Gain of Function Mutations in Membrane Region M2C2 of KtrB Open a Gate Controlling K+ Transport by the KtrAB System from *Vibrio alginolyticus* 

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KtrB, the K\(^{+}\)-translocating subunit of the Na\(^{+}\)-dependent bacterial K\(^{+}\) uptake system KtrAB, consists of four M\(_{1}\)PM\(_{2}\) domains, in which M\(_{1}\) and M\(_{2}\) are transmembrane helices and P indicates a p-loop that folds back from the external medium into the cell membrane. The transmembrane stretch M\(_{2}\)C is, with its 40 residues, unusually long. It consists of three parts, the hydrophobic helices M\(_{2}\)C\(_{1}\) and M\(_{2}\)C\(_{3}\), which are connected by a nonhelical M\(_{2}\)C\(_{2}\) region, containing conserved glycine, alanine, serine, threonine, and lysine residues. Several point mutations in M\(_{2}\)C\(_{2}\) led to a huge gain of function of K\(^{+}\) uptake by KtrB from the bacterium *Vibrio alginolyticus*. This effect was exclusively due to an increase in \(V_{\text{max}}\) for K\(^{+}\) transport. Na\(^{+}\) translocation by KtrB was not affected. Partial to complete deletions of M\(_{2}\)C\(_{2}\) also led to enhanced \(V_{\text{max}}\) values for K\(^{+}\) uptake via KtrB. However, several deletion variants also exhibited higher \(K_{m}\) values for K\(^{+}\) uptake and at least one deletion variant, KtrAP\(_{326\text{–338}}\), also transported Na\(^{+}\) faster. The presence of KtrA did not suppress any of these effects. For the deletion variants, this was due to a diminished binding of KtrA to KtrB. PhoA studies indicated that M\(_{2}\)C\(_{2}\) forms a flexible structure within the membrane allowing M\(_{2}\)C\(_{2}\) to be directed either to the cytoplasm or (artificially) to the periplasm. These data are interpreted to mean (i) that region M\(_{2}\)C\(_{2}\) forms a flexible gate controlling K\(^{+}\) translocation at the cytoplasmic side of KtrB, and (ii) that M\(_{2}\)C\(_{2}\) is required for the interaction between KtrA and KtrB.

The alkali cation potassium is the main osmolyte in the cytoplasm of prokaryotes (1). It binds close to the active center of the ribosome (2) and participates actively in pH homeostasis (3) and osmoadaptation (4, 5) by transport across the cell membrane. Hence, prokaryotes regulate their K\(^{+}\) contents and adapt them rapidly in response to changes in the environment. For this purpose, they possess a number of K\(^{+}\) channels, pumps, and transporters (1, 6). Several of the K\(^{+}\)-uptake systems contain a K\(^{+}\)-translocating subunit belonging to the superfamily of K\(^{+}\) transporters (7, 8) (termed SKT proteins (9)). These proteins may have evolved from simple K\(^{+}\) channels of the M\(_{1}\)PM\(_{2}\) type, like KcsA (10, 11) or Kir (12), by multiple gene duplications and gene fusions (7). Whereas the channels form homotetramers from four identical M\(_{1}\)PM\(_{2}\) subunits, SKT proteins consist of four covalently linked M\(_{1}\)PM\(_{2}\) motifs connected by cytoplasmic loops. The four p-loops (P) are thought to fold back from the external medium to the middle of the membrane, where they form a part of the permeation pathway for K\(^{+}\) through the channel center (7–9, 13). Within each p-loop, most SKT proteins contain one conserved glycine residue, which is part of their K\(^{+}\) selectivity filter (9, 14–17). With single conserved glycine residues in SKT proteins, this filter appears to have a simpler structure than in K\(^{+}\) channels, in which the filter is formed by the well conserved p-loop sequence TVGGY from each subunit (18).

The SKT-protein KtrB forms the K\(^{+}\)-translocating subunit of the Na\(^{+}\)-dependent K\(^{+}\)-uptake system KtrAB from bacteria (9, 14, 19–22). KtrA, the other subunit from KtrAB, is located at the cytoplasmic side of the membrane and is a member of the RCK/KTN protein family (1, 23). KtrA may regulate K\(^{+}\) transport by binding ATP (24, 25). It confers velocity, Na\(^{+}\) dependence, and K\(^{+}\) selectivity to the complex (9). KtrB alone transports K\(^{+}\) slowly in a process that is independent of Na\(^{+}\). In addition, it transports Na\(^{+}\) with relatively low affinity (\(K_{m}\) value of \(-3 \text{ mN}\) Na\(^{+}\)). The exact structure of KtrB is unknown, but it has been modeled based on the structure of KcsA (11, 13). Most of the KtrB structure was similar to that of KcsA. However, in particular, the C termini from the membrane spans M\(_{2}\)C\(_{2}\) and M\(_{2}\)D deviated from that of KcsA-M\(_{2}\). This may reflect the difference in function between the channel KcsA and the transporter KtrB (13). Subsequent cross-linking studies showed that the external half of KtrB is very similar to that of the KcsA tetramer, whereas its cytoplasmic half deviates. In addition, KtrB may form dimers (26). In their modeling studies, Durell and Guy (13) focused on membrane span M\(_{2}\)C\(_{2}\). They divided it into three regions, from M\(_{2}\)C\(_{1}\) to M\(_{2}\)C\(_{3}\) (see Fig. 1A). M\(_{2}\)C\(_{1}\) and M\(_{2}\)C\(_{3}\) can form hydrophobic α-helices. However, M\(_{2}\)C\(_{2}\) contains many conserved small and polar residues (Ala, Gly, and Ser, Thr, Lys, respectively; see Fig. 1B). It may form a random coil or β-turn structure (13). According to the first Durell and Guy model (13), M\(_{2}\)C\(_{1}\) and M\(_{2}\)C\(_{3}\) span the membrane, and M\(_{2}\)C\(_{2}\) forms a loop that fills the cavity just beneath the p-loops. Alternatively, these authors proposed (13) that M\(_{2}\)C\(_{1}\) and M\(_{2}\)C\(_{3}\) span the membrane (the latter in a coiled conformation) and helix M\(_{2}\)C\(_{3}\) is located within the inner surface of the membrane. The conserved lysine residue (Lys\(_{125}\)) in VaKtrB from *Vibrio alginolyticus*; see vertical arrow in Fig. 1B) may form a salt bridge with...
a conserved aspartate residue from the C terminus of M2B (VaKtrB Asp222, 13). Region M2C2 is conserved among the bacterial SKT protein families KtrB, TrkH, and KdpA (7, 8, 13). All material SKT protein families KtrB, TrkH, and KdpA (7, 8, 13). All of these require at least one additional subunit for activity. The interaction with the other subunit(s) (i.e. KtrA for KtrB) may modulate the conformation of M2C2, thereby allowing the passage of $K^+$ through a gate (13).

In this study, we report on the effects of point mutations and deletions in region M2C2 of KtrB from V. alginolyticus (VaKtrB) on the uptake of $K^+$ and $Na^+$ by Escherichia coli cells. Many of the changes cause a huge gain of function effect on $K^+$ transport, whereas $Na^+$ transport remained unaltered. In addition, KtrB-PhoA fusions suggest that region M2C2 possesses a flexible structure. These results are interpreted to mean that M2C2 forms a gate for $K^+$ permeation through the KtrB protein.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Growth Conditions**—The strains and plasmids used in this study are listed in the supplemental Table. Plasmids pKtrB$_{G314A}$ to pKtrB$_{S327C}$ containing point mutations in codons from M2C2 of VaKtrB were generated from plasmid pEL903-100 by PCR using the QuickChange mutagenesis kit from New England Biolabs (Frankfurt, Germany). Plasmids with deletions in region M2C of KtrB (pKtrB$_{A314-332}$ to pKtrB$_{A326-329}$ and pKtrB$_{A314-328}$Leu$^{326}$-PhoA to pKtrB$_{A326-329}$Leu$^{326}$-PhoA) were generated with the Finnzymes Phusion™ site-directed mutagenesis kit from New England Biolabs (Frankfurt, Germany) from plasmids pEL900-100 and pKtrBL340-PhoA, respectively. KtrAB plasmids with point mutations or deletions in region M2C2 from ktrB were generated by replacing a 1-kb BglI-MscI restriction fragment from ktrB in plasmid pH301 by the same fragment from a ktrB plasmid carrying the desired mutation. Plasmids encoding KtrB-PhoA fusion proteins were generated with the aid of plasmid pPAB404 as described in Ref. 9. The absolutely conserved VaKtrB residues Gly$^{290}$ (selectivity-filter residue (9, 14)), Gly$^{314}$ and Lys$^{325}$ (both M2C2 residues) are marked by vertical arrows.

**FIGURE 1. Region M2C of VaKtrB.** A, schematic representation of how M2C is supposed to fold across the membrane according to Ref. 9, subdivision of M2C into regions M2C1 to M2C3. B, conserved residues in regions PC and M2C2. Increasing degree of conservation of single residues is indicated by the background changing from white to black. The absolutely conserved VaKtrB residues Gly$^{290}$ (selectivity-filter residue (9, 14)), Gly$^{314}$ and Lys$^{325}$ (both M2C2 residues) are marked by vertical arrows.
LB medium up to an OD_{578} value of 0.5, harvested by centrifugation, washed with buffer containing 150 mM NaCl and 10 mM TrisCl, pH 8.0, and suspended in 0.5 ml of the same buffer. Alkaline phosphatase activity of the suspension was determined according to Ref. 33 and expressed in nmol/min/mg cell (dry weight).

**Overproduction and Purification of His_{10}-KtrAKtrB complexes**—Cells of strain LB2003 containing plasmid pIH301 or one of its derivatives were grown in 1 liter of K30 medium (29) in the presence of 0.2% glycerol (v/v) and 0.02% L-arabinose (w/v) up to an OD_{518} value of 1.0–1.5, harvested by centrifugation, washed once with buffer S containing 600 mM NaCl, 10% glycerol (w/v), and 50 mM TrisCl, pH 8, and resuspended at 10 mg (dry weight)/ml of the buffer S in the presence of 1 mM EDTA and some DNase. Cells were broken by sonication with a Branson 250 Sonifier II cell disruptor (Branson). Subsequently, the suspension was centrifuged for 15 min at 15,000 × g, and its supernatant was centrifuged overnight at 100,000 × g. The pellet was suspended at 10 mg protein/ml of buffer S, and its proteins were solubilized by incubation with 1% β-D-dodecylmaltoside (Anatrace) and 1% protease-inhibitor mixture P8849 (Sigma-Aldrich, Steinheim, Germany) for 1 h at 4 °C and centrifuged for 45 min at 200,000 × g in a TL100.3 Mini-ultracentrifuge (Beckmann, Frankfurt, Germany). The supernatant was incubated at a concentration of 150 mg of protein/ml packed nickel-nitrotriacetic acid-agarose (Qiagen, Hilden, Germany) in the presence of 10 mM imidazole in a 10-ml polypropylene column for 1 h. Subsequently, the agarose was washed with 50 volumes of buffer W, containing 200 mM NaCl, 20 mM TrisCl, pH 8, 0.5 mM β-mercaptoethanol, 10% glycerol (w/v), 0.04% β-D-dodecylmaltoside (w/v) plus 50 mM imidazole. His-tagged protein was eluted with 3 volumes of buffer W containing 500 mM
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**RESULTS**

*Gain of Function Changes in Region M₂C₂—Durell and Guy* (13) have proposed that region M₂C₂ plays an important role in the K⁺-transport cycle of KtrB. To obtain more information about this region, we changed single amino acids in M₂C₂ to cysteines in Cysless VaKtrB containing an N-terminal His tag, as encoded by plasmid pEL903–100 and its derivatives. However, several of these cysteine variants were inactive. This was not unexpected in view of the large degree of conservation of several amino acid residues in M₂C₂ (Fig. 1B, residues with a gray to black background). To learn more about these residues, more conservative amino acid changes were engineered in VaKtrB residues at positions Gly⁴¹³, Gly⁴¹⁵, Gly⁴¹⁷, Gly⁴²², Gly⁴²³, Lys⁴²⁵, and Val⁴²⁶ (Fig. 2E). Together with the variants KtrB₃₁₄₃, KtrB₃₁₇₇, KtrB₃₁₈₃, KtrB₃₂₀₃, KtrB₃₂₄₃, and KtrB₃₂₇₇, it was investigated whether these constructs influenced growth of *E. coli* strain LB2003 at low K⁺ concentrations. This strain lacks functional K⁺ uptake systems, and, as a consequence, it grows only at an external [K⁺] > 10 mm (28). A plasmid encoding a functional K⁺ uptake system will lower this concentration to 0.1 mm K⁺ (14). In addition, we measured the net uptake of K⁺ and Na⁺ by these strains and determined the produced amounts of these His-tagged KtrB variants by Western blotting with a monoclonal anti-Hisₙ antibody.

The following variants were not characterized further because they inhibited growth of strain LB2003 completely at all K⁺ concentrations: KtrB₃₁₄₃, KtrB₃₁₇₇, KtrB₃₁₈₃, KtrB₃₂₀₃, KtrB₃₂₄₃, and KtrB₃₂₇₇. The remaining constructs showed four phenotypes (Fig. 2, panel E). First, growth occurred equally well at all K⁺ concentrations and was similar to that of KtrB (Fig. 2, panels A and C). This applied to variants KtrB₃₁₄₃ and KtrB₃₂₅₃. Second, the cells grew well at low K⁺ concentrations, but growth was inhibited at elevated K⁺ concentrations (Fig. 2D). Growth inhibition was observed for variant KtrB₃₁₆₆ at ≥ 10 mm K⁺, for variants KtrB₃₁₆₆, KtrB₃₁₇₇, KtrB₃₂₁₇, KtrB₃₂₁₈, KtrB₃₂₁₉, KtrB₃₂₁₆, (Fig. 2D), and KtrB₃₂₅₆ at ≥ 30 mm K⁺, and for variants KtrB₃₁₅₃, KtrB₃₁₇₇, KtrB₃₂₅₃, and KtrB₃₂₅₇ at 115 mm K⁺, respectively. Third, constructs causing slow growth at all K⁺ concentrations, possibly with additional inhibition at high K⁺ concentrations (variants KtrB₃₁₄₉, KtrB₃₁₄₉, KtrB₃₁₆₉, and KtrB₃₁₇₇, respectively. Third, constructs causing slow growth at all K⁺ concentrations, possibly with additional inhibition at high K⁺ concentrations (variants KtrB₃₁₄₉, KtrB₃₁₄₉, KtrB₃₁₆₉, and KtrB₃₁₇₇, respectively. Third, constructs causing slow growth at all K⁺ concentrations, possibly with additional inhibition at high K⁺ concentrations (variants KtrB₃₁₄₉, KtrB₃₁₄₉, KtrB₃₁₆₉, and KtrB₃₁₇₇, respectively. Third, constructs causing slow growth at all K⁺ concentrations, possibly with additional inhibition at high K⁺ concentrations (variants KtrB₃₁₄₉, KtrB₃₁₄₉, KtrB₃₁₆₉, and KtrB₃₁₇₇, respectively. Third, constructs causing slow growth at all K⁺ concentrations, possibly with additional inhibition at high K⁺ concentrations (variants KtrB₃₁₄₉, KtrB₃₁₄₉, KtrB₃₁₆₉, and KtrB₃₁₇₇, respectively. Third, constructs causing slow growth at all K⁺ concentrations, possibly with additional inhibition at high K⁺ concentrations (variants KtrB₃₁₄₉, KtrB₃₁₄₉, KtrB₃₁₆₉, and KtrB₃₁₇₇, respectively. Third, constructs causing slow growth at all K⁺ concentrations, possibly with additional inhibition at high K⁺ concentrations (variants KtrB₃₁₄₉, KtrB₃₁₄₉, KtrB₃₁₆₉, and KtrB₃₁₇₇, respectively. Third, constructs causing slow growth at all K⁺ concentrations, possibly with additional inhibition at high K⁺ concentrations.
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KtrB<sub>K325H</sub> and KtrB<sub>K325Q</sub>). Fourth, constructs that neither complemented cell growth nor inhibited it at high K<sup>+</sup> concentrations (variants KtrBG<sub>321C</sub>, KtrBG<sub>323C</sub>, and KtrBG<sub>323S</sub>). Western blots of KtrB samples taken from the growing cells showed that almost all of the variants were present at amounts similar to that of unaltered KtrB. Exceptions with significantly less to no KtrB protein were cells with the variants KtrBG<sub>314C</sub> and KtrBG<sub>323A</sub>. Both grew slowly at all K<sup>+</sup> concentrations (probably due to lack of KtrB protein; Fig. 2E).

Fig. 3, panels A–D, show time courses of net K<sup>+</sup> uptake by some of the variants. Both in the absence and presence of the C90S exchange KtrB transported K<sup>+</sup> relatively slowly (results not shown and Fig. 3A, respectively). By contrast, almost all of the KtrB variants transported K<sup>+</sup> considerably faster than did KtrB (Fig. 3B for the KtrBG<sub>325C</sub> variant). For only two variants transport was somewhat faster than that of KtrB (Fig. 3C for KtrBG<sub>325R</sub> and KtrBS<sub>325C</sub>), and for two of the variants, KtrBG<sub>314A</sub> and KtrBT<sub>318C</sub> it was similar to that of KtrB (results not shown and Fig. 3D, respectively). The kinetic analysis of the transport data showed that His-tagged KtrB transported K<sup>+</sup> with a 25-fold lower affinity than did KtrB without this tag (K<sub>m</sub> values for K<sup>+</sup> uptake of ~0.5 and 0.02 mM K<sup>+</sup>, respectively; Fig. 4B and (9)). More importantly, the stimulation of K<sup>+</sup> uptake for the variants was due exclusively to an increase in V<sub>max</sub>, which increased from about 35 nmol-min<sup>−1</sup>-mg<sup>−1</sup> (dry weight) of cells for KtrB (Fig. 3A and Fig. 4A, two first columns) to 200 nmol-min<sup>−1</sup>-mg<sup>−1</sup> (dry weight) for the KtrBG<sub>316S</sub> variant (Fig. 4A). By contrast, changes in K<sub>m</sub> for K<sup>+</sup> uptake by the different variants were relatively small (Fig. 4B). The observed effects on V<sub>max</sub> were not due to an increased amount of KtrB variant protein in the cells, as Western blots showed similar amounts of KtrB in cell suspensions used for the K<sup>+</sup>-uptake experiments (results not shown).

KtrB also transports Na<sup>+</sup> ions (9). Hence, we tested whether the KtrB variants overactive in K<sup>+</sup> uptake also translocated Na<sup>+</sup> faster. For this purpose, we used ktrB-plasmid containing E. coli TO114 cells depleted of both K<sup>+</sup> and Na<sup>+</sup> as described under “Experimental Procedures” and in Ref. 9. For the Na<sup>+</sup> uptake experiment, cells were suspended at 1 mg (dry weight)/ml of medium containing 200 mM triethanolamine Hepes, pH 7.2, 0.2% glycerol, and 0.02% L-arabinose. The suspension was shaken at room temperature. After 10 min, NaCl was added at 1.3 mM, 1.9 mM, 5 mM, or 7 mM. Cell samples of 1 ml were taken at different time points and analyzed for their Na<sup>+</sup> contents by flame photometry. The nomenclature of the His-tagged KtrB variants with single amino acid changes was as in Fig. 2.
most active KtrB variant with respect to K\(^{+}\)/H\(^{+}\) translocation (Fig. 4A). However, compared with WT-KtrB, the Na\(^{+}\)/H\(^{+}\) transport of this variant was not changed. The same was true for most of the other KtrB variants. Variant KtrBK325Q shows significantly slower Na\(^{+}\)/H\(^{+}\) transport than did KtrB (Fig. 4, panels C and D). From these data, we conclude that the gain of function of the different M2C2-KtrB variants with single amino acid changes is limited to its K\(^{+}\)/H\(^{+}\) transport feature.

M2C2 Forms a Flexible Linker within the Membrane—We obtained more information about the topology of M2C with the technique of PhoA fusions (38). The fusion set behind the last of a cluster of three positive residues, Lys344 gave a relatively low alkaline-phosphatase activity (Fig. 5, line 1), suggesting a cytoplasmic location of this residue. This result is compatible with the topology model of Fig. 1A and was expected on the basis of the “internally positive rule” for the topology of transmembrane stretches of von Heijne (39). However, an unexpectedly high PhoA activity was observed, when the fusion was set just N-terminal to this topological signal of positive residues, at positions Thr338, Phe339, or Leu340 of VaKtrB (Fig. 5, lines 3–5). This result suggests that in these fusions, alkaline-phosphatase faces the periplasm. These data indicate that M2C has a flexible structure and can either cross the membrane or bend back toward the periplasm. A good candidate for the flexible part of M2C is its middle region M2C2 with its many glycine residues (Figs. 1 and 5). The result with the deletion of the complete region M2C3 (residues 329–339) from the construct in which PhoA is fused behind residue Leu340 supported this notion because this protein exhibited a low PhoA activity (Fig. 5, line 6). In addition, deletion of the complete region M2C3 from the construct in which PhoA is fused behind residue Leu340 supported this notion because this protein exhibited a low PhoA activity (Fig. 5, line 6). In addition,
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**FIGURE 6. Kinetics of K⁺ uptake by strains containing KtrB with deletions in region M₂C₂.** Plasmid-containing cells of strain LB2003 were grown at 3 mM K⁺ according to protocol 2, except for strain LB2003/pEL903-100, which was grown at 10 mM K⁺, and for strain LB2003/pKtrB₃₂₆–₃₂₈, which was grown at 30 mM K⁺ according to protocol 1. The L-arabinose concentration used is indicated in the figure. The bottom panel gives the relative amounts of KtrB variants present in the cell suspensions used for the uptake assays. His-tagged KtrB variants labeled with KtrB, variants present in the cell suspensions used for the uptake assays. His-tags indicated in the figure. The **figure** gives the relative amounts of KtrB variants present in the different suspensions used for the uptake experiments. Labeling of the KtrB variants is as outlined in the legend to Fig. 6.

**FIGURE 7. Na⁺ uptake by strains containing KtrB with deletions in region M₂C₂.** Plasmid-containing cells of strain TO114 were grown at 3 mM K⁺ and 0.02% L-arabinose in the triethanolamine-Hepes based medium described under the “Experimental Procedures.” Na⁺ uptake after the addition of 3 mM NaCl to the cell suspension was determined as outlined in the legend to Fig. 4. The ordinate gives the initial rate of Na⁺ uptake after the addition of NaCl to the cell suspension. The bottom panel gives the relative amounts of KtrB variants present in the different suspensions used for the uptake experiments. Labeling of the KtrB variants is as outlined in the legend to Fig. 6.

For this purpose, we fused parts of KtrB with either LacZ (27) or green fluorescent protein (40). However, as outlined under supplemental "Results of LacZ and GFP Fusions," both methods gave unsatisfactory results because regardless of the expected location of the fusion site, a relatively high β-galactosidase or green fluorescent protein fluorescence was almost always observed due to complete proteolysis of the fusion proteins to holo-LacZ or holo-green fluorescent protein.

**Gain of Function Deletions in Region M₂C₂—**Because parts of M₂C₂ could be deleted without changing the flexibility of this region (Fig. 5), we tested how these deletions affected cell growth and the transport of K⁺ and Na⁺ by the corresponding His-tagged KtrB variants. Remarkably, strains with a ktrB plasmid in which either region M₂C₂ or region M₂C₃ was deleted grew well at low K⁺ concentrations, and so did all of the other strains carrying plasmids with partial deletions of M₂C₂ from ktrB. In addition, we observed that several of these ktrB-M₂C₂ deletion plasmids grew at 3 mM K⁺ without induction of ktrB by added L-arabinose. Moreover, like many of the ktrB strains containing point mutations in single M₂C₂ codons (Fig. 2D), several of the ΔM₂C₂ strains showed inhibition of growth at high K⁺ concentrations after induction with 0.005 or 0.02% of L-arabinose. By contrast, a plasmid containing full-size KtrB did not grow at low K⁺ concentrations without induction, and its growth was not inhibited by high K⁺ concentrations upon full induction. Finally, strain LB2003/pKtrB₃₁₄–₃₂₈ grew poorly at high L-arabinose concentrations (supplemental Fig. S3). This effect was much smaller in a medium without Na⁺ ions (i.e. the triethanolamine-based medium to grow strain TO114 (9)). We will return to this effect below. The results from these growth experiments suggest (i) that at least some of these deletion plasmids encode overactive KtrB variants and (ii) that complementation of growth at low K⁺ concentrations of cells containing these plasmids is not limited by the preservation of the flexible region of M₂C₂ in KtrB (Fig. 5).

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and KtrB with the complete M2C2 deletion (KtrB<sub>314–328</sub>) around 5 mM. By contrast, the complete deletion of M2C3 (KtrB<sub>326–328</sub>) increased the K<sub>m</sub> value to only ~1 mM (Fig. 6).

**The KtrB<sub>326–328</sub> Variant Transports Na<sup>+</sup> Faster**—Because deletions in M2C2 and M2C3 might also increase permeation pathways through KtrB for cations other than K<sup>+</sup>, we measured Na<sup>+</sup>-uptake by some of the strains (Fig. 7). For this purpose, we focused on strains with the complete deletions of M2C2 and M2C3 as well as on those with the small deletions ∆314–320 and ∆326–328. The latter two transported K<sup>+</sup> with relatively low K<sub>m</sub> values, similar to that of KtrB (Fig. 6). For KtrB<sub>326–328</sub>, Na<sup>+</sup> transport was significantly faster than that of the KtrB control (Fig. 7). Kinetic analysis showed that this effect was due to a higher V<sub>max</sub> and a lower K<sub>m</sub> value than that of KtrB. For the other deletion variants, Na<sup>+</sup> uptake was similar to or somewhat lower than that of KtrB (Fig. 7). However, all KtrB-deletion strains produced less KtrB than did cells with intact KtrB (Fig. 7, bottom). Hence, it might be that also KtrB-deletion variants other than KtrB<sub>326–328</sub> transport Na<sup>+</sup> faster than does KtrB. In conclusion, all of the deletions in M2C2 and M2C3 cause a gain of function of K<sup>+</sup> transport via KtrB (Fig. 6), and the KtrB<sub>326–328</sub> variant transports Na<sup>+</sup> also faster (Fig. 8).

**KtrA Does Not Suppress the Gain of Function Phenotype Exerted by Point and Deletion Mutations in ktrB**—Because one of the functions of KtrA is the control of the transport cycle via KtrB (9), we tested how the presence of KtrA affects the gain of function effects described above. For this purpose, we used the pH301-encoded KtrA construct, in which KtrA carries an N-terminal extension with 10 histidine residues. In this plasmid, we engineered the ktrB<sub>G316S</sub>, ktrB<sub>T318C</sub> and ktrB<sub>K325Q</sub> mutations as well as the ∆314–328 (ΔM2C2), ∆318–329, and ∆326–328 deletions. In growth complementation tests with strain LB2003, the presence of KtrA did not influence the effect of growth of any of these changes observed for KtrB alone, indicating that KtrA does not suppress the effects of these mutations and deletions in region M<sub>2C2</sub> from KtrB. For the mutations G316S, T318C, and K325Q and the deletion Δ314–328 (ΔM2C2), we tested net K<sup>+</sup> uptake by K<sup>+</sup>-depleted LB2003 cells and came to the same conclusion, i.e. that we did not observe an effect of KtrA on K<sup>+</sup> transport by the KtrB variants (data not shown).

**M2c2 Deletions Abolish the Binding of KtrB to His-tagged KtrA**—We attempted to purify KtrAB complexes with the following changes in KtrB: G316S, K325Q, ∆314–328 (ΔM2C2), ∆318–329, and ∆326–328 by affinity chromatography on nickel-nitrilotriacetic acid-agarose. Whereas the two point mutants yielded normal complexes, the three KtrB-deletion variants did not bind to His-tagged KtrA on the affinity column (Fig. 8, and results not shown for the His<sub>10</sub>-KtrAB<sub>K325Q</sub> variant). We conclude that at least the KtrB-M2C2 residues 326–328 are essential for the interaction between KtrA and KtrB.

**DISCUSSION**

The transmembrane stretch M<sub>2C</sub> from KtrB is, with its 40 amino acid residues, much longer than most transmembrane helices. It is difficult to predict a structure for its middle part M2C2 with its conserved Gly, Ala, Ser, Thr, and Lys residues (Fig. 1B). Durell and Guy (13) have proposed that M2C2 forms either a loop within the membrane connecting the apolar membrane-spanning stretches M2C1 and M2C3 with each other, or that M2C1 and M2C2 span the membrane together (the latter in a coiled conformation) and that helix M2C3 is located within the inner surface of the membrane. Our results do not allow us to distinguish between the two models. Because of the observed flexibility of M2C2 (Fig. 5), we favor the first model. We assume that a flexible M2C2 loop fills the cavity just below the p-loop and that VaKtrB M2C2-residue Lys<sup>325</sup> forms a salt bridge with VaKtrB residue Asp<sup>222</sup> from membrane span M2B (see the model in Fig. 9) (13). By electric repulsion, K<sup>+</sup> may weaken this salt bridge during the transport cycle and thereby open the M2C2 gate, allowing K<sup>+</sup> to permeate to the cytoplasm (Fig. 9). The gain of function mutations characterized in this article (Figs. 2–4 and 6) are proposed to change the structure of M2C2 in such a manner that K<sup>+</sup> can slip through the gate without having to open it. With the point mutations, this slip-through effect was specific for K<sup>+</sup>, whereas with at least some deletions (in particular that of VaKtrB<sub>314–328</sub>) the rate of Na<sup>+</sup> transport through KtrB increased also (Fig. 7). The latter was not unexpected, since even small deletions may change the structure of a protein fundamentally and thereby alter its substrate (in the case of KtrB, transport) specificity. More importantly, either M2C2 or M2C3 could be deleted without KtrB losing its K<sup>+</sup> transport function. Apparently, both regions are not essential. We assume that the K<sup>+</sup> transport feature of KtrB is determined by or close at its selectivity filter at the periplasmic half of the protein, and that like in KcsA (11), most of its cytoplasmic half functions as a K<sup>+</sup> pore. The removal of M2C2 or M2C3 does not change the pore structure in such a manner that K<sup>+</sup> cannot permeate through it any longer.

Only those SKT proteins that require additional subunits for activity contain an M<sub>2C2</sub> region. Because KtrA controls the K<sup>+</sup> transport cycle in Ktr systems via KtrB (9), we determined whether this control by KtrA still exists for the KtrB-M<sub>2C2</sub> variants. For both the point mutations and deletions in M<sub>2C2</sub>, KtrA did not suppress the effect of these mutations (see “Results”), indicating that in these situations KtrA lost its control of the K<sup>+</sup> transport cycle. With the deletion variants, the explanation for...
Gain of Function Mutations Open a K⁺ Transport Gate in KtrB

FIGURE 9. Model explaining how M2C2 functions as a gate for K⁺ transport through KtrB. Region M2C2 is proposed to form a gate, which in its closed form prevents K⁺ translocation from the selectivity filter region (indicated by the two glycine residues) to the cytoplasm. A salt bridge between VaKtrB-M2C2 residue Lys252 and Asp222 from membrane span M2B (13) will keep the gate closed. K⁺ permeates from the periplasm into KtrB, where it is dehydrated to the enter the selectivity filter region (11). From this position, K⁺ opens the gate by electrostatic repulsion of residue Lys252, leading to a weakening of the Asp222-Lys252 salt bridge and thereby allowing K⁺ to move to the cytoplasm, followed by closing of the M2C2 gate. The gain of function amino acid changes in M2C2 allow K⁺ to slip through the gate. The presence of a second gate for K⁺ at the periplasmic site of KtrB (42) and a role for Na⁺ in the K⁺ transport cycle are not included in this simplified model.

this effect is simple because KtrA and KtrB did not interact with each other (Fig. 8), suggesting that the region M2C2 is required for the binding of the two subunits to each other. Some of the KtrB variants with a single amino acid change in M2C2 still formed KtrAB complexes (Fig. 8). In these constructs, the point mutations appear to open the M2C2 gate permanently without KtrA being able to close it during the transport cycle. In this respect, it is important to establish whether the observed gain of function effects render K⁺ transport via the KtrB variants maximally equal to that of KtrB in the KtrAB complex or that K⁺ transport by the variants exceeds that of the latter. KtrB alone and KtrB transport K⁺ with $V_{\text{max}}$ values of ~25 and 200 nmol·min⁻¹·mg⁻¹ of cell (dry weight), respectively (9). The $V_{\text{max}}$ values of some of the KtrB variants were significantly higher than that of KtrAB (Figs. 4 and 6). Moreover, several of the KtrB-deletion variants produce considerably less KtrB protein than does unaltered KtrB (Fig. 6). Finally, many of these KtrB variants transport K⁺ with considerable rates without induction of KtrB transcription by L-arabinose, a condition at which we could not detect any KtrB in the cells at all (Results not shown). All of these results suggest that several of the variant KtrB proteins are more active in K⁺ transport than is unaltered KtrB in the KtrAB complex. However it is difficult to quantify this effect. Comparison of the amounts of either unaltered KtrB or of the KtrB-deletion variants in the immuno-blot at the different L-arabinose concentrations with the observed $V_{\text{max}}$ values for K⁺ transport by these cells (Fig. 6) show that transport levels off at sub-maximal amounts of KtrB. In addition, in attempts to isolate some of the deletion variant KtrB proteins we observed that in contrast to unaltered KtrB protein they have a strong tendency to aggregate. When this also occurs within the cell membrane, the amount of active KtrB will be lower than expected on the basis of the immuno-blot data, increasing the gain of function effect per active KtrB molecule.

When then the activity of some of the KtrB variants exceeds that of KtrB in the KtrAB complex, one might expect that the loss of control due to the release of a gate for K⁺ transport within KtrB would give the protein K⁺ channel like properties. However, the $K_m$ values for K⁺ transport of almost all of the his-tagged KtrB variants did not increase in comparison to that of unaltered his-tagged KtrB (Fig. 4B). In addition, only in a few of the deletion mutants these $K_m$ values became with 5 mM significantly higher than the 0.7 mM of wild type (Fig. 6). If KtrB were to become a K⁺ channel, one would expect K⁺–translocation to be controlled by diffusion and as a consequence to occur with very low affinity. Since this was not observed, we conclude that the changes introduced into M2C2 do not transform KtrB into a K⁺ channel, but rather give it some channel-like properties.

Gadsby discusses several examples of similar transitions between (active) transporters and channels (41, 42), in particular for the Cl⁻ transporter from E. coli (43). He depicts transporters with two gates, one at each side of the membrane, controlling the uptake and release of the transported compound at the two membrane interfaces. Our conclusion that the changes in M2C2 do not transform KtrB into a K⁺ channel may suggest that these changes open only one of the two gates in KtrB, presumably the one at the cytoplasmic side of the membrane. At present no information is available, whether KtrB also contains a gate within its periplasmic half and how such a gate controls the K⁺-transport cycle.

The function of Na⁺ for K⁺ transport via the KtrAB complex is unknown. Since KtrB alone translocates Na⁺ (9), KtrAB might be a Na⁺/K⁺-symporter. However, no direct evidence exists in support of this notion. Our results suggest that the permeability pathway of the cations K⁺ and Na⁺ through KtrB differ from each other. This is concluded from the fact that the variants with the point mutations in M2C2 were specific for the increase of K⁺ transport with no effect on that of Na⁺ (Figs. 3 and 4). Hence, these mutations cause subtle changes in the KtrB structure leading to the opening of a gate for K⁺, but not for Na⁺. This notion appears to contradict a model according to which K⁺ and Na⁺ file behind each other through the same central pathway of KtrB during the transport cycle. The small KtrB1326–328 deletion caused a significant stimulation of the transport of both K⁺ and Na⁺ (Figs. 6 and 7). Hence this part of M2C2 may play a role in Na⁺ transport via KtrB as well.

Our fusion studies of KtrB with alkaline phosphatase PhoA showed that M2C2 possesses a flexible structure either allowing M2C2 to reach the cytoplasm or to bend back artificially toward the periplasm (Fig. 5 and supplemental Fig. S2B). Such a flexible structure of M2C2 is in accordance with both Durell and Guy models (13). The PhoA technique is normally used to determine whether a residue of an integral membrane protein is located at the inner or outer side of the bacterial membrane (38). It will have to be determined whether this technique can also be used for the determination of flexible structures within the membrane span of proteins other than KtrB. In conclusion, region M2C2 from membrane span M2C of KtrB forms a flexible
structure within the membrane that functions as a gate for $K^+$ translocation via the KtrB protein of V. alginolyticus.

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