp. strain PCC 6803 (*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: (i) cold acclimation of cells exposed to a suboptimal growth temperature, such as 22 to 24°C (9, 10) or 15°C (11) and (ii) survival under long-term (8 to 10 days) 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 *Synechocystis* 6803) under long-term chill-light stress.

Polyamines are polycationic hydrocarbon molecules with mul-

tiple 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^{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, 18), 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
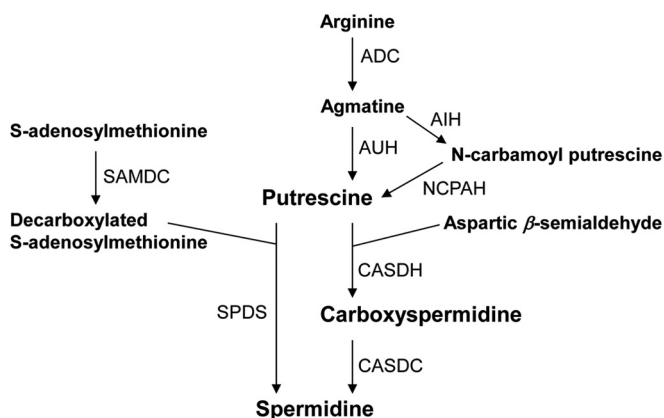
FIG 1 Pathways for synthesis of spermidine in prokaryotes. Abbreviations: ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; AUH, agmatine ureohydrolase; CASDC, carboxyspermidine decarboxylase; CASDH, carboxy-spermidine dehydrogenase; NCPAH, *N*-carbamoylputrescine amidohydrolase; SAMDC, *S*-adenosylmethionine decarboxylase; SPDS, spermidine synthase.

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, 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 the 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, from ASA and glutamate, diaminopropane (DAP) is produced via the intermediate diaminobutyrate. ASA and DAP are transformed by CASDH into carboxynorspermidine and 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 putrescine into homospermidine, in certain groups of cyanobacteria (28). Like other bacteria, cyanobacteria can take up

polyamines from the environment (29–32) and may secrete polyamines (32).

In the study of overwintering mechanism of cyanobacteria, we used microarray analysis to identify genes upregulated in *Synechocystis* 6803 exposed to chill-light stress. *CASDC*, a gene involved in synthesis of spermidine, was upregulated 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 RACLT. *Synechocystis* 6803 was a gift from J. Zhao at the Institute of Hydrobiology. The wild type and its derivative strains were grown in BG11 as previously described (33). Measurements of the relative acquired chill-light tolerance (RACLT) were performed according to the method of Yang et al. (7). Polyamines except homospermidine were purchased from Sigma Chemicals. Homospermidine was synthesized in this lab and confirmed using nuclear magnetic resonance spectroscopy. The data (means ± the standard deviations [SD]) were generated from the results of three replicate experiments.

Construction of plasmids. Molecular manipulations were performed according to standard methods or according to the manufacturers' instructions. Clones of PCR products were confirmed by sequencing. The details of plasmid construction are described in Table S1 in the supplemental material. Plasmid pHB2193 was used to inactivate *sll0873* (*CASDC* in *Synechocystis*) with a kanamycin resistance (Km^r) cassette excised from pRL446 (NCBI GenBank accession no. EU346690). Plasmid pHB4239 was used to inactivate *slr0049* (*CASDH* in *Synechocystis*) with the Km^r cassette; plasmid pHB5450 was used to inactivate *Synpcc7942_0628* (*SPDS* in *Synechococcus*) with the Km^r cassette. Plasmid pHB2972 was used to insert Ω-P$_{rbcL}$-*sll0873* into an integrative neutral platform of *Synechocystis* 6803 (34, 35) to complement the *sll0873*::Km^r mutation. Plasmid pHB4768 was used to insert Ω-P$_{slr0049}$-*slr0049* into the integrative platform to complement the *slr0049*::Km^r mutation. Plasmid pHB4324 was used to insert Ω-P$_{sll0873}$-*lacZ* into the integrative platform of *Synechocystis* 6803. Ω is a spectinomycin/streptomycin resistance cassette excised from pRL57 (36).

Generation of cyanobacterial strains. Transformation of *Synechocystis* 6803 was performed according to the method of 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 described previously by Güler et al. (37) with modifications. Fifty milliliters of *Synechococcus* cells (4 days old, optical density at 730 nm [OD$_{730}$] of ~1.2) were collected by centrifugation (4,000 rpm, 10 min), washed with 50 ml of 10 mM NaCl, and resuspended in 1.5 ml of BG11. Plasmid DNA was added to cell suspension at 2 μg of DNA/ml. After incubation overnight in the dark at 30°C on a rotating shaker, the cells were spread on BG11 plates with 5 μg of kanamycin/ml. Cyanobacterial strains are described in Table 1, and the primers are listed in Table S1 in the supplemental material.

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 v.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. The data (means ± the SD) were generated from three independent experiments, each with two 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

TABLE 1 Cyanobacterial strains

Synechocystis strain	Derivation and/or relevant characteristics[a]	Source or reference
PCC 6803		
Synechocystis 6803 (wild type)	Wild type, a glucose-tolerant strain	J. Zhao, Peking University/Institute of Hydrobiology
DRHB2193	Km^r; *sll0873*::Km^r (*CASDC* insertion mutant), *Synechocystis* 6803 transformed with pHB2193	This study
DRHB2193/DRHB2972	Km^r Sp^r, *sll0873*::Km^r complemented with wild-type *sll0873*, DRHB2193 transformed with pHB2972	This study
DRHB2193/DRHB4324	Km^r Sp^r; Ω-P$_{CASDC}$-*lacZ* integrated into the neutral platform in the genome of *CASDC* mutant, DRHB2193 transformed with pHB4324	This study
DRHB4239	Km^r; *slr0049*::Km^r (*CASDH* insertion mutant), *Synechocystis* 6803 transformed with pHB4239	This study
DRHB4239/DRHB4768	Km^r Sp^r; *slr0049*::Km^r complemented with wild-type *slr0049*, DRHB4239 transformed with pHB4768	This study
DRHB4324	Sp^r; Ω-P$_{CASDC}$-*lacZ* integrated into the neutral platform in the genome of wild type, *Synechocystis* 6803 transformed, with pHB4324	This study
PCC 7942		
Synechococcus 7942 (wild type)	Wild type	Freshwater Algae Culture Collection at the Institute of Hydrobiology
DRHB5450	Km^r; *Synpcc7942_0628*::Km^r (*SPDS* insertion mutant), *Synechococcus* 7942 transformed with pHB5450	This study

[a] Sp^r, spectinomycin resistance; Km^r, kanamycin resistance.

time points as indicated, separated by electrophoresis on an agarose-formaldehyde gel, and blotted onto an Immobilon-Ny+ membrane (Millipore) by capillary transfer. Digoxigenin-labeled DNA probes were prepared by PCR using the primer pairs sll0873n-1/sll0873n-2 and rnpBn-1/rnpBn-2. Hybridization and immunological detection were performed with a DIG High Prime DNA labeling and detection starter kit I (Roche) according to the manufacturer's protocol.

RACE, qRT-PCR, and RNA stability. Rapid amplification of cDNA ends (RACE) was performed according to the method of Zhang et al. (38). The gene-specific primer used for the first PCR was sll0873up-7, and the gene-specific primer used for the second PCR was sll0873up-2. PCR products were cloned into pMD18-T and sequenced. Quantitative reverse transcription-PCR (qRT-PCR) was performed according to the method of Gao and Xu (39). *rnpB* (RNase P subunit B) (40) was used as the internal control. RNA stability analyses were performed according to the method of Wang and Xu (41) with modifications. Cells were pretreated at 15°C for 2 days and exposed to chill-light stress for 1 days, and then rifampin was added to inhibit mRNA synthesis. Total RNA was extracted at 0, 1, and 2 h. The PCR primers are listed in Table S1 in the supplemental material.

Assays of β-galactosidase. The β-galactosidase activity of *Synechocystis* 6803 cells was measured as described by Miller (42) with modifications. Portions (1.5 ml) of cells were spun down by centrifugation (12,000 rpm, 1 min), resuspended in 1 ml of Z buffer, and permeabilized by addition of 50 μl of 0.1% sodium dodecyl sulfate (SDS) and 50 μl of chloroform, and then 200 μl of 13.28 mM ONPG (*o*-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 Na$_2$CO$_3$. Cells were removed from the reaction mixture by centrifugation (12,000 rpm, 2 min), and the absorbance at 420 nm was measured to calculate the β-galactosidase activity (OD$_{420}$ min^{-1} μl^{-1} OD$_{730}$$^{-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 phenylmethylsulfonyl fluoride (PMSF). 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. Portions (20 μg) of the proteins

were loaded into each lane. The protein concentrations were determined by the Bradford method (43).

Measurements of polyamines. Polyamines were extracted from *Synechocystis* 6803 with 5% cold HClO$_4$ and derivatized with benzoyl chloride as reported earlier (21). The derivatization of polyamine standards (Sigma Chemicals) was performed similarly. The polyamine contents were analyzed by high-performance liquid chromatography using a C$_{18}$ reversed-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 the presence of 1 mM PMSF. Cell debris was removed by centrifugation (12,000 rpm, 15 min) at 4°C. Protein carbonylation was measured spectrophotometrically by using an OxiSelect protein carbonyl spectrophotometric assay kit (Cell Biolabs).

RESULTS

Upregulation of *sll0873* (*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 the present 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 h of exposure to chill-light, including 44 genes upregulated (4°C/15°C ratio of ≥2.0) and 39 genes downregulated (4°C/15°C ratio of ≤0.5) (see Table S2 in the supplemental material).

By generating mutants of genes that are upregulated upon exposure to chill-light (denoted by *a* or *b* in Table S2 in the supplemental material) and testing their chill-light survivability, we identified *slr0083* (*crhR*) and *sll0873* (*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 to 24°C) before (9). In our study, we focused on *sll0873*, a gene predicted to encode the car-
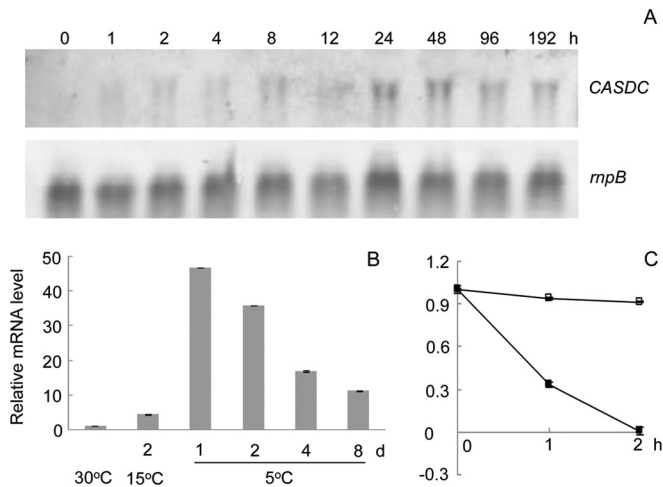
FIG 2 mRNA levels of *CASDC* in *Synechocystis* 6803 at different stages of acquisition of chill-light tolerance. (A) Northern blot analysis of expression of *CASDC* in cells exposed to chill-light stress. (B) qRT-PCR measurement of *CASDC* mRNA levels in cells grown at 30°C, treated at 15°C, and exposed to chill (5°C)-light stress. (C) qRT-PCR analysis of *CASDC* mRNA decay at 15°C (■) or under chill-light stress (□).
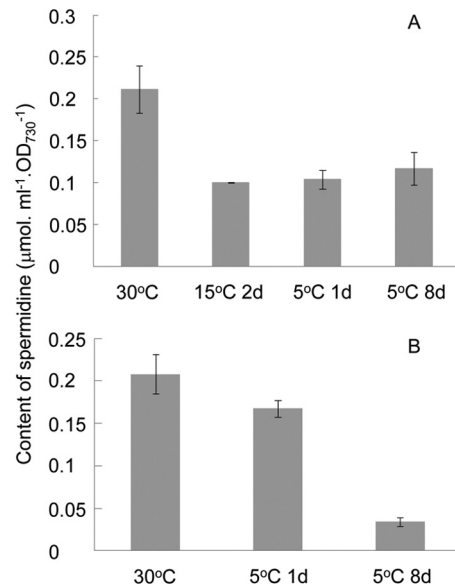

FIG 3 Content of spermidine in *Synechocystis* 6803 at different temperatures. (A) Cells grown at 30°C were treated at 15°C for 2 days before exposure to chill-light stress. (B) Cells grown at 30°C were directly exposed to chill-light stress. d, day(s).

boxy(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. qRT-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 rifampin. In cells exposed to chill light for 1 day, *sll0873* mRNA showed much more enhanced stability than 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 (see Fig. S1 in the supplemental material). Similarly, substrains of *Synechocystis* 6803 differ in tocopherol contents (7). Unlike the mRNA level of *CASDC*, the spermidine content decreased after transfer from 30 to 15°C and 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 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^r (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 (see Fig. S1 in the supplemental material). The spermidine-minus mutant lost over 50% RACLT (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*::Km^r. Spermidine was added to the culture medium at 0.01 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*::Km^r (DRHB4239). Inactivation of this gene showed similar effects on synthesis of spermidine (see Fig. S2 in the supplemental material) and chill-light survivability (Fig. 4B), and the RACLT was reduced to ca. 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 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). The addition of 0.0l or 0.1 mM spermine/homospermidine restored the RACLT of *CASDC* mutant to the wild-type level, while cadaverine and putrescine only slightly increased the RACLT (Fig. 5). Apparently, diamines cannot serve as substitutes for spermidine in promoting chill-light survivability.
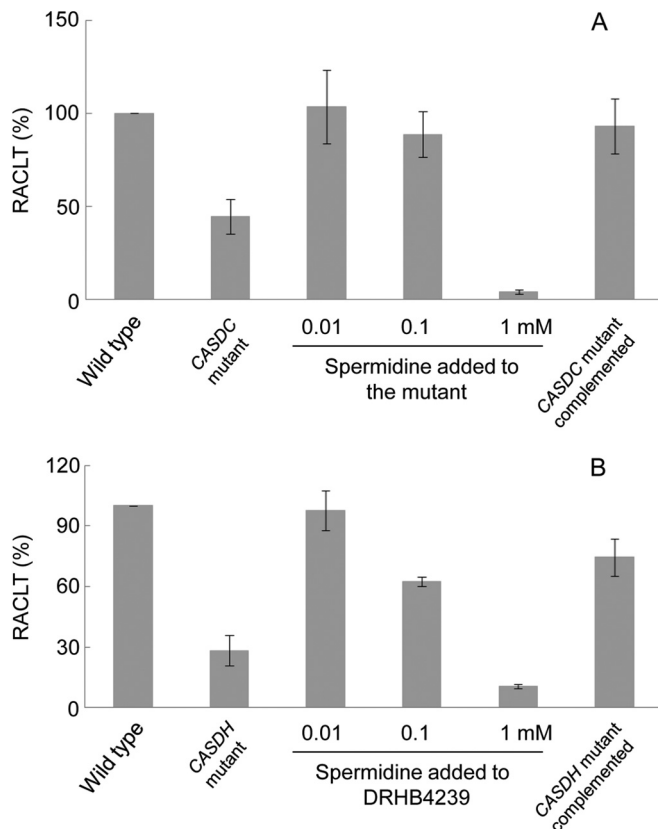
FIG 4 Effect of spermidine on the chill-light survivability of *Synechocystis* 6803. RACLT, relative acquired chill-light tolerance. (A) Requirement of *CASDC* for the chill-light survivability. (B) Requirement of *CASDH* for the chill-light survivability.

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 use the *in vitro* translation system (44) to test this hypothesis but found it technically unfeasible due to the precipitation of constituents at the low temperature. Alternatively, we used *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 324 bp ("A" at bp 1141984 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^r 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 and 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 D). 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 the transfer of cells from 30 to 15°C, *Synechocystis* 6803 *sll0873*::Km^r 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 ability 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 day 8, LacZ remained almost unchanged in the wild type with P$_{CASDC}$-*lacZ* but disappeared in the spermidine-minus strain (Fig. 7B).

The decrease in 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 results similar to when we assayed the β-galactosidase activity (Fig. 8A): the lack of spermidine led to a gradual decrease in 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
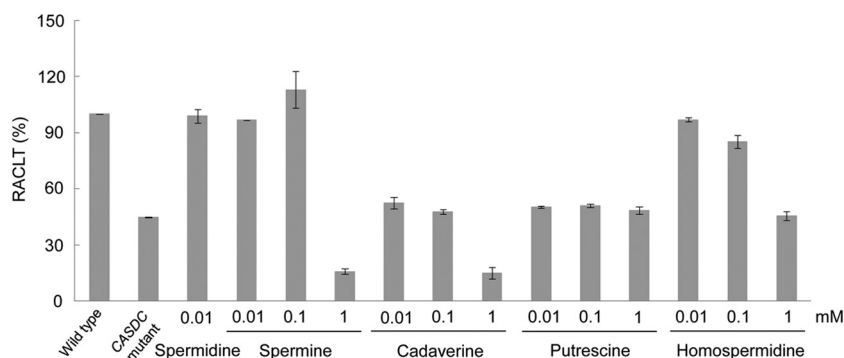

FIG 5 Effects of spermine, cadaverine, putrescine, and homospermidine on the RACLT of the *CASDC* mutant of *Synechocystis* 6803.
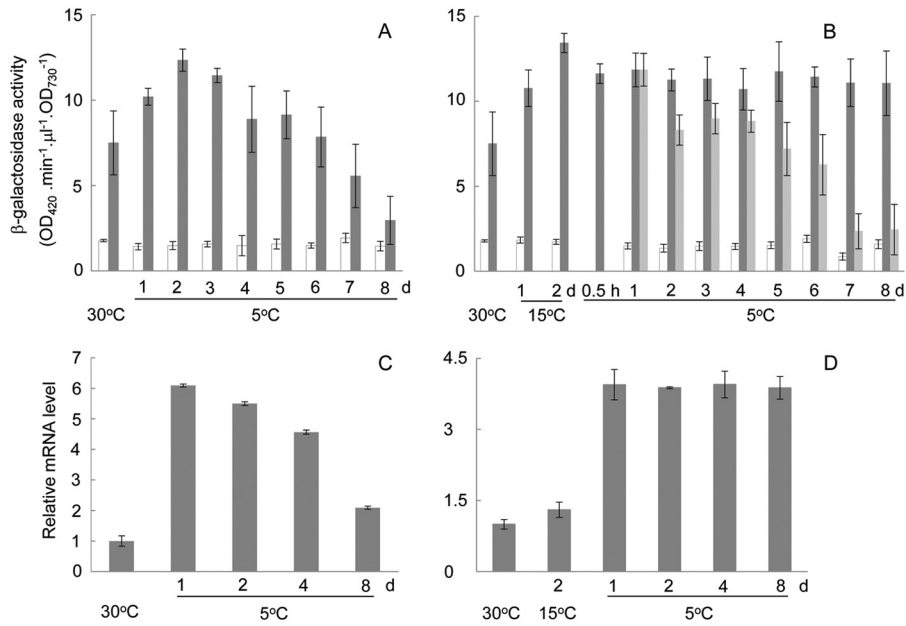
FIG 6 Expression of *lacZ* in *Synechocystis* 6803::P$_{CASDC}$-*lacZ* under the chill-light stress with (B and D) or without (A and C) preconditioning. The expression of *lacZ* was determined as shown by the β-galactosidase activity (A and B) or qRT-PCR (C, D). Bars in panels A and B: dark gray, *Synechocystis* 6803::P$_{CASDC}$-*lacZ*; light gray, *Synechocystis* 6803::P$_{CASDC}$-*lacZ* supplemented with chloramphenicol upon transfer to 5°C; open, *Synechocystis* 6803 treated in parallel (background activity).

expression under chill-light stress, we compared the relative mRNA levels of four 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 Ts (EF-Ts), RNA polymerase α-subunit, and a potential oxidoreductase, respectively. In wild-type cells transferred from 30 to 15°C and 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 (see Fig. S3 in the supplemental material). Therefore, in *Synechocystis* cells exposed to chill-light stress, spermidine enhances transcription activity rather than mRNA stability.
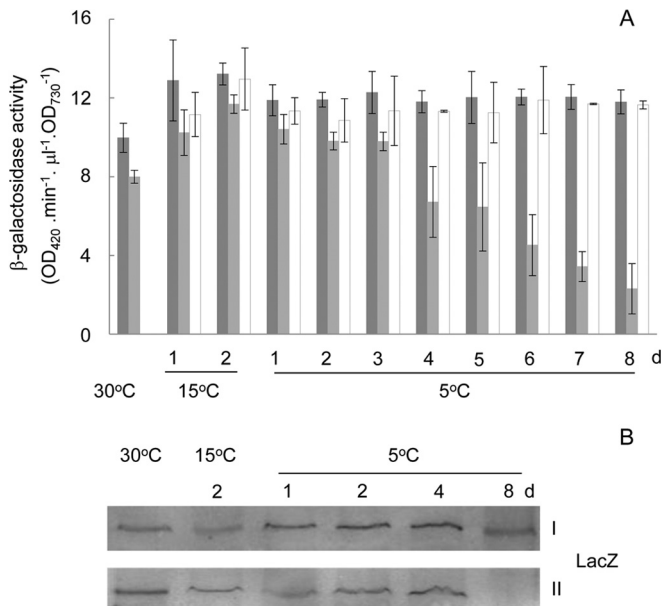

FIG 7 Assays of β-galactosidase activity and LacZ abundance showed the effect of spermidine on expression of P$_{CASDC}$-*lacZ* in *Synechocystis* strains. (A) β-Galactosidase activity in *Synechocystis* 6803::P$_{CASDC}$-*lacZ* (dark gray), *Synechocystis* 6803 DRHB2193::P$_{CASDC}$-*lacZ* without (light gray), or with (open) exogenous spermidine. (B) Western blot detection of LacZ in *Synechocystis* 6803::P$_{CASDC}$-*lacZ* (row I) and *Synechocystis* 6803 DRHB2193::P$_{CASDC}$-*lacZ* (row II). DRHB2193, the *CASDC* mutant.
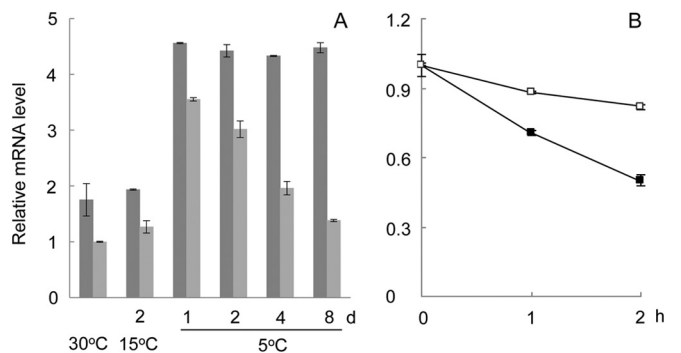

FIG 8 Measurements of *lacZ* mRNA level showing the effect of spermidine on expression of P$_{CASDC}$-*lacZ* in *Synechocystis* strains. (A) qRT-PCR analysis of *lacZ* mRNA levels in *Synechocystis* 6803::P$_{CASDC}$-*lacZ* (dark gray) and *Synechocystis* 6803 DRHB2193::P$_{CASDC}$-*lacZ* (light gray). (B) qRT-PCR analysis of *lacZ* mRNA decay in *Synechocystis* 6803::P$_{CASDC}$-*lacZ* (■) and *Synechocystis* 6803 DRHB2193::P$_{CASDC}$-*lacZ* (□) under chill-light stress. DRHB2193, the *CASDC* mutant.
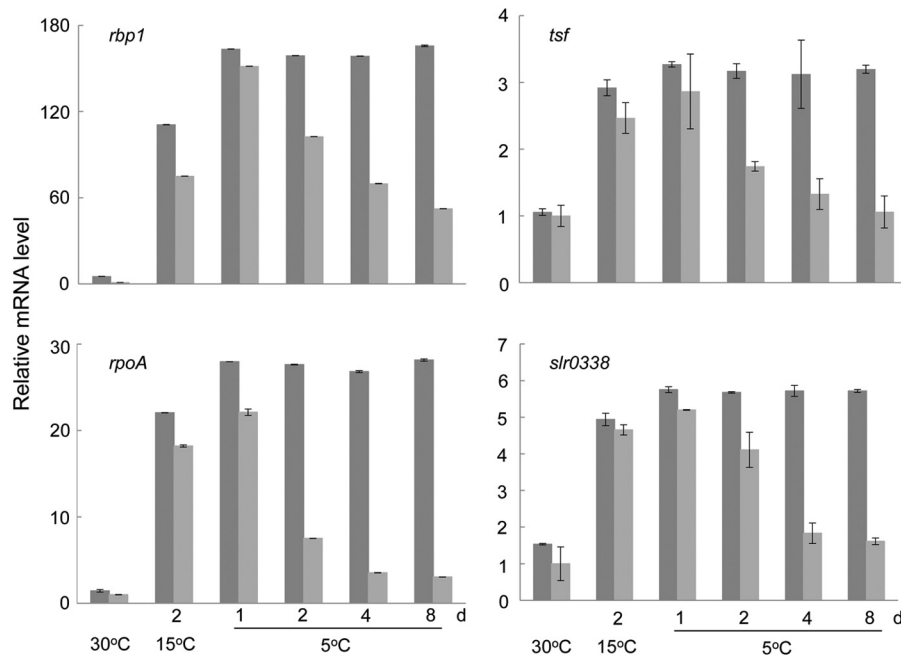
FIG 9 qRT-PCR measurement of mRNA levels showing the role of spermidine synthesis in gene expression under chill-light stress. Bars: dark gray, *Synechocystis* 6803; light gray, the *CASDC* mutant. *rbp1*, RNA-binding protein 1; *tsf*, EF-Ts; *rpoA*, α subunit of RNA polymerase; *slr0338*, probable oxidoreductase.

Unlike LacZ, the abundance of Rbp1 and EF-Ts remained unchanged within 8 days of chill-light stress in strains with or without spermidine (see Fig. S4 in the supplemental material). 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 O$_2$ 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, the 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 to 15°C and 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*::Km^r). In this mutant, spermidine was no longer synthesized, but putrescine was detected at a low level (see Fig. S5 in the supplemental material). Elimination of spermidine from *Synechococcus* 7942 reduced its RACLT to ca. 40%, and the addition of 0.1 mM spermidine to the medium restored the RACLT to ca. 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 the present 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 path-
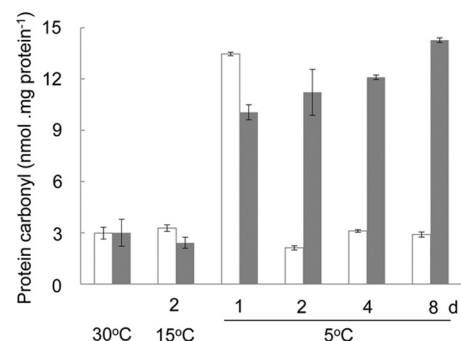

FIG 10 Comparison of protein carbonylation in the wild type and the *CASDC* mutant of *Synechocystis* 6803. Bars: open, wild type; gray, *CASDC* mutant.

TABLE 2 Distribution of genes involved in spermidine synthesis in cyanobacteria

Strain	Presence (+) or absence (−) of gene for:							
	ADC	AUH	AIH	NCPAH	SAMDC	SPDS[a]	CASDH	CASDC
Acaryochloris marina MBIC11017	+	+	+	+	−	+	−	−
Anabaena sp. PCC 7120	+	+	−	−	−	−	−	−
Anabaena variabilis ATCC 29413	+	+	−	−	−	−	−	−
Arthrospira platensis	+	+	−	−	+	+	−	−
Chamaesiphon minutus	+	+	−	−	−	−	+	+
Cyanothece sp. ATCC 51142	−	−	−	−	−	−	−	−
Cyanothece sp. PCC 7424	+	+	−	−	+	+	−	−
Cyanothece sp. PCC 7425	+	−	+	+	−	−	−	−
Cyanothece sp. PCC 8801	+	−	+	+	+	+	−	−
Fischerella sp. PCC 9605	+	+	−	−	+	+	−	−
Gloeobacter violaceus PCC 7421	+	−	+	+	−	−	−	−
Leptolyngbya boryana	+	−	−	−	−	−	+	+
Mastigocladopsis repens	+	+	−	−	+	+	−	−
Microcystis aeruginosa NIES-843	+	+	−	−	+	+	−	−
Nostoc punctiforme ATCC 29133	+	+	−	−	−	−	−	−
Prochlorococcus marinus[b]	+	+	−	−	+	+	−	−
Synechococcus sp. WH8102	+	+	−	−	+	+	−	−
Synechococcus elongatus PCC 7942	+	−	+	+	+	+	−	−
Synechococcus sp. PCC 9311	+	+	−	−	+	+	−	−
Synechocystis sp. PCC 6803	+	+	−	−	−	−	+	+
Synechocystis sp. PCC 6714	+	+	−	−	−	−	+	+
Scytonema hofmanni	+	+	−	−	+	+	+	+
Trichodesmium erythraeum IMS101	+	+	−	−	+	+	−	−

[a] 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).

[b] *Prochlorococcus marinus* includes all 12 strains listed in the Cyanobase (http://genome.microbedb.jp/cyanobase/).

way in bacteria (15), spermidine synthesis in *Synechocystis* 6803 was thought to occur 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 the 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. [27], with no information of axenicity of these strains).
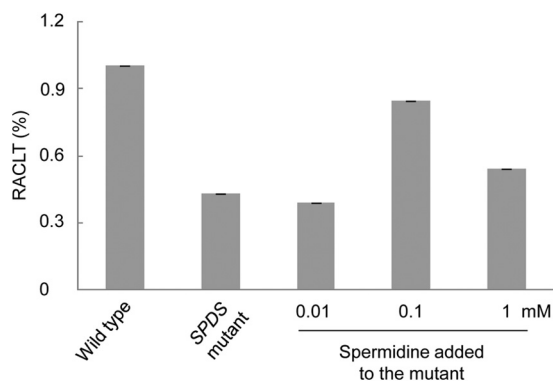

FIG 11 Effect of spermidine on chill-light survivability of *Synechococcus* 7942.

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 the 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 one strain that lacks both AUH and AIH/NCPAH (*Leptolyngbya boryana*) and one strain that lacks all of the enzymes involved in spermidine synthesis (*Cyanothece* sp. strain ATCC 51142). 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 upregulation of *CASDC* gene in response to chill and light. Preconditioning at 15°C enabled *Synechocystis* cells to maintain a much higher level of spermidine after 8 days of chill-light stress. Similar effects of preconditioning had been found on α-tocopherol (7) and Rbp1 (12) in *Synechocystis*. A downshift of the temperature to 5°C greatly increased the stability of *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 a slowed uptake of nitrate (46) because the 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 *CASDC* were not upregulated.

Our results clearly indicated that spermidine promoted the chill-light survivability. Inactivation of either *CASDH* or *CASDC* in *Synechocystis* 6803 or *SPDS* in *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 *Synechocystis* *CASDC* mutant could be restored by spermine (a tetramine) and spermidine and homospermidine (triamines) but not putrescine and cadaverine (both 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 P_{CASDC}-*lacZ* into the wild type and the *CASDC* mutant of *Synechocystis* 6803. After preconditioning, the wild type with P_{CASDC}-*lacZ* showed a high level of β-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 in β-galactosidase activity to a very low level. On one hand, this showed the persistence of protein synthesis activity in *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 the β-galactosidase activity, the abundance of *lacZ* mRNA decreased without compromised mRNA stability, and examination of the mRNA abundance for *tsf*, *rbp1*, *rpoA*, and *slr0338* showed similar results. The decrease in 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 to 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 its 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 the 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 a *Synechocystis* spermidine-minus background, the transcrip-

tion activity (as seen with mRNA abundance in Fig. 8 and 9) and β-galactosidase activity (Fig. 7) continued to decrease within 8 days, and LacZ was even completely degraded between days 4 and 8 (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, 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

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