Comparative Analysis of kdp and ktr Mutants Reveals Distinct Roles of the Potassium Transporters in the Model Cyanobacterium Synechocystis sp. PCC 6803

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Running Head: Distinct roles of Kdp and Ktr in Synechocystis

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ABSTRACT

Photoautotrophic bacteria have developed mechanisms to maintain K\(^+\) homeostasis under changing ionic concentrations in the environment. *Synechocystis* sp. PCC 6803 contains genes encoding for a well-characterized Ktr-type K\(^+\) uptake transporter (Ktr) and for a putative ATP-dependent transporter specific for K\(^+\) (Kdp). The contributions of each of these K\(^+\) transport systems to cellular K\(^+\) homeostasis have not yet been defined conclusively. To verify the functionality of Kdp, *kdp* genes were expressed in *E. coli*, where Kdp conferred K\(^+\) uptake albeit with lower rates than that of Ktr. The on-chip microfluidic device enabled to monitor the biphasic initial volume recovery of single *Synechocystis* cells after hyperosmotic shock. Here, Ktr functioned as the primary K\(^+\) uptake system during the first recovery phase, whereas Kdp did not significantly contribute. Expression of the *kdp* operon was induced by extracellular K\(^+\) depletion in *Synechocystis*. Correspondingly, Kdp-mediated K\(^+\) uptake supported the cell growth at trace amounts of external potassium. This induction of *kdp* expression depended on two adjacent genes, *hik20* and *rre19*, encoding for a putative two-component system. The circadian expression of *kdp* and *ktr* peaked at subjective dawn, which may support the acquisition of K\(^+\) required for the regular diurnal photosynthetic metabolism. These results indicate that Kdp contributes to maintain a basal intracellular K\(^+\) concentration at limited K\(^+\) in natural environments, whereas Ktr mediates fast potassium movements in the presence of higher K\(^+\) availability. Through their distinct activities both Ktr and Kdp coordinate the responses of *Synechocystis* to changes in K\(^+\) levels under fluctuating environmental conditions.
INTRODUCTION

Living cells have developed specific responses to hyperosmotic shock. Upon exposure to this stress cells initially lose water and their volume shrinks. In all living cells, K$^+$ is the major intracellular cation used for the maintenance of turgor pressure, cytosolic osmolarity, protein structuring, and membrane potential (1-3). In contrast to animals, Na$^+$/K$^+$-ATP pumps are generally missing in bacteria and plants. Hence, these cells possess K$^+$ uptake transporters to supply K$^+$ to the cells. Particularly after hyperosmotic stress, cells quickly take up K$^+$ from the medium to increase the intracellular osmolarity, which prevents water efflux from the cell. Data from genetic and biochemical experiments indicate that the activity and the expression of these transporters respond to hyperosmotic stress. In the later phase of acclimation to hyperosmotic stress, cells also induce the synthesis of osmoprotective molecules such as glutamate, trehalose, proline, and glucosylglycerol (4, 5). Despite an increasing amount of data on cellular osmoregulation involving ion flux across the membrane, direct evidence for the involvement of specific transporters in the cellular response to osmotic up-shock is lacking for photoautotrophic organisms.

The cyanobacterium Synechocystis sp. PCC 6803 (hereafter referred to as Synechocystis) is a frequently used unicellular photosynthetic prokaryote that can survive a wide range of environmental conditions (6). Unlike E. coli, Synechocystis possesses an internal thylakoid membrane system, which is mainly involved in photosynthesis. Based on their membrane structure and the nature of their K$^+$ selective filters, prokaryotes express three families of K$^+$ uptake transporters, Ktr/Trk/HKT, Kup/HAK/KT, and Kdp (7-12). Genes coding proteins of the Kup family are absent from the Synechocystis genome, while it contains K$^+$ transporter homologs Ktr and Kdp as well as the characterized K$^+$ channels, SynK and SynCaK (13-16). Previous studies have shown that the Ktr system is essential for the acclimation of the Synechocystis cell to...
osmotic stress caused by either ionic compounds like NaCl or non-ionic compounds like sorbitol (17, 18). The recent study on the transcription level of ktr and kdp in Staphylococcus aureus revealed that both transporters are active systems in the acclimation towards salt stress (19, 20). These studies indicate that the Ktr system plays the dominant role in the accumulation of K\(^+\) as a quick response to hyperosmotic shock.

In *E. coli*, the K\(^+\) refill at high osmolality is performed by the Kdp transporter, a P-type ion-translocating ATPase that has been characterized in great detail (7, 21-25). Kdp is an inducible K\(^+\) uptake system with high affinity and specificity for K\(^+\) (24, 26). It consists of four subunits that are encoded by the *kdpFABC* operon in *E. coli*. KdpA binds and transports K\(^+\), and KdpB catalyzes the hydrolysis of ATP in cooperation with KdpC. The gene products of the *kdpDE* operon have a regulatory role and form a typical two component system (26). The tandem gene arrangement of two component system and *kdp* operon was also found in *Staphylococcus aureus* (19, 27). The Kdp system functions as an emergency K\(^+\) uptake system that is only expressed when the cell experiences osmotic stress or K\(^+\) limitation and becomes rapidly degraded when no longer required (28). In *Synechocystis*, the Kdp homologous proteins are encoded by the *kdpABGCD* operon. KdpA is predicted to be a membrane protein containing K\(^+\)-conducting pore regions (8). Growth tests using a *kdpA* mutant showed that in *Synechocystis* KdpA seems to play only a minor role in K\(^+\) uptake compared to the Ktr-type transporter (17). The regulatory two component system of the *Synechocystis* Kdp differs in its arrangement from the well-characterized situation in *E. coli*, since the histidine kinase KdpD seems to be split into two separate parts. The same situation has been observed in the desiccation-tolerant strain *Anabaena* sp. L-31(28). In contrast to *Synechocystis*, this cyanobacterial strain harbors two operons encoding components of Kdp. Only one of these operons also includes a gene for the truncated KdpD. Thus, various composition of two component system of Kdp has been found in
Osmoadaptation of the cells including cyanobacteria to external hyperosmotic shock entails solute flux across the plasma membrane causing the sequential cell volume changes (4, 29). Conventional experimental batch-culture approaches provide an average of many individual responses from many different cells, some of which may not actually undergo osmoadaptation. In this study, to dissect the individual contributions of the Ktr and Kdp transport systems under rapidly changing environmental conditions, we developed a microfluidic device that can be used to monitor discrete dynamic processes in single cells in real-time. These studies revealed the low importance of kdp in the acclimation to hyperosmotic stress. Therefore, we characterized the kdp operon of *Synechocystis* in more detail, studied its circadian expression and determined the physiological functions of Ktr and Kdp by heterologous expression in *E. coli* as well as through analysis of *Synechocystis* mutants.

**MATERIALS AND METHODS**

**Cells and growth conditions.** *Synechocystis* sp. PCC 6803 and mutant cells were grown at 30°C in BG11 medium containing 20 mM TES-KOH (pH 8.0) (18). Solid medium consisted of BG11 buffered at pH 8.0, 1.5% agar or agarose, and 0.3% sodium thiosulfate. Continuous illumination was provided by fluorescent lamps (50 μmol of photons m⁻² s⁻¹; 400–700 nm). Transformants of *E. coli* LB2003 (*F*, *thi*, *lacZ*, *gal*, *rha*, ΔkdpFABC5, trkΔ1, ΔtrkA) were cultured in a medium consisting of 1% tryptone, 0.5% yeast extract, 30 mM KCl, 1% glucose, 0.25 mM IPTG and the required antibiotics at 30°C (30).

**Microfluidic analysis.** The microfluidic device consisted of a cover slip made of polydimethylsiloxane (PDMS) and hydrogel cages on a glass slide. The cover slip was produced by replica molding with a master mold fabricated by photolithography. The positive type
photoresist (OFPR-800, Tokyo Ohka Kogyo) was spin-coated onto the silicon wafer (1000 rpm, 20 s), prebaked at 90°C for 15 min, and irradiated by UV light (wavelength: 405 nm) using a mask aligner (MA6, SÜSS MicroTec AG). Then the UV-irradiated area of the OFPR was removed by the NMD-3 reagent (Tokyo Ohka Kogyo) and rinsed with deionized water. Final thickness of the OFPR was 2 μm. The developed pattern was baked at 145°C for 30 min for hardening, and used as a master mold for the following steps. The PDMS (SILPOT 184 and CATALYST SILPOT 184, Toray) was poured into the master mold and hardened in an oven at 90°C for 15 min. Then the PDMS replica was peeled from the master mold. For the connection with tubing and a syringe pump holes were made on the PDMS patterned cover slip. The hydrogel cages on the glass were produced by photolithography. The 4.0% (w/v) photo-crosslinkable polymer (azide-unit pendant water-soluble photopolymer: AWP, Toyo Gosei) was spin-coated (500 rpm, 10 s) onto the glass preheated at 145°C for 10 s, baked at 65°C for 20 min and irradiated by UV light with photomask using the mask aligner to harden the photo-exposed AWP. The resultant hydrogel cages on the glass were cleaned with deionized water. Thickness of the hydrogel was 2-2.5 μm. Then, the PDMS cover slip and the hydrogel cages on the glass were connected by plasma treatment (plasma ion bombarder, PIB-10, Vacuum Device) followed by heat treatment on a hot plate at 120°C to seal them completely. Cells in the hydrogel cages were observed with a CCD camera (WAT-221S, Watec) through an objective lens (UPLSAPO 100XO, N.A. 1.4, Olympus). The simulation of the flow was performed according to finite element algorithm using the analysis software by COMSOL (COMSOL INC., Palo Alto, CA). To set up the experiment, a *Synechocystis* cell was transported into the patterned hydrogel cage through in-port (a) at a flow rate of 300 μl/h established by a syringe pump. Determination of cell volume during hyperosmotic stress was performed by using Image J (NIH; http://rsb.info.nih.gov/ij/index.html) to measure the cell area, followed by converting area into...
volume under the assumption that the volume change was isotropic.

**E. coli complementation assay.** The *Synechocystis* kdpABGCD (*slr1728-1729, ssr2912, slr1730-1731*) region was amplified by PCR using the *Sac*I site-containing forward primer, 5’-CAGTGGATCTCATGGCAAGTTTTT-3’, and the *Pst*I site-containing reverse primer, 5’-CAGTCTGAGTATGAGCCATGGCAATGA-3’ with *Synechocystis* genomic DNA as template. The PCR fragment was digested with *Sac*I and *Pst*I and ligated into the corresponding sites of plasmid pPAB404 (31, 32). pSTV28-*ktrA* and pPAB404-*ktrEB* were used to express the Ktr K⁺ transport system in *E. coli* (18). *E. coli* LB2003, which lacks the three K⁺ uptake systems, Trk, Kup, and Kdp (31), containing empty vector, pSTV28-*ktrA* and pPAB404-*ktrEB* or pPAB404-*kdpABGCD* was grown in synthetic medium (46 mM Na₂HPO₄, 23 mM NaH₂PO₄, 8 mM (NH₄)₂SO₄, 0.4 mM MgSO₄, 6 μM FeSO₄, 10 μg/ml thiamine, 1% glucose, 1.2% agarose) (18) in the presence of 30 mM KCl, 0.25 mM IPTG, 25 μg/ml of ampicillin, and/or 15 μg/ml of chloramphenicol for 24 h at 30°C. Then these pre-cultures were washed with synthetic medium without KCl and diluted to an OD₆₀₀ of 0.005 with the same medium. Ten-fold serial dilutions were plated on synthetic agar medium containing 7.5 mM, 10 mM, 15 mM or 20 mM KCl and incubated for 2 days at 30°C to test for growth. For growth curves, *E. coli* LB2003 cells expressing *ktr* or *kdp* were grown in synthetic medium containing 15 mM KCl.

**Measurement of K⁺ uptake in E. coli.** K⁺ influx was measured essentially as described by Matsuda et al. (18, 33, 34). The net uptake of K⁺ was measured by the silicone filtration technique, the K⁺ contents of the cell pellets were determined by flame photometry.

**Construction of Synechocystis kdpA deletion strains.** The gene encoding *kdpA* was disrupted by insertion of a spectinomycin resistance gene into the *kdpA* gene in both the wild type and in a *ktrB* disruption mutant. The transformants were grown on BG11 solid medium supplemented with spectinomycin (20 μg/ml) and buffered at pH 8.0. The disruption of *kdpA* in
the transformants after homogeneity segregation by successive-streak was confirmed by PCR amplification. In addition, the \textit{kdpA} genes in each mutant were amplified by PCR and sequenced to confirm the correct disruption.

**Growth assays with \textit{Synechocystis}**. For hyperosmotic stress assays in \textit{Synechocystis} strains, wild type, \textit{ΔktrB}, \textit{ΔkdpA}, and the \textit{ΔktrB/ΔkdpA} double mutant were grown in BG11 to an OD\textsubscript{730} of 0.8-1.0, incubated in medium without (control) or with (osmotic shock) 3 M sorbitol for 4 h. Cells were washed by BG11 medium twice and resuspended with BG11 medium to an OD\textsubscript{730} of 1.0 and then spotted onto standard BG11 medium. For K\textsuperscript{+}-deficiency stress assays, cells were grown in BG11 to an OD\textsubscript{730} of 0.8-1.0. After two-times washing of the cells with K\textsuperscript{+}-depleted BG11 medium, cells were diluted into K\textsuperscript{+}-depleted BG11 medium containing 5 mM KCl or without addition of KCl to an OD\textsubscript{730} of 0.05, and growth was monitored.

\textbf{β-Galactosidase assays}. To study the regulation of \textit{kdpA} expression, a 0.5 kb \textit{kdpA} promoter sequence (\textit{P}_{\textit{kdpA}}) was fused to the bacterial \textit{β}-galactosidase gene at the \textit{AflII} and \textit{NdeI} sites of pNS1::\textit{lacZ}+\textit{Cm}\textsuperscript{r} and the resulting \textit{P}_{\textit{kdpA}}::\textit{lacZ} construct was inserted into the TS1 region in the wild type or \textit{ΔkdpA} mutant genome using a set of PCR primers:

5’-GGGCTTAAGATCGCCGTAGTTTACCAATG-3’ and 5’-GGGCATATGAAATTGAGATGA CGAAGATGG-3’. In addition, \textit{kdpD} (\textit{slr1731}), \textit{hik20} (\textit{sll1592}, homologous to histidine kinase domain in KdpD of \textit{E. coli}) and \textit{rre19} (\textit{sll1590}, homologous to KdpE of \textit{E. coli}) deletion strains were generated by insertion of a kanamycin resistance cassette into these genes in the \textit{ΔkdpA} background containing \textit{P}_{\textit{kdpA}}::\textit{lacZ}. In order to determine whether deletion of \textit{hik20} affected expression of \textit{rre19}, the \textit{rre19} gene was placed under the control of the constitutive \textit{sphS} promoter (35) and inserted by homologous recombination into the TS4 region of the \textit{ΔkdpA/Δhik20} strain containing \textit{P}_{\textit{kdpA}}::\textit{lacZ} using erythromycin resistance as a marker (+\textit{rre19} strain). Cells resistant to erythromycin (20 μg/ml) were isolated, and the correct integration of the
gene confirmed by PCR with genomic DNA. *Synechocystis* wild type, ΔkdpA, ΔkdpD, Δrre19, Δhik20 and +rre19 strains carrying the integrated P<sub>kdpA</sub>::lacZ construct were assayed for β-galactosidase activity in exponentially growing cultures. Quantitative evaluation of β-galactosidase activity was carried out using permeabilized cells obtained from exponential phase cultures incubated with o-nitrophenyl-β-D-galactopyranoside substrate as previously described by Miller (36). Average (± standard deviation) activity units were calculated from three independent assays.

**Measurement of circadian rhythm of kdpA and ktrB promoter activity in *Synechocystis***.

The coding region of a modified firefly luciferase gene (*luc<sup>+</sup>*, Promega Japan, Tokyo) was substituted for the *lacZ* sequence using the NdeI and XbaI sites in the P<sub>kdpA</sub>::lacZ construct. The resultant P<sub>kdpA</sub>::*luc<sup>+</sup>* construct was inserted into the TS1 region in the *Synechocystis* genome (37). The P<sub>trEB</sub>::*luc<sup>+</sup>* containing approximately 0.5 kb of *ktrEB* promoter was also constructed with the same manner using 5'-GGGCTTAAGCTCAAACGGGTACAGACC-3' and 5'-GGGCATATGGCAGATAACCAGCGTGAA-3'. Cells were grown in BG11 liquid medium to a density of 6-7 × 10<sup>8</sup> cells/ml at 30 °C under white fluorescent lamps (42 μmol of photons/m<sup>2</sup> s) (i.e. continuous light conditions; LL) with shaking at 180 rpm and diluted with BG11 to 2.5 × 10<sup>8</sup> cells/ml. 180 μl aliquots (4.5 × 10<sup>7</sup> cells) of the suspension were mixed with 20 μl of 5 mM D-luciferin (Promega Japan) dissolved in BG11 and transferred to 96-well microplates (CulturePlate-96; Perkin-Elmer Life Sciences Japan, Tokyo). The plates were sealed with a plate seal (Perkin-Elmer Life Sciences Japan). To reset the circadian clock of the cells, the plates were placed in the dark for 12 h at 30°C and then returned to LL conditions. The bioluminescence from each well was measured automatically every hour using an automated bioluminescence-monitoring apparatus (K. Onai, N. Siraki and M. Ishiura, unpublished; model CL96-4; Churitsu Electric Co., Nagoya, Japan). The data were analyzed using
bioluminescence-analyzing software (K. Onai, N. Shiraki and M. Ishiura, unpublished; SL00-01; Churitsu Electric Co., Nagoya, Japan). Circadian time (CT) was calculated by dividing the peak-phase value by the period and multiplying by 24. The amplitude of rhythms was calculated as the average ratio of peak to trough in each cycle.

RESULTS

**Kdp functions as an additional K⁺ uptake transport system.** *Synechocystis* contains two K⁺ uptake systems, Ktr and Kdp. To elucidate the role of the *Synechocystis* Kdp transporter in K⁺ uptake, the *kdpABGCD* operon was heterologously expressed in the K⁺-uptake deficient *E. coli* strain LB2003 (31). Expression of *kdpABGCD* restored K⁺ uptake in the LB2003 strain on solid medium containing 15 mM and 20 mM KCl but not 10 mM KCl (Fig. 1A). The KtrABE potassium transporter of *Synechocystis* was used as positive control. Its expression enabled growth at 10, 15 and 20 mM KCl. In liquid medium containing 12.5 mM KCl, the growth of the *kdpABGCD*-expressing strain was delayed compared with that of the *ktrABE*-expressing strain but stimulated in comparison to the *E. coli* strain containing the empty vector (Fig. 1B). To further characterize the Kdp system, we examined the K⁺ uptake activity of cells incubated in 200 mM HEPES-NaOH buffer in the presence of glucose. When 10 mM KCl was added, a low rate of K⁺ uptake activity was measured in *E. coli* mutant cells expressing the *Synechocystis kdpABGCD* operon (Fig. 1C). However, much higher rates were observed in the strain expressing *ktrABE* as reported previously (17, 18). These results indicate that the *Synechocystis* Kdp system mediates K⁺ uptake albeit with lower rates compared to that of Ktr.

**The Ktr-driven K⁺ uptake is required for initial cell volume recovery after hyperosmotic shock.** Since standard biochemical techniques are ineffective in monitoring changes of cell volume and ionic homeostasis in microorganisms, a direct approach was needed
assessing these dynamics in combination with studying the involved membrane transport systems
in single *Synechocystis* cells. Hence we developed a microfluidic device, which allows the fluid
exchange to be performed gently and smoothly without perturbing the cell’s structural integrity.
During the experiment, the cells were confined within a cage surrounded by a patterned hydrogel
wall (Figs. 2A-E and Supplemental Fig. S1). Figs. 2D-E show that the water-permeable hydrogel
wall used to trap the cells in the slots allowed replacement of the liquid without influencing the
cells mechanically. To determine the requirement of K$^+$ for cell recovery after hyperosmotic
shock, wild-type *Synechocystis* cells were incubated in the microfluidic device in either standard
BG11 or in potassium-depleted K0 medium (0.1 OsM) and then subjected to hyperosmotic shock
by addition of 3 M sorbitol (3.6 OsM) to the respective medium (Fig. 2F-G). In standard BG11
medium, cell volume rapidly decreased by 54.8% at 120 s after hyperosmotic shock (Fig. 2F-G).
The subsequent cell volume recovery occurred in a biphasic response. During the first recovery
phase, from 120 s to 300 s, cell volume increased by 12.2%, and during the second recovery
phase, from 300 s to 3,600 s, by 13.4%. When the same experiment was performed with cells in
potassium-free K0 medium (which contained Na$^+$ instead of K$^+$ in the BG11), the same initial
rapid decrease of cell volume was recorded within 120 s. However, no cell volume recovery was
observed from 120 s to 300 s. The cell volume seemed even to decrease slightly further by 2.8%
at 300 s (Figs. 2G and Table 1). While the first recovery phase was absent in K0 medium, a
relatively quick recovery of cell volume started at 600 s (Fig. 2G and Table 1). These results
show that cell volume recovery after a hyperosmotic stress was biphasic and depended on the K$^+$
uptake by the *Synechocystis* cells.

Ktr contributes more to high osmolarity acclimation than Kdp in *Synechocystis*. To
distinguish which transporter is responsible for the K$^+$ uptake ensuring rapid volume recovery
after hyperosmotic stress of *Synechocystis*, mutants defective in the two K$^+$-transport systems
were analyzed. In addition to the already described mutant ΔktrB (17, 18), we generated the mutant ΔkdpA, which is defective in the kdpA gene encoding the permease subunit of Kdp. The Synechocystis KdpA shares 59% identity with E. coli KdpA. In addition, the double mutant, ΔktrB/ΔkdpA, was generated. In a first experiment, the survival of these cells toward harsh osmotic shocks was investigated. After their incubation in liquid medium containing 3 M sorbitol for 4 h, cells were then spotted onto plates with solid BG11 without sorbitol (Fig. 3A). The treatment strongly decreased the growth of both Ktr mutants, the ΔktrB strain and the ΔktrB/ΔkdpA strain, while the wild type grew well. Growth of the ΔkdpA strain was better than that of the other two mutants but less well than that of the wild type (Fig. 3A).

In a second experiment, the microfluidic device was used to evaluate changes in cell volume in response to hyperosmotic stress in BG11 medium containing sufficient amounts of K⁺ (Fig. 3B). The cell volume of the ΔktrB strain, the ΔkdpA strain and the ΔktrB/ΔkdpA strain had shrunk to 40.9-48.4% after 120 s, similar to the wild type (Figs. 2G, 3C and Table 2). Cells of the wild type began to swell again during the first phase of recovery (120-300 s). In contrast, the cell volume of the ΔktrB/ΔkdpA mutant remained at a low level in the first phase of recovery (35.0-40.9%), and started to increase to 66.1% only much later at 3,600 s. Using the two single mutants, the individual contributions of Ktr and Kdp to the cell volume changes could be estimated (Fig. 3C and Table 2). The ΔktrB cells, like the double mutant, continued to shrink during the time span when the wild type showed its first recovery phase (120-300 s). Then, after 600 s the volume of the ΔktrB cells increased, eventually reaching a similar level to the wild type at 3,600 s (Fig. 3C). The ΔkdpA cells recovered faster than the ΔktrB cells but less rapidly than the wild type; volume recovery towards the original volume initiated within 180-300 s. Based on these results, we conclude that Ktr represents the major contributor to K⁺ uptake for initial cell volume recovery from hyperosmotic stress, while Kdp was likely not or only to a very low extent.
KdpA functions as uptake system at trace K\textsuperscript{+} amounts in *Synechocystis*. To further elucidate the function of Kdp in *Synechocystis*, we compared the growth of the wild type, the \( \Delta ktrB \) strain and the double mutant, \( \Delta ktrB/\Delta kdpA \) in K\textsuperscript{+}-limited medium (Fig. 4). The growth of all the strains was comparable in the medium supplemented with 5 mM K\textsuperscript{+} (Fig. 4A). However, in K\textsuperscript{+}-depleted medium containing just trace amounts of K\textsuperscript{+}, the Kdp-expressing cells (the wild type and \( \Delta ktrB \) strain) grew better than the \( \Delta kdpA \) strain (Fig. 4D). These results suggest that Kdp mediated K\textsuperscript{+} uptake at very low K\textsuperscript{+} concentrations.

Hence, expression of *kdpA* under various abiotic stress conditions including K\textsuperscript{+} deficiency was determined by a reporter gene approach. To this end, we generated *Synechocystis* cells expressing the lacZ gene under control of the *kdpA* promoter (0.5 kb long). The reporter constructs were integrated into the neutral TS1 regions, both in wild-type and \( \Delta kdpA \) backgrounds. In the wild-type background expression of the *kdpA* promoter construct was enhanced approximately 3.1-fold at K\textsuperscript{+} deficiency conditions after 24 h (Figs. 5A and 5B). Under the same conditions, more than two-times higher expression (approximately 7.9-fold) of the *kdpA* promoter was measured in the \( \Delta kdpA \) strain. This result suggested that the decreased intracellular K\textsuperscript{+} level due to the lack of KdpA-mediated K\textsuperscript{+} uptake activity in the \( \Delta kdpA \) background led to enhanced induction of the *kdpA* promoter. In contrast, the *kdpA* promoter showed only a small response to either salinity stress or hyperosmotic shock in the wild-type and \( \Delta kdpA \) background (Figs. 5C and 5D).

Distinct regulation of *kdp* expression in *Synechocystis*. The arrangement of the *kdp* operon in *Synechocystis* differs from the situation in *E. coli*, where the expression of the *kdp* operon is mediated by a two component system comprising the sensor kinase KdpD and the response regulator KdpE (Fig. 6A) (28). An additional gene, *kdpG*, is situated in between *kdpB* and *kdpC*
in *Synechocystis*. The genes encoding for the corresponding two component system also are differently arranged compared to *E. coli*. The *Synechocystis* KdpD homolog is divided into two separate proteins. The annotated KdpD in the *kdpABGCD* operon (CyanoBase: http://genome.microbedb.jp/cyanobase/Synechocystis/genes/slr1731) corresponds only to the N-terminal region of the canonical histidine kinase. Another *Synechocystis* gene, annotated as histidine kinase 20 (Hik20) corresponds to the C-terminal region of KdpD in *E. coli*. The *hik20* gene forms a putative operon with the gene for the response regulator homolog KdpE, named *rr*19 in *Synechocystis*. The *hik20-rr*19 gene operon encoding truncated KdpD-like and KdpE proteins is situated in reverse orientation upstream of the *kdpA* gene (Fig. 6A). To study the regulation of *kdp* gene expression in *Synechocystis*, we deleted *kdpD, hik20* or *rr*19 in the ΔkdpA strain containing the reporter construct *P*<sub>kdpA</sub>::*lacZ*. The ΔkdpA strain was chosen as the parental strain, because it showed the most sensitive reaction toward low concentrations of K⁺ (Figs. 5B and 6B). Disruption of *kdpD* had only a small effect on the *kdpA* promoter activity, when compared with the parental strain. However, the deletion of either *hik20* or *rr*19 completely prevented induction of the *kdpA* promoter driven reporter gene (Fig. 6B). Since the *rr*19 gene is located downstream of *hik20* (Fig. 6A), we tested whether the observed effect of the *hik20* deletion was indirectly caused by polar effects disrupting *rr*19 expression and not by the deletion of *hik20* itself. Thus, a Δ*hik20* mutant was generated that constitutively expressed *rr*19 under control of the *sphS* promoter (35). The constitutive expression of *rr*19 in the Δ*hik20* strain did not restore the activity of the *kdpA* promoter at low K⁺ concentrations. Collectively, these results strongly suggested that the expression of the *kdp* operon was positively regulated by both, Hik20 and Rre19, but not by KdpD.

The *kdp* and *ktr* genes show circadian expression in *Synechocystis*. Photoautotrophic organisms are exposed to daily periodic changes of light in their natural environment, K⁺
homeostasis is controlled by the circadian oscillation of K\(^+\) transport in various species including plant cells (38-40). Synechocystis may also be faced to periodically requirements of K\(^+\), which is taken up by Kdp and Ktr in environments containing low amounts of K\(^+\). The data base on the circadian oscillation in Synechocystis did not show apparent rhythms of the genes encoding subunits of Kdp and Ktr, namely KdpA and KtrB (37) (KEGG EXPRESSION Database http://www.genome.jp/kegg/expression/). The circadian oscillation of kdp and ktr expression in Synechocystis was tested using a real-time bioluminescence monitoring system. Cells expressing firefly luciferase driven by the circadian-controlled promoter of dnaK, a gene encoding a heat shock protein, were used as controls (Fig. 7A, upper panel) (41). The expression of kdpA exhibited circadian oscillation with a circadian period (wave length of the cosine curve) of 23.0 ± 0.2 h, close to the period length of dnaK expression (23.0 ± 0.4 h). The kdpA expression reached its highest level at circadian time of 23.7 ± 0.5 h, corresponding to subjective dawn (Fig. 7A and Table 3). The genes ktrE and ktrB consist of the same cistron due to overlapping of the stop codon of ktrE and the initiation codon of ktrB (18, 42). The expression of ktrEB showed circadian rhythm with the peak of 23.0 ± 0.5 h, almost consistent to that of kdpA (Table 3).

DISCUSSION

Although K\(^+\) uptake transporters overall contribute to maintain K\(^+\) as the major cation in the cell, each type of K\(^+\) transporter is likely to have a divergent individual physiological role. Synechocystis contains two of the three classes of K\(^+\) uptake systems found in E. coli, the well-characterized Ktr (18, 28, 33, 34, 43) and the less-characterized Kdp (17). Despite many efforts analyzing Kdp in Anabaena strains (28), in E. coli (21-25), and in Staphylococcus aureus (19, 27), its function in the important model cyanobacterium Synechocystis is only scarcely known due to the existence of Ktr-type K\(^+\) uptake system and the absence of specific mutants (17,
Preliminary investigations with *Synechocystis* mutants bearing defects in ktr or kdp genes indicated for a major role of Ktr for K\(^+\) uptake in salt-shocked *Synechocystis* cells (17). This finding could indicate that the kdp genes encode an inactive transporter in *Synechocystis*. Hence, we first aimed to proof the potential role of the *Synechocystis* Kdp in K\(^+\) transport by complementing a K\(^+\) uptake mutant of *E. coli*. These experiments showed that the expressed cyanobacterial Kdp allowed growth of the K\(^+\) uptake deficient *E. coli* (Fig. 1A and B). However, even in the *E. coli* system, the Ktr-mediated activity was dominating because the growth promotion and particularly the K\(^+\) uptake activity were much higher in *ktrABE*-expressing cells compared with the *kdpABGCD* expressors (Fig. 1B). Nevertheless, these experiments clearly revealed that the *Synechocystis* kdp operon encodes for an active K\(^+\) transporter.

To identify the role of the different K\(^+\) transport systems, we first aimed to study cellular response to sudden shocks of high osmolarity, since the osmostress acclimation involves dramatic changes in K\(^+\) fluxes (5). Conventional experimental approaches to elucidate the physiological responses of cells to abiotic and biotic stress commonly make use of large populations of cells that are transferred from one medium into another. The data obtained from those experiments are therefore an average of many individual responses from many different cells, some of which may not actually undergo osmo-acclimation. In contrast, microfluidic devices make it possible to characterize and monitor cellular responses in individual living cells in real-time. To realize a single cell analytical approach on a microfluidic platform with real-time monitoring, physical stress to the cells caused by the fluid exchange must be minimized. Approaches using lab-on-a-chip devices enable the monitoring and manipulation of nanoparticles and biomolecules (44). Device design and the materials used in the construction of the device are of critical importance to successfully monitor individual cells over extended periods of time. There have been reports of cages made of permeable membranes (45). Although a monitoring device for
animal cell cultures has been reported (46-48), it was not applicable to microorganisms like 
Synechocystis due to its smaller size of 2-4 μm in diameter. The size of cells is a critical aspect 
since the physical force exerted by the flow of the medium can affect the cells in the course of 
observing cell volume changes. Therefore we developed a new microfluidic device and used it to 
confirm that individual Synechocystis cells required K\(^+\) to protect itself against hyperosmotic 
shock (Fig. 2). The data in Tables 1 and 2, and the in Fig. 2G illustrate how water and K\(^+\) flux 
accompanied volume changes of single cells under hyperosmotic stress conditions. As a first 
response to the stress, water flowed out of the cells, resulting in cell shrinkage (54.8% decrease at 
120 s). K\(^+\) ions in the liquid medium were taken up to ensure a fast volume recovery (Fig. 2G). 
This rapid K\(^+\) uptake is mainly driven via the Ktr transport system, when the medium contained 
sufficient concentrations of K\(^+\) (Fig. 3C, and Table 2). This finding was consistent with the idea 
that Ktr functions as a K\(^+\) uptake system during hyperosmotic stress in Synechocystis (18). This 
conclusion is based on two findings: (i) the wild-type cells lack the first phase of recovery 
(120-300 s) in a K\(^+\) depleted media (Fig. 2G and Table 1), and (ii) the cells defective in Ktr, i.e. 
the ΔktrB and the ΔktrB/ΔkdpA mutants, also lack the first recovery phase even at sufficient K\(^+\) 
conditions (Fig. 3C and Table 2), whereas the mutant Δkdp behave like wild type under this 
conditions. In the second phase of cell volume recovery, the wild-type cell volume increased very 
slowly by 13.4% to a moderate level at only 3,600 s in BG11 medium (Fig. 2G and Table 1). Full 
volume recovery probably needs the synthesis and accumulation of the compatible solute 
glicosylglycerol in the second or later phase of osmo-acclimation, which induced more water 
influx and a cell volume recovery (4, 5). During the second phase of cell adaptation, not only the 
compatible solute biosynthesis and accumulation but also an influx of sorbitol into the cells may 
have occurred. Sorbitol influx has been shown to occur in cells of the Synechocystis wild type 
and a mutant unable to synthesize the compatible solute glucosylglycerol treated by high external
Taken together, these results suggest that the uptake of K\(^+\) into the cells via K\(^+\) transporters was required for the first phase of recovery, and that especially Ktr but not Kdp contributed significantly to this first recovery phase.

The values of the cell volume are presented as relative cell volume by conversion of each area of the light microscopic image (Figs. 2-3 and Table 1-2). The oval cells which are not isotropic shaped-cells might bear some errors to some extents. Moreover, it is possible that during these experiments the plasma membrane could be separated from cell wall similarly to plasmolysis seen in the plant cells, which may somehow decrease the total extent of cell shrinkage (50).

Under the experimental conditions used here, we cannot rule out these possible influences on the exact measurement of the relative cell volumes.

To reveal a physiological function separate from osmo-acclimation of Kdp in Synechocystis, we compared the growth rates of wild type, ΔktrB, and the double mutant ΔkdpA/ΔktrB at different external K\(^+\) levels (Fig. 4). These experiments illustrated the physiological importance of Kdp at trace concentrations of K\(^+\). The specific role of Kdp at trace K\(^+\) amounts is indirectly supported by the notion that the kdp expression was induced in the low µmol K\(^+\) level in our reporter gene experiments with Synechocystis similarly as reported before in Anabaena (51) and Staphylococcus aureus (19, 21). The presence of two transport systems, Ktr and Kdp with different affinities for K\(^+\) may contribute to an increase in the survival rate of Synechocystis and lead to an expansion of its habitat.

Next, we investigated the expression regulation of the Synechocystis kdp operon. It showed a clear response to K\(^+\) limited conditions analogous to the situation in many other microorganisms (Fig. 5B) (28). In E. coli, addition of both ionic or non-ionic impermeable solutes also induced kdp operon expression (52). However, the Anabaena L-31 kdp2 operon did not respond to increases in osmolarity imposed by salinity stress (28, 53). Similarly, the Synechocystis kdp
operon was not induced by salt or osmotic stress (Figs. 5C and 5D). Sensing of the extracellular K⁺ concentration is critical for the regulation of *Synechocystis* and *Anabaena kdp* expression but not osmotic stress. In *E. coli*, the stress and K⁺ dependent upregulation of *kdp* depends on the two component system coded by *kdpDE* (24, 26). Similarly, *kdpA* was induced by 2 M NaCl in *Staphylococcus aureus* (19). In the latter bacterium, the high-salt inducible Kdp and the constitutively expressed Ktr cooperatively participated in the alleviation of salt and osmotic challenges. In *Synechocystis*, Kdp showed a lower contribution to high osmolality acclimation at least under our study conditions (Fig. 3 and Fig. 5). The *Synechocystis kdp* operon has a unique organization that is different from that of the *E. coli* and *Staphylococcus aureus kdp* operon (Fig. 6A) (28). Particularly, the histidine kinase KdpD of *E. coli* is divided into two parts, as was reported before for the homologous operon in *Anabaena L-31* (28). Deletion of either *hik20* or *kdpD* revealed that Hik20 but not KdpD was required for sensing low extracellular K⁺ concentrations and the induction of the *kdpABGCD* operon under these conditions (Fig. 6B). The function of the truncated KdpD in *Synechocystis* remains unclear. In *E. coli*, the N-terminal domain of KdpD, corresponding to the *Synechocystis* KdpD, was shown to be involved in modulating the activity of the Kdp transport system *in vivo* and *in vitro* by enhancing *kdp* expression (28). Moreover, the functionality of the truncated KdpD form of *Anabaena* sp. L-31 has been shown after its fusion to the C-terminal domain of the *E. coli* KdpD. The resulting *Anabaena-E-coli* chimeric KdpD was able to complement an *E. coli kdpD* mutation regarding sensing of K⁺ deficiency (54). Moreover, the recombinant KdpE of *Anabaena* sp. L-31, which is highly similar to Rre19, was able to bind in a phosphorylation dependent manner to the promoter of the *Anabaena kdp2* operon. Thus, the cyanobacterial two component system, which is encoded by *hik20* and *rre19* in *Synechocystis*, is responsible for the K⁺-dependent expression of the *kdpABGCD* operon. Possibly the missing N-terminal part of the *E. coli* KdpD in Hik20 could be
responsible to the missing induction of the system by hyperosmotic stress in contrast to *E. coli* (28). However, the *kdp* operon coding the structural proteins is induced by desiccation stress in *Anabaena* strains (53, 55). Further studies are still needed to clarify the physiological role of the two component system of Kdp.

In their natural environment, microorganisms regularly have to cope not only with changing salinities but also with a scarcity of essential elements such as K⁺. The circadian oscillation of *kdp* and *ktr* expression found in this study suggests an involvement of Kdp and Ktr in the regulation of diurnal potassium homeostasis in *Synechocystis* (Table 3 and Fig. 7). Despite of apparent difference in the function between Kdp and Ktr, their peaks of circadian oscillations were consistent to each other but occurred at a different time than that of other membrane transport proteins involved in the acclimation to environmental changes (56-58) (Fig. 7B). By increasing *kdp* and *ktr* expression and possibly K⁺ uptake at the beginning of the light period, cells may prepare themselves for the acquisition of K⁺ required to support processes of the regular diurnal metabolism, for instance photosynthesis, respiration, and/or cell division (59). Consequently, the circadian rhythm of the two transporters-mediated K⁺ uptake activities may have evolved in order to meet K⁺ requirements that fluctuate with daily rhythmic changes. Such changes in cellular K⁺ transport have been shown for eukaryotic algae. *Gonyaulax*, a unicellular marine photosynthetic dinoflagellate, exhibited a two-fold increase of K⁺ content in the cells at the end of the light period compared with the beginning (60), whereas light-stimulated K⁺ uptake played a crucial role for growth in the red alga *Porphyra leucosticta* (61). This alga showed daily cycles in K⁺ enrichment and subsequent turgor-driven enlargement that were disturbed by K⁺ limitation or inhibitors of K⁺ transport. In plants the K⁺ concentration in the guard cells starts to increase in the morning, reaching the highest level at dusk (62). In the motor cells of the tropical tree species *Samanea saman* expression of K⁺ channels oscillated rhythmically during light-dark
cycles (63). It can be argued that the circadian control system of cells must not only be able to respond to environmental changes but also to prepare for such conditions in advance (64). Phototrophic organisms like *Synechocystis* therefore induce the synthesis of their K\(^+\) uptake system at dawn, in order to prepare themselves for their control of turgor pressure and membrane potential in their cells for diurnal activity.

Collectively, our study clearly revealed that each type of K\(^+\) transporter is likely to have an individual physiological role. We demonstrated that Ktr-mediated K\(^+\) uptake activity dominated under high osmolarity conditions in the presence of relatively high external K\(^+\) compared with Kdp. However, K\(^+\)-deficiency frequently occurs in the natural environment of cyanobacteria, which have been found in harsh habitats (65). Under such conditions, Kdp will become the main transporter supplying K\(^+\) to the cells, while Ktr only plays a minor role. This scenario suggests that Kdp may function to keep the intracellular K\(^+\) concentration above the basal level. The two *Synechocystis* K\(^+\) uptake systems, Ktr and Kdp therefore fulfill distinct, unique roles that help to contribute to the survival of the cyanobacteria living in a variable, stressful light-dark cycle environment.

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division in *Synechococcus elongatus*. Cell. 140, 529-539


FIGURE LEGENDS

FIG 1 Synechocystis Kdp-mediated K\(^+\) uptake plays a minor role compared to that of Ktr. (A) Complementation of the K\(^+\) uptake-deficient *E. coli* strain LB2003 by *Synechocystis* *kdpABGCD*. LB2003 cells containing *kdpABGCD*, *ktrABE* (as a positive control) (18) or the empty vector were grown on solid synthetic medium supplemented without added KCl or with 10 mM, 15 mM or 20 mM KCl. The first spot of each row represents OD\(_{600}\) of 0.005, which is diluted 1:10 for each spot thereafter. (B) Growth of *E. coli* LB2003 containing empty vector, *kdpABGCD* or *ktrABE* in liquid synthetic medium with 12.5 mM KCl at 5 h (open), 21.5 h (gray), 25.5 h (black). Each value is the mean and SD (n = 3 for empty vector, n = 10 for *kdpABGCD*, n = 4 for *ktrABE*). (C) K\(^+\) uptake activities by *E. coli* LB2003 containing KdpABGCD (triangle), KtrABE (square) or the empty vector (circle). K\(^+\)-depleted cells were energized with glucose, at *t* = 0 min 10 mM KCl was added to the medium. KtrABE or the empty vector was the same constructs as reported previously (18). Samples were taken at the times indicated in the figure. Error bars denote SD from four independent experiments.

FIG 2 K\(^+\) dependent dynamic changes of the wild-type cell volume subjected to hyperosmotic stress. Microfluidic device for on-chip continuous monitoring of individual cells of *Synechocystis* sp. PCC 6803 (A-E). (A and B) Ten cages were placed into the microfluid channel at a time. (B) is a detail of (A), showing two of the cages. (C) Photograph of the cage surrounded by the hydrogel wall. The arrow is pointing at one of the slots that hold the cells during the measurements. (D and E) Images of a *Synechocystis* cell inside the slot. (E) is a detail of (D). (F and G) K\(^+\) uptake is required for the recovery of cell volume during hyperosmotic stress. Wild-type cells inside the microfluidic device were incubated either in standard BG11 or in K\(^+\)-depleted K0 medium (K0) and then (corresponding to time = 0) subjected to hyperosmotic...
shock by addition of 3 M sorbitol (in the same medium). (F) Images of single cells recorded over time during the hyperosmolarity treatment. (G) Volume changes of *Synechocystis* wild-type cells over time calculated from data recorded by the device in standard BG11 (filled diamonds) or in K0 medium (open diamonds). The different phases are indicated by broken vertical lines: Cell shrinkage after the hyperosmotic shock, first phase of recovery (120-300 sec), and second phase of recovery (300-3,600 sec). The cell volume was calculated from the cell diameter using Image J as described in Materials and Methods. The relative cell volume at 0 seconds was set at 100% in each case. Error bars denote SD from at least 7 cells. The percentages in G correspond to the data obtained in standard BG11 for the different phases following hyperosmotic shock (Table 1). Diagram depicting biphasic cell volume changes of *Synechocystis* during hyperosmotic stress. Transfer of cells from isotonic to hyperosmotic medium triggered efflux of water from the cells, resulting in reduction of cell volume. This quick adaptation occurred in the first phase of the cell recovery response in the K⁺-containing medium (BG11), but not in K⁺-depleted medium (K0). During the second phase of cell adaptation compatible solute biosynthesis and accumulation may have occurred, which induced more water influx and a cell volume recovery (4, 5). Arrows indicate the trend of volume changes.

**FIG 3** Effect of disruption of ktrB and kdpA in *Synechocystis* on the hyperosmotic stress response. (A) Growth of *Synechocystis* wild type, ΔktrB, ΔkdpA and ΔktrB/ΔkdpA double mutant spotted onto standard BG11 medium with the serial dilution of 1:5 from an OD₇₅₀ of 1 after incubation in medium containing 3 M sorbitol for 0 h (left) or 4 h (right). (B) Representative time course of single cells (ΔktrB, ΔkdpA and ΔktrB/ΔkdpA double mutant) inside the microfluidic device showing shrinkage and recovery after being subjected to hyperosmotic stress by replacing the standard BG11 medium with the medium containing 3 M sorbitol at 0 s. (C) Profile of volume
changes of ΔktrB (open circle, green), ΔkdpA (filled circle, pink) and ΔktrB/ΔkdpA double mutant (open diamond, orange) cells determined from the microscope images taken by the camera attached to the microfluidic device. The lower panel shows a subset of the data in the upper panel (0-600 s). The dashed line (dark blue) corresponds to the volume changes of the wild type, the data are the same as shown in Fig 2G. Error bars denote SD from 5-8 cells (see Table 2).

**FIG 4** Significance of Kdp-mediated K⁺ uptake at K⁺-depleted medium. *Synechocystis* wild type (circle), ΔktrB (triangle) and ΔktrB/DkdpA double mutant (square) were grown in K⁺-depleted medium without (A) or with 5 mM KCl (B). Error bars denote SD from six independent experiments.

**FIG 5** Induction of the *kdp* operon by abiotic stress. *Synechocystis* wild type and ΔkdpA carrying the reporter construct *P*<sub>kdpA</sub>::*lacZ* in their genome were grown in BG11 to an OD<sub>730</sub> of 0.6–2.0. β-Galactosidase activity was measured at the beginning of the assay (0h, gray bar) or after (black bar) 24 h incubation of the cells in BG11 (i.e. no stress) (A), in K⁺ depleted medium (B), in BG11 medium containing 500 mM NaCl (salt stress) (C) or 400 mM sorbitol (osmotic stress) (D).

**FIG 6** Regulation of *kdp operon* expression by histidine kinase and response regulator. (A) Comparison of the gene arrangements of the *E. coli* and the *Synechocystis* *kdp* operons. Homologous genes are denoted by the same color or pattern. (B) β-Galactosidase activity was measured in exponentially growing *Synechocystis* wild type or various mutant strains carrying *P*<sub>kdpA</sub>::*lacZ* before (gray bars) or after (black bars) incubation in K⁺ depleted medium for 24 h. The deletions present in the strains tested are indicated under the bars. +rre19 designates the reintroduction of rre19 under control of a constitutive promoter into the ΔkdpA/Δhik20
background to control for a possible effect of the deletion of hik20 on rre19 expression. Results are means ± S.E. for three experiments.

**FIG 7** Circadian rhythm of *kdpA* expression in *Synechocystis*. (A) Representative circadian oscillation profiles of bioluminescence measurements from *Synechocystis* strains containing *P dnaK::luc*, *P kdpB::luc*, and *P ktrEB::luc* are shown. The cell cultures were preincubated in the dark for 12 h (filled square) and then transferred to continuous light conditions (open square). Essentially the same profiles were obtained from three independent experiments. Detailed values are listed in Table 1. (B) Model of the circadian rhythm of *kdpA* and *ktrB* expression in *Synechocystis*. The expression peak of the gene coding membranous transport proteins, *kdpA* and *ktrB* are marked by open circles at the corresponding circadian time. As reported previously, the peaks of *aqpZ* (aquaporin) (57), *nhaS3* (Na⁺/H⁺ antiporter) (56) and *mscL* (mechanosensitive channel large conductance) (58) are also indicated by filled circles.
TABLE 1. Contribution of extracellular K⁺ to cell volume recovery in the wild-type *Synechocystis* after hyperosmotic shock (3 M sorbitol). The percentage of the cell volume at each time point compared to the volume at 0 s (set at 100%) is shown. The lowest cell volume for each condition is underlined.

<table>
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<th>Time (s)</th>
<th>0</th>
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<th>120</th>
<th>180</th>
<th>240</th>
<th>300</th>
<th>600</th>
<th>1200</th>
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<td>47.3</td>
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* Values are summaries of the results shown in Fig. 2G.

* SD is shown in Fig. 2G, based on at least 7 cells.

* P<0.05 versus wild type in BG11

* P<0.01 versus wild type in BG11
TABLE 2. Changes in cell volume of the K⁺ transporter mutants after hyperosmotic shock (3M sorbitol). The percentage of the cell volume compared to the volume at 0 s (set at 100%) is shown, and the lowest cell volume for each condition is underlined.

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<th>120</th>
<th>180</th>
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<tr>
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<td>ΔktrB (%)(^c)</td>
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<td>44.4</td>
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<td>ΔkdpA (%)(^c)</td>
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<td>44.0(^e)</td>
<td>60.3</td>
<td>66.1</td>
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</table>

\(^a\) Values are summaries of the results shown in Fig. 3C.

\(^b\) The wild type data is shown as a control, and it is the same as the data shown in Table 1.

\(^c\) SD is shown in Fig. 3C, based on 5-8 cells.

\(^d\) P<0.05 versus wild type

\(^e\) P<0.01 versus wild type
TABLE 3 Circadian expression of *kdp* and *ktr*.  

<table>
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<th>Strain</th>
<th>Period (h)</th>
<th>Peak phase (h)</th>
<th>Peak phase (CT)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Amplitude</th>
<th>n</th>
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<tbody>
<tr>
<td><em>P</em>&lt;sub&gt;dnak&lt;/sub&gt;:&lt;sup&gt;Luc&lt;/sup&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>22.8 ± 0.4</td>
<td>21.4 ± 0.6</td>
<td>22.5 ± 0.6</td>
<td>1.40 ± 0.03</td>
<td>11</td>
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<tr>
<td><em>P</em>&lt;sub&gt;kdpA&lt;/sub&gt;:&lt;sup&gt;Luc&lt;/sup&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>23.0 ± 0.2</td>
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<td>1.70 ± 0.03</td>
<td>7</td>
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<tr>
<td><em>P</em>&lt;sub&gt;ktrEB&lt;/sub&gt;:&lt;sup&gt;Luc&lt;/sup&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>22.5 ± 0.7</td>
<td>21.6 ± 0.5</td>
<td>23.0 ± 0.5</td>
<td>1.18 ± 0.10</td>
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</table>

<sup>a</sup> Values are summaries of the results shown in Fig. 7.

<sup>b</sup> CT, circadian time
**Figure 1**

**A**

No addition  
K10  
K15  
K20  
Ktr  
Kdp  
vector  
Ktr  
Kdp  
vector  
Figure 1

**B**

Growth (OD600)  
0  
0.2  
0.4  
0.6  
0.8  
1.0  
vector  
Kdp  
Ktr

**C**

K+ uptake  
(nmol/mg protein)  
0  
50  
100  
150  
200  
vector  
Kdp  
Ktr

**FIG 1** *Synechocystis* Kdp-mediated K+ uptake plays a minor role compared to that of Ktr. (A) Complementation of the K+ uptake-deficient *E. coli* strain LB2003 by *Synechocystis kdpABGCD*. LB2003 cells containing *kdpABGCD, ktrABE* (as a positive control) (18) or the empty vector were grown on solid synthetic medium supplemented without added KCl or with 10 mM, 15 mM or 20 mM KCl. The first spot of each row represents OD600 of 0.005, which is diluted 1:10 for each spot thereafter. (B) Growth of *E. coli* LB2003 containing empty vector, *kdpABGCD* or *ktrABE* in liquid synthetic medium with 12.5 mM KCl at 5 h (open), 21.5 h (gray), 25.5 h (black). Each value is the mean and SD (n = 3 for empty vector, n = 10 for *kdpABGCD*, n = 4 for *ktrABE*). (C) K+ uptake activities by *E. coli* LB2003 containing KdpABGCD (triangle), KtrABE (square) or the empty vector (circle). K+ -depleted cells were energized with glucose, at $t = 0$ min 10 mM KCl was added to the medium. KtrABE or the empty vector was the same constructs as reported previously (18). Samples were taken at the times indicated in the figure. Error bars denote SD from four independent experiments.
**Figure 2**

**FIG 2** $K^+$ dependent dynamic changes of the wild-type cell volume subjected to hyperosmotic stress. Microfluidic device for on-chip continuous monitoring of individual cells of *Synechocystis* sp. PCC 6803 (A-E). (A and B) Ten cages were placed into the microfluid channel at a time. (B) is a detail of (A), showing two of the cages. (C) Photograph of the cage surrounded by the hydrogel wall. The arrow is pointing at one of the slots that hold the cells during the measurements. (D and E) Images of a *Synechocystis* cell inside the slot. (E) is a detail of (D). (F and G) $K^+$ uptake is required for the recovery of cell volume during hyperosmotic stress. Wild-type cells inside the microfluidic device were incubated either in standard BG11 or in $K^+$-depleted K0 medium (K0) and then (corresponding to time = 0) subjected to hyperosmotic shock by addition of 3 M sorbitol (in the same medium). (F) Images of single cells recorded over time during the hyperosmolarity treatment. (G) Volume changes of *Synechocystis* wild-type cells over time calculated from data recorded by the device in standard BG11 (filled diamonds) or in K0 medium (open diamonds). The different phases are indicated by broken vertical lines: Cell shrinkage after the hyperosmotic shock, first phase of recovery (120-300 sec), and second phase of recovery (300-3,600 sec). The cell volume was calculated from the cell diameter using Image J as described in Materials and Methods. The relative cell volume at 0 seconds was set at 100% in each case. Error bars denote SD from at least 7 cells. The percentages in G correspond to the data obtained in standard BG11 for the different phases following hyperosmotic shock (Table 1). Diagram depicting biphasic cell volume changes of *Synechocystis* during hyperosmotic stress. Transfer of cells from isotonic to hyperosmotic medium triggered efflux of water from the cells, resulting in reduction of cell volume. This quick adaptation occurred in the first phase of the cell recovery response in the $K^+$-containing medium (BG11), but not in $K^+$-depleted medium (K0). During the second phase of cell adaptation compatible solute biosynthesis and accumulation may have occurred, which induced more water influx and a cell volume recovery (4, 5). Arrows indicate the trend of volume changes.
FIG 3 Effect of disruption of ktrB and kdpA in Synechocystis on the hyperosmotic stress response. 
(A) Growth of Synechocystis wild type, ΔktrB, ΔkdpA and ΔktrB/ΔkdpA double mutant spotted onto standard BG11 medium with the serial dilution of 1:5 from an OD730 of 1 after incubation in medium containing 3 M sorbitol for 0 h (left) or 4 h (right). (B) Representative time course of single cells (ΔktrB, ΔkdpA and ΔktrB/ΔkdpA double mutant) inside the microfluidic device showing shrinkage and recovery after being subjected to hyperosmotic stress by replacing the standard BG11 medium with the medium containing 3 M sorbitol at 0 s. (C) Profile of volume changes of ΔktrB (open circle, green), ΔkdpA (filled circle, pink) and ΔktrB/ΔkdpA double mutant (open diamond, orange) cells determined from the microscope images taken by the camera attached to the microfluidic device. The lower panel shows a subset of the data in the upper panel (0-600 s). The dashed line (dark blue) corresponds to the volume changes of the wild type, the data are the same as shown in Fig 2G. Error bars denote SD from 5-8 cells (see Table 2).
FIG 4 Significance of Kdp-mediated K\(^+\) uptake at K\(^+\)-depleted medium. *Synechocystis* wild type (circle), ΔktrB (triangle) and ΔktrB/DkdpA double mutant (square) were grown in K\(^+\)-depleted medium without (A) or with 5 mM KCl (B). Error bars denote SD from six independent experiments.
FIG 5 Induction of the kdp operon by abiotic stress. Synechocystis wild type and ΔkdpA carrying the reporter construct P_{kdpA}:lacZ in their genome were grown in BG11 to an OD_{730} of 0.6–2.0. β-Galactosidase activity was measured at the beginning of the assay (0h, gray bar) or after (black bar) 24 h incubation of the cells in BG11 (i.e. no stress) (A), in K+ depleted medium (B), in BG11 medium containing 500 mM NaCl (salt stress) (C) or 400 mM sorbitol (osmotic stress) (D).
**Figure 6**

**Regulation of kdp operon expression by histidine kinase and response regulator.**

(A) Comparison of the gene arrangements of the *E. coli* and the *Synechocystis* kdp operons. Homologous genes are denoted by the same color or pattern. (B) β-Galactosidase activity was measured in exponentially growing *Synechocystis* wild type or various mutant strains carrying $P_{kdpA}:lacZ$ before (gray bars) or after (black bars) incubation in K+ depleted medium for 24 h. The deletions present in the strains tested are indicated under the bars. $+rre19$ designates the reintroduction of *rre19* under control of a constitutive promoter into the Δ*kdpA/Δhik20* background to control for a possible effect of the deletion of *hik20* on *rre19* expression. Results are means ± S.E. for three experiments.
Circadian rhythm of \( kdpA \) expression in \( Synechocystis \). (A) Representative circadian oscillation profiles of bioluminescence measurements from \( Synechocystis \) strains containing \( P_{dnaK}^{::luc+}, P_{kdpA}^{::luc+}, \) and \( P_{ktrEB}^{::luc+} \) are shown. The cell cultures were preincubated in the dark for 12 h (filled square) and then transferred to continuous light conditions (open square). Essentially the same profiles were obtained from three independent experiments. Detailed values are listed in Table 3. (B) Model of the circadian rhythm of \( kdpA \) and \( ktrB \) expression in \( Synechocystis \). The expression peak of the gene coding membranous transport proteins, \( kdpA \) and \( ktrB \) are marked by open circles at the corresponding circadian time. As reported previously, the peaks of \( aqpZ \) (aquaporin) (53), \( nhaS3 \) (\( Na^+/H^+ \) antiporter) (52) and \( mscL \) (mechanosensitive channel large conductance) (54) are also indicated by filled circles.