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The KtrA and KtrE Subunits Are Required for Na^+-Dependent K^+ Uptake by KtrB across the Plasma Membrane in *Synechocystis* sp. Strain PCC 6803▽†

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The Na^+-dependent K^+ uptake KtrABE system is essential for the adaptation of *Synechocystis* to salinity stress and high osmolality. While KtrB forms the K^+-translocating pore, the role of the subunits KtrA and KtrE for Ktr function remains elusive. Here we characterized the role of KtrA and KtrE in Ktr-mediated K^+ uptake and in modulating Na^+ dependency. Expression of KtrB alone in a K^+ uptake-deficient *Escherichia coli* strain conferred low K^+ uptake activity that was not stimulated by Na^+. Coexpression of both KtrA and KtrE with KtrB increased the K^+ transport activity in a Na^+-dependent manner. KtrA and KtrE were found to be localized to the plasma membrane in *Synechocystis*. Site-directed mutagenesis was used to analyze the role of single charged residues in KtrB for Ktr function. Replacing negatively charged residues facing the extracellular space with residues of the opposite charge increased the apparent K_m for K^+ in all cases. However, none of the mutations eliminated the Na^+ dependency of Ktr-mediated K^+ transport. Mutations of residues on the cytoplasmic side had larger effects on K^+ uptake activity than those of residues on the extracellular side. Further analysis revealed that replacement of R262, which is well conserved among Ktr/Trk/HKT transporters in the third extracellular loop, by Glu abolished transport activity. The atomic-scale homology model indicated that R262 might interact with E247 and D261. Based on these data, interaction of KtrA and KtrE with KtrB increased the K^+ uptake rate and conferred Na^+ dependency.

Cyanobacterium *Synechocystis* sp. strain PCC 6803 contains a number of different K^+ uptake systems that may contribute to satisfying its requirement of K^+ (3, 19, 36). Among these systems, Ktr has been shown to have a major role not only in K^+ uptake but also in adaptation against high-osmolarity stress (3, 19). Inactivation of the *ktr* gene renders the cells hypersensitive to high concentrations of NaCl and the nonionic compound sorbitol. Ktr-mediated K^+ uptake depends on the presence of Na^+ in the medium, which is likely to be an adaptation to salinity stress. A requirement of Na^+ for K^+ transport activity has also been found in the homologous protein from *Vibrio alginolyticus* (21). This dependency on Na^+ is a unique property of Ktr-type transporters and has not been found in other types of K^+ transporters or channels (32). The structure and function of Ktr-type transporters have been studied in a number of organisms (3, 6, 7, 9, 11–14, 18–20, 30, 32–34). The Ktr system from *Synechocystis* consists of three subunits, KtrA, KtrB, and KtrE (19). The KtrE gene and the KtrB gene form a cistron, whereas the KtrA gene resides at a site distant from the KtrEB genes in the *Synechocystis* genome (19). KtrB, the K^+-translocating subunit, is a member of the Ktr/Trk/HKT family of K^+ transporters. These transporters have been proposed to have evolved from two membrane-spanning K^+ channels (6, 7). According to the model, this type of transporter contains eight transmembrane domains, which consist of a 4-fold-repeated membrane-pore-membrane (M1-P-M2) motif (6, 7, 13, 18). An intramolecular electrostatic interaction of *Synechocystis* KtrB has been proposed to stabilize the protein in its active configuration (12). In addition, a conserved His in the external region in *Synechocystis* KtrB has been shown to be crucial for KtrB function (39). The region of the *Vibrio* Ktr protein responsible for gating of ion permeation has been identified (9). However, not much is known about the mechanism of Na^+ binding to KtrB in *Synechocystis*.

The KtrA subunit belongs to the family of KTR (K^+-transport nucleotide binding)/RCK (regulating the conductance of K^+ channels) proteins, which contain a Rossmann-fold sequence encoding β-α protein structure for NAD^+/NADH binding (17). Accordingly KtrA has been proposed to regulate the K^+ transport activity of KtrB by changing its binding from NAD^+ to NADH through a ligand-mediated conformational switch mechanism (25). It has also been shown that ATP promotes complex formation between KtrA and KtrB and that KtrAB from *V. alginolyticus* when expressed in *Escherichia coli* cells requires both ATP and the membrane potential for its activity (17).

KtrE is a unique subunit found only in *Synechocystis*; it is not involved in KtrB-mediated K^+ transport in *V. alginolyticus* and

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Bacillus subtilis (11, 32). The termination codon of *ktrE* overlaps the initiation codon of *ktrB* in the same cistron, which has not been found in other bacterial *ktrB*-related genes. Coexpression of KtrA with KtrB alone does not complement the growth defect of an *E. coli* K^+ uptake mutant. However, introduction of KtrE into the same mutant background in addition to KtrA and KtrB complements the mutation of the K^+ uptake system (19). Interestingly, the KtrE protein has been shown to function as a digalactosyldiacylglycerol (DGDG) synthase (EC 2.4.6.241), an enzyme that produces DGDG from monogalactosyldiacylglycerol (MGDG). KtrE has therefore also been designated DgdA (1). Under nonstress conditions, DGDG is found in the thylakoid membranes, which helps stabilize the photosystem II complex in *Synechocystis* (29). Under phosphate-limited conditions, DGDG is synthesized instead of phospholipids in *Synechocystis* (1). However, KtrB functions as a major K^+-conducting transport pore in the *Synechocystis* plasma membrane. The subcellular localization of KtrE has not been identified directly. Inactivation of *ktrE* (also called *dgdA*) in *Synechocystis* does not result in sensitivity to osmotic stress imposed by 300 mM sorbitol (1). This may be inconsistent with the requirement of KtrE for KtrB-mediated K^+ uptake in the presence of KtrA in the *E. coli* expression system (19).

Because of these uncertainties about the roles of the KtrA and KtrE subunits in K^+ uptake by KtrB in *Synechocystis* and about the identity of the Na^+ binding site in KtrB, we examined the subcellular localization and membrane association of KtrA and KtrE, the requirement of these subunits for KtrB-mediated K^+ uptake, and the primary target for Na^+ binding in KtrB.

MATERIALS AND METHODS

Construction of plasmids. The *ktrE* and *ktrB* genes, which exist in the same cistron, were subcloned into separate plasmids for expression in *E. coli*. To this end, both genes were amplified using primers containing appropriate restriction sites. For *ktrB*, the primers used were 5′-GAGTCTAGAAGGAATCTGCATG ACTATTTCC-3′ (XbaI site underlined) and 5′-ATTCTGCAGTTAGCCTAC CAGCAA-3′ (PstI site underlined); for subcloning of *ktrE*, the primers used were 5′-GAGGGTACCAGGAATCTGCATGCATATTGCT-3′ (KpnI site underlined) and 5′-ATTCTGCAGTCATTCCAGCAGAT-3′ (PstI site underlined). The resulting PCR products were cloned downstream of the *tac* promoter into pPAB404 (4) or pSTV28 (Takara, Japan), at the XbaI/PstI or the KpnI/PstI sites, respectively. Selective markers used were resistance to ampicillin (pPAB404) or to chloramphenicol (pSTV28). The sequences were verified by sequencing. The constructs were used to transform *E. coli* LB2003 (F⁻ *thi lacZ gal rha ΔkdpFABC5 trk01 ΔtrkA*), which lacks the three K^+ uptake systems, Trk, Kup, and Kdp and is unable to grow at K^+ concentrations below approximately 15 mM (31). Point mutation variants of KtrB were created by site-directed mutagenesis of charged residues located in the extracellular pore loop and in the cytoplasmic domain of KtrB using overlap extension PCR (39).

Complementation growth test of *E. coli* LB2003. *E. coli* strain LB2003 containing a specific mutated version of KtrB or containing different combinations of Ktr subunits was grown at 30°C overnight on synthetic solid medium (39) in the presence of 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 40 μg/ml ampicillin, 20 μg/ml chloramphenicol, and different concentrations of KCl.

K^+ uptake assay. K^+ uptake was measured essentially as described elsewhere (39). *E. coli* LB2003 cells harboring a specific KtrB variant or different combinations of Ktr subunits were cultured in a synthetic medium at 30°C. The cells were collected by centrifugation, resuspended in 120 mM Tris-HCl (pH 8.0) to an optical density at 578 nm (OD_{578}) of 30, and then EDTA was added to a final concentration of 1 mM. Subsequently, the cell suspension was shaken for 10 min at 37°C, collected by centrifugation, washed twice with 200 mM HEPES-NaOH (pH 7.5) or 200 mM HEPES-triethanolamine (pH 7.5), and then resuspended in the same buffer. After the suspension was shaken for 20 min at room temperature, the cell density was adjusted to an OD_{578} of 3 with the same buffer. Ten minutes prior to the start of the K^+ uptake measurement, 10 mM glucose was added to the suspension. To start the uptake assay, KCl was added to the cells (for concentrations, see figure legends). For determination of the initial velocity of K^+ uptake, 1 ml of cell suspension was removed 1 min after the addition of KCl, transferred to a tube containing 150 μl of silicon oil, and centrifuged at 12,000 rpm for 1 min. For time course experiments, 1-ml aliquots were withdrawn at the times indicated in the figure legend. The potassium content of the cell pellet was determined by flame photometry.

Membrane localization of KtrA and KtrB in *E. coli*. The *E. coli* strain BL21 containing KtrA or KtrE in pPAB404 was grown to an OD_{610} of 0.5 to 0.6 at 30°C; expression of *ktrA* or *ktrE* was induced by addition of IPTG (final concentration of 0.5 mM) to the cells, which were then cultured for an additional 3 h. Cells were harvested by centrifugation, and the cell pellet was resuspended with 20 mM Tris-HCl (pH 8)–0.1 M NaCl. The cells were sonicated and then centrifuged at 15,000 rpm for 1 min. The supernatant was transferred to a new tube and ultracentrifuged at 100,000 rpm for 15 min. The soluble fraction was resuspended with the same buffer as the membrane fraction. The proteins were separated by SDS-PAGE, followed by immunoblotting. Antibodies against KtrA and KtrE were raised against synthetic peptides with the sequences NH_2-IVDR FKLDPNNSIV-COOH for KtrA, and NH_2-CPGQNDLKKEYQAER-COOH for KtrE (Operon Biotechnologies, Japan).

Membrane localization of KtrA and KtrE in *Synechocystis*. Thylakoid and plasma membrane fractions were prepared from *Synechocystis* cells as described previously (22). Polyclonal antibodies raised against the plasma membrane nitrate transporter NrtA (24) and against the thylakoid membrane proteins NdhD3 and NdhF3 (38) were used as markers for the plasma membrane fraction or for the thylakoid membrane fraction in *Synechocystis*, respectively. Proteins were separated by SDS-PAGE on a 12.5% gel and then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated for 1 h with the primary antibody (1:1,000 in blocking buffer), followed by incubation for 30 min with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG [1:1,000; Amersham Pharmacia]) and subsequently developed by chemiluminescence detection (ECL; Amersham Pharmacia).

RESULTS

The KtrA and KtrE subunits enhance Ktr-mediated K^+ uptake rate and contribute to its Na^+ dependency. Previously, we had used a plasmid containing the *ktrE-ktrB* cistron to express *ktrB* and *ktrE* in the K^+ uptake-deficient *E. coli* strain LB2003 and to perform K^+ transport assays and test the growth rate of the cells in medium containing 5 mM KCl (19). For this study, *ktrB* and *ktrE* were separated into different plasmids under the control of individual promoters (see Materials and Methods). The LB2003 cells containing *ktrA*, *ktrE*, and *ktrB* in different combinations were tested for growth on solid medium containing various concentrations of KCl (Fig. 1A). At 5 mM KCl only the cells containing all three subunits, KtrABE, were able to grow while cells expressing only *ktrA* or *ktrE* behaved like cells containing only the empty vector. This confirmed that KtrB required both KtrA and KtrE to be present in order to mediate K^+ uptake at low K^+ concentrations (19). However, at higher K^+ concentrations (10 mM KCl), KtrB alone was able to sustain growth of the *E. coli* cells. Similar results were obtained when K^+ uptake activity at low K^+ concentrations (5 mM) was measured directly (Fig. 1B). In this assay the role of the subunits on the Na^+ dependency of K^+ transport was also analyzed. K^+ uptake was strongly stimulated by Na^+ when all three subunits, KtrABE, were present. Cells containing only KtrB or KtrB together with either KtrA or KtrE showed low K^+ transport activity that was independent of the addition of Na^+. These results indicate that absence of either KtrA or KtrE eliminated the Na^+ dependency of K^+ transport. However, KtrB alone retained some K^+ uptake activity that was Na^+ independent, which is also found in the KtrB protein from *V. alginolyticus* (3, 6, 7, 9, 11–14, 18, 19, 32, 33). However, in contrast to the *Vibrio* KtrB protein (3, 6,

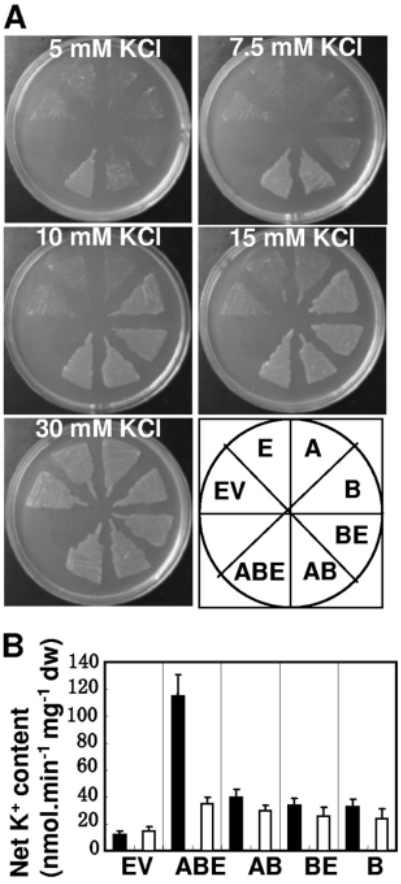


FIG. 1. Role of the KtrA and KtrE subunits for Ktr-mediated K^+ uptake in *E. coli*. (A) Growth of *E. coli* strain LB2003 expressing various combinations of KtrA (A), KtrB (B), and KtrE (E) on solid medium supplemented with the indicated KCl concentrations. EV, empty vector. (B) Determination of the initial rate of K^+ uptake by *E. coli* LB2003 expressing KtrB (B) with KtrA (A) or KtrE (E). Assays were performed either in 200 mM HEPES-NaOH buffer (black bars) or Na^+-free buffer (white bars) in the presence of 10 mM glucose and 5 mM KCl. Values are averages of results obtained from three independent experiments. Error bars represent standard deviations. dw, dry weight.

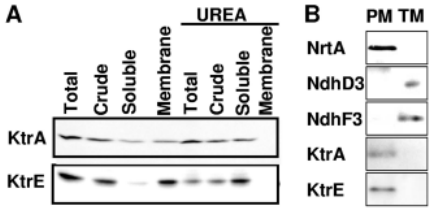


FIG. 2. Association of KtrA or KtrE with the plasma membrane. (A) KtrA and KtrE were detected on Western blots using specific antibodies. Samples in the right half were treated with sample buffer containing 4 M urea to separate peripheral proteins from the membranes. (B) KtrA and KtrE are localized in the plasma membrane in *Synechocystis* sp. strain PCC 6803. PM, plasma membrane; TM, thylakoid membrane. NtrA is a marker protein for the PM (24). NdhD3 and NdhF3 are marker proteins for the TM (38).

7, 9, 11–14, 18, 19, 32, 33), *Synechocystis* KtrB did not transport Na^+ (19; also data not shown).

Membrane localization of KtrA and KtrE in *Synechocystis*. Based on their hydropathy plots, KtrA and KtrE are unlikely to be membrane-integral proteins since they are lacking strong hydrophobic domains. However, previous results (19) as well as the data shown in Fig. 1 indicate that KtrA and KtrE are functionally interacting with KtrB, which is a transmembrane protein. To determine the subcellular localization of the KtrA and KtrE subunits, anti-KtrA and anti-KtrE antibodies were generated and used to probe fractions of proteins isolated from *E. coli* cells expressing KtrA or KtrE. The signal for KtrA protein was detected not only in the plasma membrane fraction but also in the soluble, cytoplasmic fraction (Fig. 2A). In contrast, the majority of KtrE was associated with the plasma membrane fraction. When urea was added to the protein fractions, both KtrA and KtrE disappeared completely from the membrane fraction, indicating that they are peripherally associated with the plasma membrane but are not membrane-integral proteins.

Based on their roles as subunits of the Ktr K^+ uptake system, KtrE and KtrA would be expected to be colocalized with KtrB in the plasma membrane in *Synechocystis*. However, KtrE has previously been described as a DGDG synthase (1). In *Synechocystis* and in chloroplasts of higher plants, DGDG usually accumulates in the thylakoid membrane (2, 15). Therefore, in order to clarify the subcellular localization of KtrE and KtrA in *Synechocystis*, we performed immunolocalization experiments using specific antibodies. Membrane fractions of thylakoid and plasma membranes were prepared by aqueous polymer two-phase partitioning, followed by sucrose density gradient centrifugation (22). As shown in Fig. 2B, protein bands of the corresponding molecular mass of KtrE or KtrA were found only in the plasma membrane fraction, which was identified by the presence of the plasma membrane marker proteins NrtA (24). No signal for KtrE or KtrA was found in the thylakoid membrane fraction, identified by the marker proteins NdhD3 and NdhF3 (23, 38). These results indicate that KtrE and KtrA are exclusively associated with the plasma membrane fraction in *Synechocystis*.

Replacement of negatively charged residues in the extracellular or cytoplasmic regions of KtrB with positively charged residues had no effect on the Na^+ activation of KtrB. Although KtrB-mediated K^+ uptake activity was strongly stimulated by Na^+, information on the identity of the Na^+ binding site necessary for this activation of KtrB-mediated transport is lacking. For some cation transport proteins, it has been shown that Na^+ binding involves negatively charged amino acids (10, 37). To evaluate the possibility of the involvement of negatively charged residues in Na^+ activation, the influence of Na^+ on K^+ uptake activity was tested in mutated versions of KtrB. In those variants of KtrB, all of the negatively charged residues (Glu-32, Asp-56, Glu-66, Asp-172, Asp-261, Asp-282, Glu-285, Glu-374, Glu-381, and Glu-398) predicted to be on the extracellular side of the transporter were individually changed to residues of the opposite charge by mutating them to Lys (Fig. 3 and 4). These mutant versions of KtrB were introduced into *E. coli* LB2003 cells harboring KtrA and KtrE. In medium

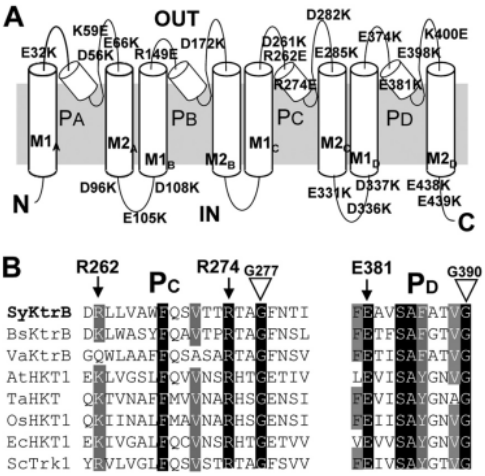
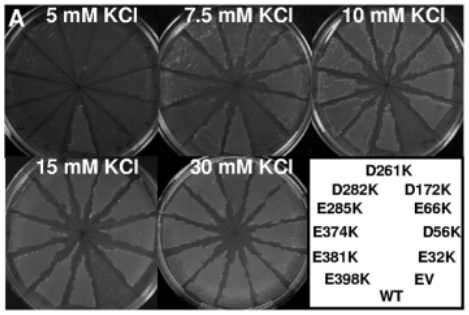


FIG. 3. Localization of charged residues in KtrB. (A) The pore region (P) in P_A, P_B, P_C, and P_D contains conserved Gly residues (shown in larger font) which form a K^+-selective filter. Fourteen charged residues located in the extracellular space (shown in bold) were changed to residues of the opposite charge. The negatively charged residues Asp (D) and Glu (E) were converted to Lys (K), and the positively charged residues Lys (K) or Arg (R) were mutated to Glu (E). Eight negatively charged residues (in bold) located on the cytoplasmic side were converted to Lys. (B) Sy, *Synechocystis*; Bs, *B. subtilis*; Va, *V. alginolyticus*; Ec, *Eucalyptus camaldulensis*; Sc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana*; Ta, *Triticum aestivum*; Os, *Oryza sativa*.



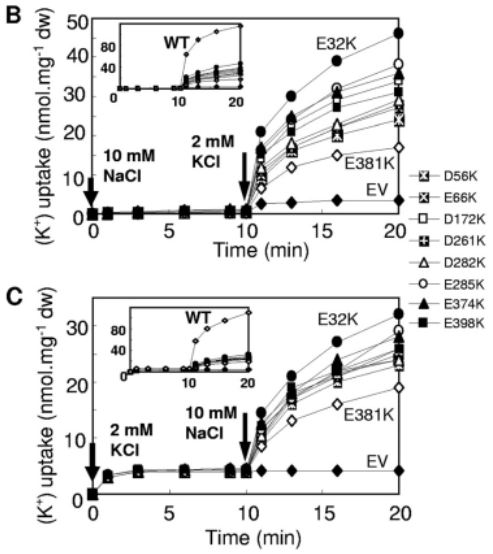


FIG. 4. Effects of mutations of negatively charged residues in the extracellular region of KtrB on the Na^+ dependency of K^+ uptake activity. (A) K^+ uptake-deficient *E. coli* containing wild-type KtrB (WT), KtrB variants, or the empty vector (EV) were grown on synthetic solid medium containing different concentrations of KCl. (B) Time course of K^+ uptake by *E. coli* LB2003 cells expressing KtrB variants with mutations in the extracellular loops. Cells were preincubated in HEPES-triethanolamine buffer with 10 mM glucose and 10 mM NaCl. At 10 min, 2 mM KCl was added to the assay; samples were removed, and the amount of K^+ taken up was determined at the times indicated. Na^+-dependent K^+ uptake activity by wild-type (WT) KtrB is shown in the inset panel. The points shown are the average of three independent experiments. (C) Time course of K^+ uptake by *E. coli* LB2003 cells expressing the same KtrB variants as in the experiment shown in panel B, preincubated with 2 mM KCl in Na^+-free buffer (HEPES-triethanolamine, 10 mM glucose). At 10 min, 10 mM NaCl was added to the assay. Na^+-dependent K^+ uptake activity by wild-type (WT) KtrB is shown in the inset panel. The points shown are the average of three independent experiments.

supplemented with 5 mM KCl, only cells expressing the wild-type KtrB were able to grow, whereas all mutations in KtrB diminished the ability to complement the growth of *E. coli* LB2003 (Fig. 4A). On medium containing 7.5 mM K^+, only the cells containing the E381K variant showed less growth while all other variants were not different from cells containing the wild-type protein (Fig. 4A). While the overall K^+ uptake of the KtrB variants was significantly lower than that of the wild-type KtrB, they all still showed Na^+-dependent activation of K^+ transport (Fig. 4B and C). The rate of increase of K^+ transport was similar whether the cells were preincubated with 10 mM NaCl and then treated with 2 mM KCl (Fig. 4B) or whether the cells were preincubated with 2 mM KCl and then treated with 10 mM NaCl (Fig. 4C). None of the mutations resulted in a complete loss of Na^+ activation.

A negative residue in the C-terminal cytosolic region of animal G protein-gated inwardly rectifying K^+ channels serves as a binding site for Na^+ to modulate the channel gating property (10, 37). In order to test whether the same could be true for KtrB, we replaced negatively charged residues on the cytoplasmic side of KtrB with positive charges. Based on the topology model (Fig. 3A), eight KtrB variants (D96K, E105K, D108K, E331K, D336K, D337K, E438K, and E439K) were generated, and their functionality was tested in *E. coli* strain LB2003 containing KtrA and KtrE. None of the KtrB variants was able to complement growth on medium supplemented with 5 mM KCl. On medium containing 7.5 mM KCl, only the E331K variant could grow. On medium containing 10 mM KCl, the D336K and D337K variants grew very slowly, whereas all other variants grew as well as the wild type (Fig. 5A). In order to assess the K^+ transport rate of the mutant proteins, 2 mM KCl was added into the assay buffer; however this did not increase K^+ uptake sufficiently (data now shown). Hence, the K^+ uptake assay was performed by adding 4 mM KCl to cells preincubated with 10 mM NaCl. In all cases K^+ uptake was stimulated by the presence of Na^+, but there were some dif-

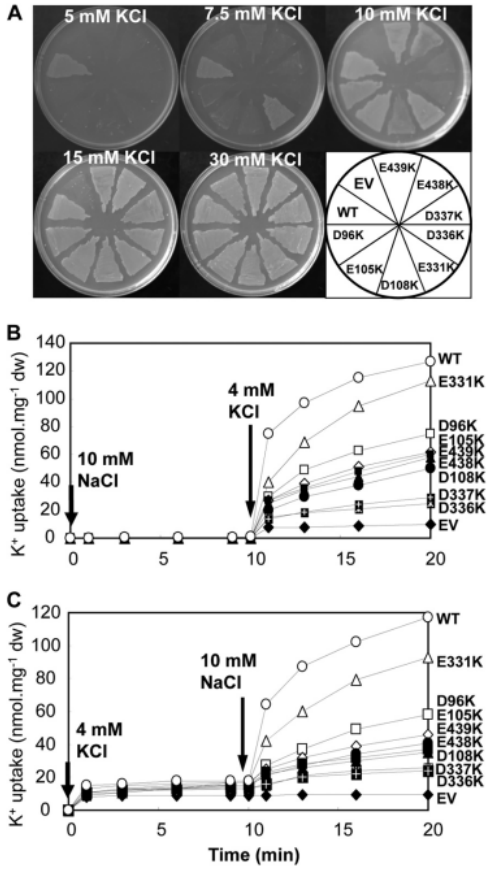


FIG. 5. Effects of mutations of negatively charged residues in the cytoplasmic region of KtrB on the Na^+ dependency of K^+ uptake activity. (A) K^+ uptake-deficient *E. coli* containing wild-type (WT) KtrB, variants, or empty vector (EV) was grown on synthetic solid medium containing different concentrations of KCl. (B) Time course of K^+ uptake by *E. coli* LB2003 cells expressing KtrB variants with mutations in the cytoplasmic region. Cells were preincubated in HEPES-triethanolamine buffer containing 10 mM glucose and 10 mM NaCl. At 10 min, 4 mM KCl was added to the assay; samples were removed, and the amount of K^+ taken up was determined at the times indicated. The points shown are the average of three independent experiments. (C) Time course of K^+ uptake by *E. coli* LB2003 cells expressing the same KtrB variants as in the experiment shown in panel B preincubated with 4 mM KCl in Na^+-free buffer (HEPES-triethanolamine with 10 mM glucose). At 10 min, 10 mM NaCl was added to the assay buffer. The points shown are the average of three independent experiments.

ferences between the variants. The D336K and D337K variants showed the lowest K^+ uptake rates (Fig. 5B). The results were essentially the same when cells were preincubated with 4 mM KCl before the addition of 10 mM NaCl (Fig. 5C). None of the mutations apparently eliminated Na^+ binding. However, the overall effect on K^+ uptake activity was greater in the case where mutations were introduced on the cytoplasmic side of KtrB instead of on the extracellular side. It is possible that these mutations interfered with binding of KtrA and/or KtrE to KtrB (12).

Replacement of Arg262 by Glu abolished KtrB-mediated K^+ uptake activity. The highly conserved positively charged residues on the extracellular side of Ktr/HKT-type transporters have been proposed to be involved in electrostatic interactions with highly conserved, negatively charged residues in the first and fourth pore loop (12). It has also been reported that the His at position 157 on the extracellular side is essential for maintaining the K^+ uptake transporter in an active form (39). Here, the contributions of five positive residues in the extracellular region of KtrB to its function were examined by replacing them with residues of the opposite charge (Lys/Arg to Glu; K59E, R149E, R262E, R274E, and K400E). As before, *E. coli* strain LB2003 was transformed with either wild-type KtrB or with a KtrB variant or the empty vector, and growth on solid medium supplemented with different amounts of KCl was assayed (Fig. 6A). The R149E variant was used as a reference because in a previous study no strong reduction in K^+ transport activity had been found for this variant (39). The growth assay showed that cells expressing the K59E variant were able to grow as well as those expressing the R149E variant on medium supplemented with 5 mM KCl. The R274E and K400E variants were able to complement growth on medium containing 15 mM KCl; however, the R262E variant did not rescue the growth defect of *E. coli* LB2003 on medium containing KCl in concentrations ranging from 5 mM to 15 mM KCl (Fig. 6A).

To analyze the effects of the mutations in the extracellular region of KtrB on the kinetic parameters of K^+ transport in more detail, the initial velocity of K^+ uptake was determined, and K_m and V_{max} were calculated (Table 1). The K_m values were consistent with the results of the growth test shown in Fig. 6A. To compare the effects of mutating D261, the residue immediately next to R262, we also determined K_m and V_{max} for the 10 KtrB variants with changes in negatively charged residues in the extracellular region shown in Fig. 3A. The D261K variant had an increased apparent K_m, compared with the wild type. However, D261K retained K^+ uptake activity. The apparent K_m for K^+ uptake of the E381K variant was the highest after that of the R274E variant. This finding supports the suggested role of E381 located in the fourth pore region (Fig. 3A, P_D) in forming electrostatic interactions with R415 in the eighth transmembrane domain (Fig. 3A, $M2_D$) (12). R262 is highly conserved among Ktr/HKT transporters (Fig. 3B). To further evaluate the role of R262 in KtrB, we replaced R262 with Ala, Gln, or Lys. Replacement of R262 with Lys (K) was able to rescue the growth of *E. coli* LB2003 on medium supplemented with 5 mM KCl (Fig. 6C) and restored K^+ uptake activity (Fig. 6D). Replacing R262 with the neutral residues Ala (A) or Gln (Q) somewhat compensated for the loss of Arg, but the R262E variant was inactive (Fig. 6A). This loss of activity suggests that the R262 is a critical site for the KtrB-mediated K^+ uptake activity; however, it may also be due to protein degradation and/or misfolding. To investigate the possibility of R262 being important for transport function, we examined the atomic-scale model of *Synechocystis* KtrB (Fig. 7). The model indicates that the positively charged guanidium group of R262 in the middle of the third external loop can interact with carboxyl groups of E247 located at the end of $M1_C$ and/or with D261 in the same loop.

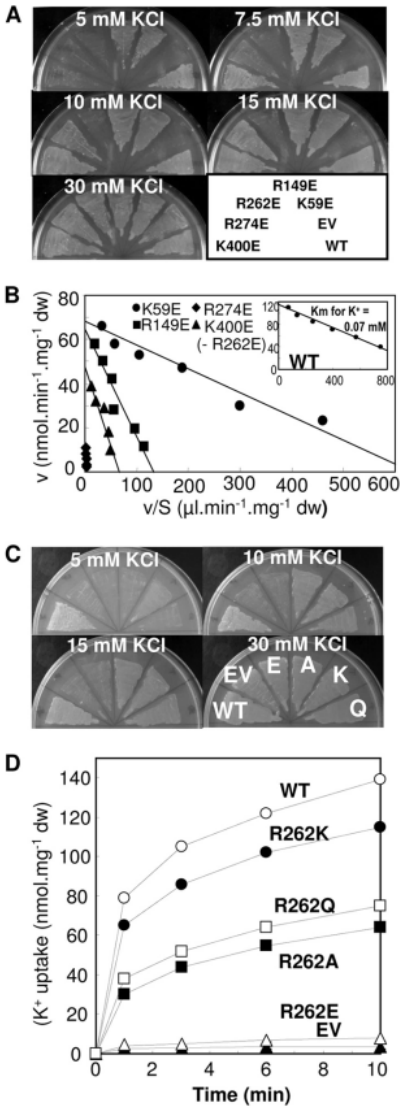


FIG. 6. Functional characterization of the role of positive residues located in the extracellular region of KtrB. (A) K^+ uptake-deficient *E. coli* cells containing wild-type (WT) KtrB, KtrB variants with changes in positive residues in the extracellular loops, or empty vector (EV) were grown on synthetic solid medium containing different concentrations of KCl. The R149E variant was used as a reference (39). (B) Eadie-Hofstee plots of the initial velocity of K^+ uptake obtained from *E. coli* cells expressing either wild-type KtrB or KtrB variants. Assays were done in HEPES-NaOH buffer. Values shown are averages from at least three independent experiments. The details of the kinetic parameters are presented in Table 1. R262E did not rescue the growth defect of *E. coli* LB2003 (see A), which was consistent with the loss of activity in the assay as shown in Table 1. The inset panel shows the initial velocity of K^+ uptake for the wild type. (C) Effect of replacement of R262 with different amino acids: Ala (A), Lys (K), Gln (Q), and Glu (E). K^+ uptake-deficient *E. coli* LB2003 containing wild-type KtrB, the different R262 variants, or the empty vector (EV) was grown on synthetic solid medium with different concentrations of KCl. (D) Determination of the K^+ uptake rate by *E. coli* LB2003 cells harboring wild-type KtrB or the KtrB variants (R262A, R262E, R262K, and R262Q). For the time course experiments, cells were incubated in 200 mM HEPES-NaOH with 10 mM glucose. At time zero, 2 mM KCl was added to the assay buffer. Values shown are averages of results obtained from three independent experiments.

TABLE 1. Kinetic parameters of K^+ uptake by *E. coli* LB2003 containing wild-type KtrB or various KtrB variants[a]

KtrB type	Apparent K_m for K^+ (mM)	V_{max} (nmol min^{-1} mg^{-1} dw)
Wild type	0.07 ± 0.009	116 ± 9
Variants with positive residues replaced by Glu		
K59E	0.1 ± 0.02	65 ± 6
R149E	0.5 ± 0.1[b]	70 ± 5[b]
R262E	ND	ND
R274E	3.6 ± 0.7	16 ± 3
K400E	0.7 ± 0.2	48 ± 5
Variants with negative residues replaced by Lys		
E32K	0.9 ± 0.2	47 ± 6
D56K	1.9 ± 0.4	20 ± 3
E66K	1.8 ± 0.3	28 ± 3
D172K	1.7 ± 0.3	34 ± 7
D261K	1.3 ± 0.4	23 ± 4
D282K	1.5 ± 0.4	30 ± 5
E285K	0.9 ± 0.2	35 ± 5
E374K	0.8 ± 0.2	35 ± 6
E381K	3.3 ± 0.6	18 ± 3
E398K	1.1 ± 0.3	25 ± 3

[a] The values were obtained by use of Eadie-Hofstee plots of the initial rate of K^+ uptake. K^+ uptake assays were performed in 200 mM HEPES-NaOH buffer. The values are averages for at least three independent experiments ± standard deviations. ND, not detected; dw, dry weight.
[b] K_m and V_{max} were measured previously (39).

DISCUSSION

This study shows that coexpression of both KtrA and KtrE together with KtrB was required for full K^+ transport activity and susceptibility to stimulation by Na^+. In the absence of KtrA and KtrE, KtrB retained low K^+ uptake activity that was Na^+ independent and selective for K^+ over Na^+ when expressed in *E. coli*. This selectivity was different from that of *Vibrio* KtrAB, which showed Na^+ transport activity in the absence of its subunits (33). The KtrE subunit has not been found in any other members of the K^+ transporter superfamily (19). KtrE (or DgdA) has enzyme activity and catalyzes the synthesis of digalactosyldiacylglycerol (DGDG) from monogalactosyldiacylglycerol (MGDG) in *Synechocystis* (1, 29). In *Arabidopsis thaliana*, DGDG is synthesized by enzymes associated with the chloroplast envelope (2, 15) and is believed to move to the thylakoid membrane via a vesicle transport system (2, 15). Here, KtrE/DgdA was shown to be a peripheral membrane protein associated with the plasma membrane of *Synechocystis* (Fig. 2), where KtrA and KtrB are located as well. These findings raise the question as to what role KtrE has in KtrB-mediated K^+ uptake. Since *E. coli* has no MGDG in its membranes, KtrE/DgdA is unlikely to generate DGDG when expressed in *E. coli* (1, 29). Consistent with previously pub-

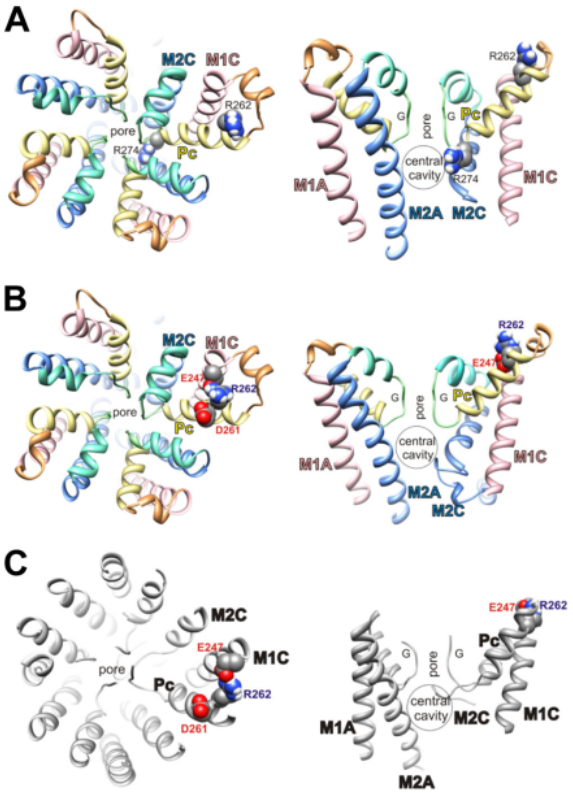


FIG. 7. Model of the localization of R262 and R274 within KtrB. (A) Position of R274 in the vicinity of the central cavity and R262 in an exposed loop. KtrB contains a 4-fold membrane-pore-membrane (MPM) motif, $M1_AP_AM2_A$-$M1_BP_BM2_B$-$M1_CP_CM2_C$-$M1_DP_DM2_D$ (6, 7). $M1_A$, $M2_A$, and $M2_C$ are the first, second, and sixth transmembrane domains, respectively. (B) The positively charged guanidium group of R262 is likely to interact with the carboxyl groups of E247 and D261. (C) A simplified version of panel B showing a top view from the extracellular space (left) and a side view (right).

lished results (1, 29), no change of the lipid composition of the *E. coli* inner membrane and no accumulation of DGDG or MGDG were found (16) (see Fig. S1 in the supplemental material). Therefore, synthesis of DGDG lipid is probably not part of the function of KtrB when it is expressed in *E. coli*.

Na^+ activation of K^+ uptake is a unique feature of Trk/Ktr/HKT transporters that distinguishes them from K^+ channels and Kdp-type transporters (19, 27, 30, 32). A mechanism for Na^+ binding and K^+ binding has been proposed for the Na^+/K^+-coupled transporter TaHKT2;1 from wheat (8). By performing an extensive random mutagenesis approach, residues affecting the Na^+/K^+ ratio of transport as well as residues determining Na^+ affinity were identified for that transporter (27, 28). Site-directed mutagenesis also revealed that replacing E464 with Gln (corresponding to E381 in the fourth pore region, P_D in KtrB) resulted in decreasing the affinity of TaHKT2;1 for Na^+ (5). In spite of these efforts, direct information on which sites are involved in Na^+ binding is still lacking. In animal ion channels, such as Kir-type channels and *N*-methyl-D-aspartate (NMDA) receptors, Na^+ acts as a second signaling factor for upregulating the activity of the channels (35). In Kir-type K^+ channels, the negative residues in the cytosolic region have been identified as playing a crucial role in Na^+-dependent activation (10, 37). Results obtained by an interactive computational-experimental approach based on the crystal structure of the cytosolic domain of Kir3.1 suggest that the Na^+ binding motif in Kir-type channels is DXRXXH (26). However, this sequence is not present in KtrB. Therefore, our mutagenesis study was focused on the possibility that a negative residue on either the external or the internal side of KtrB may be responsible for interaction with the cation. All 18 mutations introduced in this study reduced the K^+ transport activity of KtrB (Fig. 3A and 4), but none of them completely eliminated K^+ transport activity or Na^+ dependency. Considering that KtrB alone was able to mediate K^+ uptake and that absence of either KtrA or KtrE led to a loss of Na^+ dependency (Fig. 1), it seems likely that KtrA and KtrE have a role in the Na^+-dependent activation.

Although the mutational analysis of the negative residues (Fig. 4) did not reveal the identity of the primary sites for Na^+ binding (Fig. 4 and 5), the results provided new insights into structure and function of KtrABE. (i) Mutation of negatively charged residues in the extracellular region of KtrB to positive charges had a range of effects, from very small (E32K) to severe (E381K) (Fig. 4 and Table 1). The fact that the E381K mutation had the largest effect of all changes in the extracellular region supports the hypothesis that E381 in the fourth pore region (P_D) forms an electrostatic interaction with R415 in the eighth transmembrane domain ($M2_D$) (12). (ii) Mutations of residues in the cytoplasmic region of KtrB had a larger effect on transport activity than those of residues in the external space (Fig. 3A and 4). This indicates that these intracellular mutations may interfere with the association of KtrA and KtrE with KtrB. Note that we currently cannot exclude the possibility that the mutations may change the amount of functional protein in the membrane. Complementation growth tests and determination of K^+ uptake activity showed that the D336K and the D337K mutations had the most severe effects. These two residues are located in the internal loop between $M2_C$ and $M1_D$ on the cytoplasmic side of KtrB (Fig. 5). Recent work on *Vibrio* KtrAB showed that the C-terminal region of $M2_C$ was essential for the interaction between KtrA and KtrB (9). The positions of the C terminus of $M2_C$ and the internal loop of $M2_C$ and $M1_D$ are likely part of the mechanism by which Na^+ promotes K^+ transport activity.

Exchanging either of two positively charged residues with a negative charge (R262E and R274E) decreased K^+ uptake activity (Fig. 6). R274E, affecting a residue adjacent to the selective filter in the third pore region, P_C, retained some activity, whereas R262E abolished the activity completely (Fig. 6A and B). Interestingly, the D261K mutation affecting the position next to R262 retained a low level of K^+ uptake activity (Fig. 4 and Table 1). In the atomic-scale homology model, R262 resides in an electronegative environment where its positively charged guanidium group can bind to the negatively charged carboxyl groups of E247 located at the end of $M1_C$ and/or to D261 next to R262 in the same external loop (Fig. 7). This is similar to the effects of the H157 mutation, which may not be located at the selective filter (39) but led to a marked

decrease in transport activity. Together, these intramolecular electrostatic interactions probably contribute to stabilize the Ktr-transporter conformation.

This study showed that KtrA and KtrE are associated with the plasma membrane in *Synechocystis* although the enzymatic product of KtrE had been reported to be present in thylakoid membrane. The interaction of KtrA and KtrE with KtrB increased the K^+ transport rate and was required for the Na^+ dependency of KtrB when it was expressed in *E. coli*. Mutational analysis of charged residues in KtrB identified a new possible electrostatic interaction of R262 with negative residues that is proposed to contribute to maintain KtrB in an active form.

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