Characterization of the role of a mechanosensitive channel in osmotic down shock adaptation in Synechocystis sp PCC 6803

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**Abbreviations:** MS, mechanosensitive; Spe, spectinomycin; TM, thylakoid membrane; PM, plasma membrane

Synechocystis sp strain PCC 6803 contains one gene encoding a putative large conductance mechanosensitive channel homolog [named SyMscL (slr0875)]. However, it is unclear whether SyMscL contributes to the adaptation to hypoosmotic stress in Synechocystis. Here we report the in vivo characteristics of SyMscL. SyMscL was mainly expressed in the plasma membrane of Synechocystis. Cell volume monitoring using stopped-flow spectrophotometry showed that ΔsymscL cells swelled more rapidly than wild-type cells under hypoosmotic stress conditions. Expression of symscL was under circadian control, and its peak corresponded to the beginning of subjective night. These results indicate that SyMscL functioned as one component of the osmotic homeostatic regulatory system of the cell coordinating the response of Synechocystis to daily metabolic osmotic fluctuations and environmental changes.

**Introduction**

Microorganisms have developed special adaptation mechanisms to survive under different environmental stress conditions, such as shifts in temperature, pH or extracellular osmotic pressure. Many studies have focused on the response to osmotic stress in *Escherichia coli* and in lactic acid bacteria.1,5 Upon an increase in external osmolarity (osmotic-upshift), water flows out of the cells and plasmolysis occurs. To maintain intracellular turgor pressure, microbes increase their content of K+ and other osmolytes (glutamate, betaine, proline and trehalose) in the cytosol.1,6,7 In contrast, a decrease in external osmolarity (osmotic-downshift) triggers passive influx of water, leading to an increase in turgor pressure. To achieve a rapid reduction of the turgor pressure under these conditions, mechanosensitive (MS) channels are used to release excess cytoplasmic solutes.8 MS channels are classified according to their single-channel properties as mechanosensitive ion channels with large conductance (MscL; TC 1.A.22), with small conductance (MscS; TC 1.A.23), with mini conductance (MscM) or with potassium-dependent conductance (MscK). There is a specific correlation between the size of the conductance and the mid-point of activation of each MS channel type. Based on this characteristic, these channels are thought to function in response to osmotic challenges of different magnitudes. Both MscL and MscS, the two major MS channels, are known to sense tension within the membrane. These two channels are distinct in their structural and functional properties. The MscL channel is highly conserved with only a single gene found in many bacteria. The MscL protein of *E. coli* has two transmembrane (TM) helices and forms a homopentameric channel with ten TMs.9 It seems to be activated as a final survival mechanism. The MscS family in contrast is quite diverse and a single bacterium may encode multiple MscSs in its genome. The MscS proteins are predicted to have a different topology and be expressed and/or activated under distinct environmental condition from MscL.10

The cyanobacterium Synechocystis sp strain PCC 6803 is a unicellular photosynthetic prokaryote that can survive a wide range of environmental changes.13 Since the determination of the nucleotide sequence of its genome,14 Synechocystis has been considered a model photosynthetic microorganisms for studying the molecular response mechanisms to various stresses.11,13 Synechocystis contains nine genes encoding putative MS channels.14 One of them, *symscL* (slr0875), is the single gene encoding a large-conductance MS channel (MscL) in the Synechocystis genome. Nazarenko et al. reported that *symscL* encodes a protein involved in calcium release in response to plasma membrane depolarization during temperature stress.15 However, the physiological role of any of these putative MS channels during hypo-osmotic stress, has not yet been examined. In this study we focused on the role of SyMscL in the osmotic down shock adaptation mechanism of Synechocystis.
We determined the subcellular localization of the SyMscL protein in *Synechocystis* as well as the circadian expression pattern of the *symscL* gene. We also studied the effects of loss of function of SyMscL on cell volume changes during hypo-osmotic stress. The results obtained in this study indicate that SyMscL contributes to improving survival of the cells during osmotic downshock stress imposed by daily environmental changes.

**Results**

Thylakoid and plasma membrane fractions were prepared from wild-type *Synechocystis* cells by aqueous polymer two-phase partitioning followed by sucrose density gradient centrifugation. The subcellular localization of SyMscL was then determined by western blot using antibodies specific for SyMscL. As shown in Figure 1A, the majority of SyMscL protein was detected in the plasma membrane fraction, validated by the presence of the plasma membrane nitrate transporter NrtA. The small amount of SyMscL protein detected in the thylakoid membrane fraction, which contained the thylakoid marker proteins NdhD3 and NdhF3, was most likely due to a slight contamination with plasma membrane. This indicates that SyMscL was mainly present in the plasma membrane in *Synechocystis*.

The contributions of SyMscL to cell volume recovery in *Synechocystis* was evaluated using stopped-flow spectrophotometry measurements. With this method a decrease in light scattering corresponds to an increase in cell volume. To compare the cellular volume recovery rate between the wild type and the mutant cells lacking SyMscL, a *Synechocystis* Δ*symscL* (Δslr0875) strain was generated by insertion of a Spe resistance cassette into *symscL* (Fig. 1B). Disruption of the gene was confirmed by PCR (Fig. 1C) and absence of the SyMscL protein was confirmed by western blot using an anti-SyMscL antibody (Fig. 1D). Figure 2 shows the analysis of the kinetics of the swelling rate of wild-type *Synechocystis* and the deletion mutants (Δ*symscL*) in response to osmotic downshock from medium containing sorbitol. The time constants (τ) determined by fitting a curve to the data were 21.06 ± 0.74 ms (wild type) and 8.69 ± 0.80 ms (Δ*symscL*) (average ± SD, n = 3 for each, p < 0.01 (Student’s t test)). This clearly showed that the decrease in light scattering due to hypoosmotic swelling was considerably faster in the Δ*symscL* than in the wild type, i.e., the Δ*symscL* cells were swelling more rapidly. The light scattering profile of the Δ*symscL* cells (Fig. 2B) also showed an increase in scattered light intensity (broken line) after the initial rapid decrease, which may indicate a bursting of the cells due to a lack of SyMscL-mediated osmolyte efflux. As a control experiment, the cells were mixed with the same media (BG11 containing 1 M sorbitol); in this case with no change in osmolality no significant change of cell volume was observed (Fig. 2) for both strains.

*Synechocystis* has an internal biological clock, which maintains an approximately 24-h periodicity of global gene expression patterns. Since the cellular osmolarity of photosynthetic bacteria is likely to change in response to regular daily changes in the environment, which would not be relevant in *E. coli*, the expression of *symscL* may be under control of the circadian clock. Hence we examined the circadian oscillation of *symscL* expression in *Synechocystis* using a real-time bioluminescence monitoring system. Cells expressing bacterial luciferase driven by the promoter of *kaiA*, a gene encoding an endogenous oscillator, were used as controls. The expression of the *symscL* gene showed circadian oscillation (Fig. 3). The circadian period (wavelength of the cosine curve) of the cells containing *P* *symscL*::luxAB was 22.9 ± 0.1 h (average ± SD, n = 12), which is very close to the standard period (22.8 h) of *Synechocystis* and to the period of *kaiA* expression (23.0 ± 0.1 h, n = 6) (Table 1). The peak-phase of the expression of *symscL* was at circadian time (CT) 13.8 ± 0.2 h (n = 12), which corresponds to the beginning of subjective night, whereas the peak-phase of the expression of *kaiA* was at CT 7.0 ± 0.2 h (n = 6) corresponding to the middle of the day.

**Discussion**

*Synechocystis* cells lacking the large conductance MS channel SyMscL were impaired in their ability to control cell volume changes during osmotic downshock, i.e., their cells swelled more rapidly than those of the wild type as revealed by stopped-flow
spectrophotometry measurements (Fig. 2). Moreover, only in ΔsymscL cells an increase in light-scattering following the initial decrease was observed (Fig. 2B, broken line). A possible explanation for this observation is that lack of SyMscL function and thereby lack of controlled osmolyte efflux eventually lead to bursting of the cells. This suggests that even though there are nine genes encoding putative mechanosensitive channels in the Synechocystis genome, SyMscL is a strong contributor to in vivo osmoadaptation, a process that is a regulated response to naturally occurring daily environmental changes. Further evidence for this physiological role of SyMscL was the finding that SyMscL was localized to the plasma membrane (Fig. 1A). Overall these results are consistent with SyMscL functioning as part of the MS machinery in the plasma membrane that is responsible for the release of cytoplasmic solutes; similar to the role of EcMscL in E. coli.22 On the other hand, Moe et al. reported that SyMscL is the most divergent of the known MscL proteins, with a highly distinct hydrophilic carboxy terminus.23 It has been reported that MscL proteins cloned from several different bacteria are similar in their channel properties; the pressure dependence recorded for these homologous proteins is about 1.5 times larger than that required to activate the EcMscS channel.24 However, SyMscL requires three times higher pressure for its activation than EcMscS. In addition, it has been reported that an E. coli mutant lacking EcMscL has no apparent physiological phenotype,24 although in patch-clamp recordings, EcMscL displays a non-selective conductance of ~3 nanosiemens (nS).22 These data suggest that SyMscL may have evolved as an adaptation to survival in a harsher environment with respect to hypooosmolarity stress. Nazarenko et al. reported that SyMscL functions as an outward calcium channel under temperature stress conditions and has a role in regulating cytosolic calcium homeostasis.15 SyMscL therefore seems to have important roles during a wide range of environmental changes.

Osmoadaptation mechanisms are likely to be essential for the survival of photosynthetic microorganisms like Synechocystis, because they may be exposed to daily rhythmic changes in temperature, humidity, salinity and UV light intensity. The biological circadian control system of the cells must not only respond to these changes but also be prepared for such conditions in advance.27 The finding that symscL expression was under control of the circadian clock (Fig. 3; Table 1) suggests an involvement of SyMscL in the osmotic homeostatic regulatory system of the cell. The circadian clock in Synechocystis plays an important role in the response to environmental changes occurring during dark and light periods, something which is not relevant in E. coli.28 The peak of symscL expression at the beginning of subjective night at CT 13.8 h (Fig. 3; Table 1) was similar to the peaks of the expression of the Na+/H+ antiporter NhaS3 (CT 12.5 h)29 and of the aquaporin AqpZ (CT 15.3 h),30 which are both involved in cellular osmo-adaptation and/or glucose metabolism. Considering the daily metabolism of photosynthetic bacteria, we propose that decomposition and conversion of photosynthetic compounds produced during the daytime may result in changes of the intracellular osmolarity followed by stretch-activation of the cellular membrane at the beginning of the night. Adjustment of the intracellular osmolarity via SyMscL therefore contributes to supporting the maintenance of the daily carbon metabolism as well as assisting the survival of cyanobacteria subjected to a wide range of environmental changes.

**Material and Methods**

**Cells and growth conditions.** Synechocystis strain sp PCC 6803 and mutant cells were grown at 29°C in BG11 medium containing 20 mM TES/KOH (pH 8.0).30 Continuous illumination...
Table 1. Circadian bioluminescence rhythms in the P<sub>symscL::luxAB</sub> reporter strain

| Strain         | Period (h) | Peak phase (h) | CT<sup>†</sup> of peak phase (h) | Amplitude | Average bioluminescence (cps)<sup>‡</sup> | N   |
|----------------|------------|----------------|----------------------------------|-----------|------------------------------------------|-----|
| P<sub>luxAB::luxAB</sub> | 23.0 ± 0.1 | 6.7 ± 0.1      | 7.0 ± 0.1                        | 1.18 ± 0.02 | 56 ± 25                                   | 6   |
| P<sub>symscL::luxAB</sub> | 22.9 ± 0.1 | 13.2 ± 0.2     | 13.8 ± 0.2                       | 1.30 ± 0.01 | 728 ± 161                                 | 12  |

*Values are summaries of the results shown in Figure 3. 'CT, circadian time. †Mean intensity of bioluminescence rhythms from time 0 h to time 192 h.

Figure 3. Circadian rhythm of <i>symscL</i> expression in <i>Synechocystis</i>. Representative circadian oscillation profiles of bioluminescence measurements from <i>Synechocystis</i> strains containing P<sub>symscL::luxAB</sub> or P<sub>luxAB::luxAB</sub> reporter gene are shown. Essentially the same profiles were obtained in three independent experiments. Detailed values are listed in Table 1. The vertical axis indicates the intensity of bioluminescence (cps (counts per second) per colony). White and gray boxes in the lower panel represent subjective day and subjective night, respectively. The bottom panel shows a detail of the bioluminescence measurement from <i>Synechocystis</i> strains containing P<sub>symscL::luxAB</sub> from 0–72 h. Each point with error bar indicates mean with standard deviation from 6 (P<sub>luxAB::luxAB</sub>) or 12 (P<sub>symscL::luxAB</sub>) independent samples as shown in Table 1.

An anti-SyMscL rabbit polyclonal antibody was prepared by Operon using synthetic polypeptides (CDSINRLITT LENQQ) as antigens. Polyclonal antibodies raised against the plasma membrane nitrate transporter NrtA<sup>†</sup> or against the thylakoid membrane proteins NdhlD<sub>3</sub> and NdhlF<sub>1</sub> were used as markers for the plasma membrane fraction or the thylakoid membrane fraction in <i>Synechocystis</i>, respectively.

Construction of <i>Synechocystis</i> MS channel deletion strains. <i>Synechocystis ∆symscL</i> (str0875) strain was generated by insertion of a Spe resistance cassette into <i>symscL</i>.<sup>31</sup> The transformant was grown on BG11 solid medium supplemented with Spe (20 μg/ml) buffered at pH 8.0 at 29°C. The disruption of symscL in the transformants after homogeneity segregation by successive-streak was confirmed by PCR amplification. In addition, the symscL gene in each mutant was amplified by PCR and sequenced to confirm the correct disruption.

Stopped-flow spectrophotometry of cell suspensions. <i>Synechocystis</i> cells were cultured in BG11 for 1 week at 29°C. Then these pre-cultures were diluted to an OD<sub>750</sub> of 0.05 with the same medium. The cells were allowed to grow at 29°C to an OD<sub>750</sub> of 0.8. For osmotic-downshock, the cells were centrifuged at 4°C and resuspended in BG11 medium containing 1 M sorbitol, and the OD<sub>750</sub> of the suspensions was adjusted to 17. The cell suspensions were incubated for 2 h at 29°C. Stopped-flow spectrophotometry was performed on a temperature-controlled stopped-flow apparatus with a dead time of < 0.5 ms (Unisoku). The cell suspension (100 μl) was mixed in the stopped-flow device with 100 μl of BG11 medium. The changes in 90° light scattering resulting from changes in cell volume were recorded at 575 nm. Averaged data from multiple determinations were fitted to single or double exponential curves with the aid of IGOR Pro 5.03J software (WaveMetrics).

Measurement of circadian rhythm of <i>symscL</i> promoter activity in <i>Synechocystis</i>. A 1000 bp symscL promoter sequence (P<sub>symscL</sub>) was fused to the bacterial luciferase gene set luxAB at the BglIII and Nde I sites of p68TS1.1ΩLuxAB/PLNK (Onai and Ishiura, unpublished) carrying adaA as a selectable marker in <i>Synechocystis</i>, and the construct was inserted into the TS1 region in the <i>Synechocystis</i> genome by homologous recombination.<sup>32</sup> Cells were grown to colonies on solid BG11 at 30°C under white fluorescent lamps (42 μmol of photons·m<sup>−2</sup>·s<sup>−1</sup>) placed in the dark for 2 h to reset the circadian clock and then kept under continuous light conditions. Bioluminescence from colonies was measured automatically every hour as described previously.<sup>33,34</sup> using a commercially available automated bioluminescence-monitoring apparatus (K. Onai, Y. Hasegawa, N. Siraki and M. Ishiura, unpublished; model CL24-W; Churitsu Electric Corp.) with a robotic plate conveyor (K. Onai, N. Siraki and M. Ishiura, unpublished; model CI-08L; Churitsu Electric Company).

Membrane localization of SyMscL in <i>Synechocystis</i>. Thylakoid membrane and plasma membrane fractions were prepared from <i>Synechocystis</i> cells as described previously.<sup>20,21</sup> For SDS-PAGE 30 μg of protein from either the plasma membrane or the thylakoid membrane fraction was used. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by semidry electrophoretic blotting (Bio-Rad Laboratories). The PVDF membranes were incubated for 12 h with the primary antibody (1:1000 in blocking buffer) at 4°C, followed by incubation for 1 h with the secondary antibody [horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Pharmacia, 1:1000)] and subsequently developed by chemiluminescence detection (SuperSignal, Thermo Scientific).
Corps.). Bioluminescence data was analyzed using a commercially available bioluminescence-analyzing software (Onai, Shiraki and Ishiura, unpublished; SL00-01; Churitsu Electric Corp.). Circadian time (CT) was calculated by dividing the peak-phase value by the period and multiplying by 24. Amplitude of rhythms was calculated as average ratios of peak to trough in each cycle.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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