The peptidoglycan (PG) cell wall is an essential extracellular chitin polymer that safeguards bacteria against osmotic lysis and determines cellular morphology. Bacteria use multiprotein machineries for the synthesis of the PG cell wall during cell division and elongation that can be targeted by antibiotics such as the β-lactams. Lipid II, the lipid-linked precursor for PG biogenesis, is synthesized in the inner leaflet of the cytoplasmic membrane and then translocated across the bilayer, where it is ultimately polymerized into PG. In *Escherichia coli*, MurJ, a member of the MOP exporter superfamily, has been recently shown to have lipid II flippase activity that depends on membrane potential. Because of its essentiality, MurJ could potentially be targeted by much needed novel antibiotics. Recent structural information suggests that a central cavity in MurJ alternates between inward- and outward-facing conformations to flip lipid II, but how these conformational changes occur are unknown. Here, we utilized structure-guided cysteine cross-linking and proteolysis-coupled gel analysis to probe the conformational changes of MurJ in *E. coli* cells. We found that paired cysteine substitutions in transmembrane domains 2 and 8 and periplasmic loops of MurJ could be cross-linked with homobifunctional cysteine cross-linkers, indicating that MurJ can adopt both inward- and outward-facing conformations in vivo. Furthermore, we show that dissipating the membrane potential with an ionophore decreases the prevalence of the inward-facing, but not the outward-facing state. Our study provides in vivo evidence that MurJ uses an alternating-access mechanism during the lipid II transport cycle.

The cell envelope of Gram-negative bacteria such as *Escherichia coli* contains two membranes, the outer membrane, which marks the boundaries of the cell, and the inner membrane (IM), which surrounds the cytoplasm. In between these two membranes, there is an aqueous compartment, known as the periplasm, that houses an essential cell wall made of peptidoglycan (PG) (1). The PG cell wall, which is present in most bacteria, is a polymeric mesh-like structure composed of glycan strands of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) units; in addition, peptides stemming from MurNAc can be cross-linked to those from adjacent glycan strands. The resulting structure provides shape to the cell and the structural rigidity needed for protection from osmotic lysis in hypotonic environments (2, 3). Because inhibition of the PG biogenesis pathway causes cell lysis and humans do not synthesize PG, PG synthesis is one of the most clinically and economically important targets for antibiotic development (3).

PG biogenesis is a complex process that takes place in three stages. In the first stage, nucleotide precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide are synthesized by the GlmSMU and MurABCDEF enzymes, respectively, in the cytoplasm (4). In the second stage, MraY and MurG use these nucleotide precursors and undecaprenyl phosphate (Und-P) to synthesize the lipid-linked precursors lipid I (Und-PP-MurNAc-pentapeptide) and, subsequently, lipid II (Und-PP-MurNAc-pentapeptide-GlcNAc), respectively, in the inner leaflet of the IM (5). Once lipid II is made, a “flipase” translocates it across the IM (5–7). In the third and final stage, flipped lipid II molecules are utilized by glycosyltransferases to make glycan chains, which are then cross-linked by transpeptidases to form the PG layer in the periplasm (3, 8, 9). This overall strategy is conserved across nature to synthesize glycoconjugates such as bacterial exopolysaccharides and capsules and the oligosaccharides used in N-glycosylation (10, 11).

Most of the enzymes participating in PG biogenesis were identified decades ago, but the identity of MurJ as the lipid II flippase in *Escherichia coli* was only recently proposed (12, 13). MurJ belongs to the multidrug/oligosaccharidyl lipid/polysaccharide (MOP) transporter family, which includes the Wzx flipases that transport Und-PP–linked oligosaccharides structurally similar to lipid II across bacterial membranes (14, 15). MurJ was initially proposed to be the lipid II flippase based on this.

This work was supported by NIGMS, National Institutes of Health Grant R01 GM1000951 (to N.R.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Figs. S1–S7 and Tables S1 and S2.

1 Supported by National Institutes of Health Grants U19 AI109764 and R01 GM076710.
2 To whom correspondence should be addressed. E-mail: ruiz.82@osu.edu.
3 The abbreviations used are: IM, inner membrane; PG, peptidoglycan; GlcNAc, N-acetylgalactosamine; MurNAc, N-acetylmuramic acid; Und-P, undecaprenyl phosphate; lipid I, Und-PP-MurNAc-pentapeptide; lipid II, Und-PP-MurNAc-pentapeptide-GlcNAc; MOP, multidrug/oligosaccharidyl lipid/polysaccharide; SCAM, substituted-cysteine accessibility method; TM, transmembrane domain; MATE, multidrug and toxic compound extrusion; YT, yeast tryptone; BMH, 1,6-bis(maleimido)hexane; BMOE, 1,2-bis(maleimido)ethane; o-PDM, o-N,N’-phenylene)dimalimide; PL, periplasmic loop; NEM, N-ethylmaleimide; TCS, 3,3’,4’,5-tetraclorosalicylanilide; Δψ, membrane potential; LB, lysogeny broth; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; SDM, site-directed mutagenesis; CL, cytoplasmic loop.

© 2019 Kumar et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.
Probing the alternating-access mechanism for MurJ

homology and because its depletion causes PG precursor buildup and eventually cell lysis (13). Importantly, depletion or chemical inhibition of MurJ leads to lipid II accumulation in the inner leaflet of the IM of E. coli cells, demonstrating that lipid II flipping across the IM requires MurJ (16–18). More recently, it has been shown that MurJ binds lipid II in vitro (19) and that its cellular localization is governed by the presence of lipid II (20). Interestingly, although MurJ is the only lipid II flippase in E. coli and is essential for PG biogenesis in other bacteria (21–23), the structurally unrelated proteins Amj and Wzk can also translocate lipid II in Bacillus subtilis and Helicobacter pylori, respectively, and can substitute for native MurJ in E. coli (24, 25).

Earlier studies probing the structure of MurJ in vivo using the substituted-cysteine accessibility method (SCAM) revealed the presence of 14 transmembrane domains (TMs) in MurJ, that are arranged into a V-shaped structure similar to that of the multidrug and toxic compound extrusion (MATE) transporters of the MOP exporter superfamily (26, 27). It was predicted that TMs 1, 2, 7, and 8 of MurJ form a central hydrophilic cavity, and, in agreement with its role as a lipid II flippase, several charged residues in this cavity were shown to be required for MurJ function (26, 28). This suggested that MurJ might function similarly to MATE exporters, which utilize an antipor mechanism that relies on a gradient of either cations or protons across the membrane to translocate toxic drugs from the cytoplasm to the periplasm (14, 17). Indeed, this model was further supported by the recent discovery that lipid II translocation into the IM depends on the membrane potential (17).

Recent crystal structures of MurJ from Thermosiphon africanus and E. coli were reported (29, 30) showing the central cavity formed by the N-terminal (N) lobe (TMs 1–6) and the C-terminal (C) lobe (TMs 7–14) in an inward-open conformation (i.e., with the cavity open to the cytoplasm). This conformation was surprising because all crystal structures of related MATE transporters reveal an outward-open conformation (27, 31–33). It is also worth noting that MurJ has 14 TMs, whereas most members of the MOP exporter superfamily have 12 TMs (14, 26, 29, 30). At present, the only in vivo structural studies on MurJ have used SCAM probing to suggest that the protein can adopt an outward-open state (17, 34). This periplasmic-open conformation was also recently suggested by evolutionary coupling analysis (30).

In this study, we used structure- and evolutionary coupling-guided in vivo cysteine cross-linking to investigate the conformational changes that MurJ undergoes during the lipid II flipping cycle in live cells. Our data demonstrate that MurJ can adopt both inward- and outward-facing conformations in vivo. Furthermore, we show that the membrane potential is only required for the formation of the inward-facing conformation. Based on our data, we propose that MurJ functions by a cation-antiport, alternating-access mechanism to translocate lipid II across the inner membrane.

Results

Experimental rationale

We wanted to use site-directed cysteine in vivo cross-linking to test the proposed alternating-access model for the transport of lipid II by MurJ. According to this model, the central cavity of MurJ formed by the N and C lobes alternates between inward-open (i.e., open to the cytoplasm) and outward-open (i.e., open to the periplasm) conformations (29, 30). For this strategy, we needed to introduce a cysteine residue into each of the lobes of MurJ and use homobifunctional cross-linking reagents that could cross-link specific cysteine pairs when the two lobes adopted only one of the two conformations. This method has been successfully used to probe the structure of proteins such as LacY (35–37) and YidC (38). To guide us in the selection of residues to replace with cysteine, we used the crystal structure of the inward-open state and the in silico homology model predicting the outward-open state (30). Based on this rationale, we identified 11 pairs of residues in the cytoplasmic side of MurJ whose Cα positions allowed us to predict that cysteine replacements would be too far to be cross-linked in the inward-open state, yet would be within cross-linking distance in the outward-open state (Fig. 1 and Table 1). We also selected two pairs of residues in the periplasmic side of MurJ we predicted would be close enough to be cross-linked in the inward-open structure, but not in the outward-open state (Fig. 1 and Table 1). Furthermore, we also selected two pairs of residues which should not cross-link in any conformation to validate the physiological relevance of our cross-linking strategy (Fig. 1 and Table 1). These residues had previously been shown to be fully accessible to mono-reactive maleimides when substituted with cysteine (26).
Probing the alternating-access mechanism for MurJ

Construction of FLAG-MurJΔCys-thrombin variants

To aid in differentiating cross-linked and unlinked proteins in the strategy described above, we constructed a functional variant of MurJ in which the loop connecting the N and C lobes could be cleaved by the protease thrombin after cells were treated with cross-linkers (Fig. 2A). In the absence of cross-linking, thrombin should cleave this construct into two fragments: one corresponding to the N lobe (TMs 1–6) and the other corresponding to the C lobe (TMs 7–14). However, the two fragments should remain covalently linked after protease cleavage if the two lobes were cross-linked prior to proteolysis. The two native cysteine residues of MurJ were replaced with serine residues as described previously (26), and a FLAG tag was appended to the N terminus of MurJ for detection by immunoblotting. The plasmid-encoded FLAG-MurJΔCys-thrombin variant was functional as it complemented the deletion of chromosomal murJ and fully supported growth on yeast tryptone (YT) agar, a low-osmolarity medium that can be used to detect partial loss-of-function defects in MurJ (for more details, see “Experimental procedures”) (26, 28). In addition, FLAG-MurJΔCys-thrombin was detected in whole-cell samples by immunoblotting (Fig. 2B). As expected, when membrane fractions from haploid cells producing the FLAG-MurJΔCys-thrombin protein were treated with thrombin, we observed complete cleavage and only detected the N lobe containing the FLAG tag (Fig. 2C). In contrast, the membrane fraction from cells producing a FLAG-MurJΔCys variant lacking the thrombin cleavage sequence showed no proteolysis, demonstrating that nonspecific cleavage does not occur during sample preparation and thrombin protease treatment (Fig. 2C).

We then substituted single residues or pairs of residues in the FLAG-MurJΔCys-thrombin variant with cysteines at selected locations as discussed above. Altogether, 19 monocysteine and 15 double-cysteine mutant alleles were constructed by site-directed mutagenesis of flag-murJΔCys-thrombin. FLAG immunoblot analysis revealed that all variants, except five monocysteine (N155C, K293C, S290C, E302C, and A344C) and eight double-cysteine (I67C/S290C, A69C/K293C, A69C/A344C, K72C/K293C, S73C/K293C, S73C/S297C, S73C/E302C, and S73C/V65C) alleles were constructed by site-directed mutagenesis of flag-murJΔCys-thrombin. FLAG-MurJΔCys thrombin variants were treated with cross-linkers (Fig. 2A). As expected, when membranes prepared from exponential-phase cultures of haploid flag-murJΔCys-thrombin protein were treated with thrombin, we observed complete cleavage and only detected the N lobe containing the FLAG tag (Fig. 2C). In contrast, the membrane fraction from cells producing a FLAG-MurJΔCys variant lacking the thrombin cleavage sequence showed no proteolysis, demonstrating that nonspecific cleavage does not occur during sample preparation and thrombin protease treatment (Fig. 2C).

Table 1

| Cysteine substitution location | Ca–Cα distances in structurea | Cross-linkersb | Expected conformation that allows cross-linking |
|-------------------------------|--------------------------------|----------------|-----------------------------------------------|
| N lobe | C lobe | MurJ (In) | MurJ (Out) | BMH (16 Å) | BMOE (8 Å) | ω-PDM (6 Å) |
| S73C (TM2) | E302C (TM9) | 31.9 | 11.8 | Yes | No | No | Outward-open |
| S73C (TM2) | K293C (TM8) | 28.7 | 4.6 | Yes | Yes | Yes | Outward-open |
| S73C (TM2) | A296C (TM8) | 21.0 | 4.7 | Yes | Yes | Yes | Outward-open |
| S73C (TM2) | K293C (TM8) | 22.2 | 4.2 | Yes | Yes | Yes | Outward-open |
| K72C (TM2) | K293C (TM8) | 23.5 | 6.5 | Yes | Yes | Yes | Outward-open |
| E70C (TM2) | A296C (TM8) | 19.3 | 5.0 | Yes | Yes | Yes | Outward-open |
| E70C (TM2) | Q378C (CL10–11) | 17.4 | 5.6 | No | No | No | Neither |
| A69C (TM2) | K293C (TM8) | 19.8 | 5.2 | No | No | No | Neither |
| A69C (TM2) | S292C (TM8) | 18.8 | 5.4 | No | No | No | Neither |
| A69C (TM2) | A344C (PL9–10) | 52.4 | 49.4 | No | No | No | Neither |
| I67C (TM2) | S290C (TM8) | 24.8 | 11.6 | Yes | Yes | Yes | Outward-open |
| V65C (TM2) | L288C (TM8) | 19.3 | 7.4 | Yes | Yes | Yes | Outward-open |
| V43C (TM2) | T251C (TM7) | 7.8 | 17.1 | Yes | Yes | Yes | Outward-open |
| A344C (PL1–2) | A344C (PL9–10) | 10.8 | 19.1 | Yes | Yes | Yes | Outward-open |
| N155C (CL4–5) | S290C (TM8) | 24.4 | 8.4 | Yes | Yes | Yes | Outward-open |

a MurJ (In) refers to the crystal structure PDB code 6CC4. MurJ (Out) refers to the structural homology model by Zheng et al. (30).
b Distances in Å refers to spacer arm length of the cross-linker. Yes indicates cross-linking was observed in our study. No indicates cross-linking was not observed.

Figure 2. Proteolytic analysis of the FLAG-MurJΔCys-thrombin variant used to probe conformational states of MurJ in vivo. A, cartoon representation of the structure of the FLAG-MurJΔCys-thrombin variant and its proteolytic analysis. TM1, TM2, TM5, TM8, and TM9 and the location of the insertion of the thrombin cleavage sequence are labeled on the inward-open MurJ structure (PDB code 6CC4). The thrombin protease site (green box) was introduced in cytoplasmic loop 6-7 that links the N lobe (pink) and C lobe (gold) of MurJΔCys. An N-terminal FLAG tag used for detection by immunoblotting (panel on the right) is displayed as an orange flag on the N lobe. In the absence of thrombin treatment, the full-length FLAG-MurJΔCys-thrombin variant should appear as an ∼37-kDa band in a FLAG immunoblot. Thrombin treatment should cleave the two lobes, and only the N lobe (∼18 kDa) should be detected by FLAG immunoblotting. B, samples from overnight cultures of haploid murJ strains producing FLAG-MurJΔCys (WT), its cysteine-less derivative FLAG-MurJΔCys (ΔCys), and FLAG-MurJΔCys-thrombin (ΔCys-Th) were subjected to FLAG immunoblotting. As reported earlier (26), FLAG-MurJΔCys migrates more slowly than FLAG-MurJ on SDS-polyacrylamide gels. LptB immunoblotting (bottom panel) was used to check for equal loading of samples. C, membranes prepared from exponential-phase cultures of haploid murJ strains producing FLAG-MurJΔCys and FLAG-MurJΔCys-thrombin proteins, 12% gels must be used to detect the much smaller N lobe. The positions of molecular mass markers (in kDa) are indicated. Data are representative of three independent experiments.
Probing the alternating-access mechanism for MurJ

N155C/S290C) variants, were present at similar levels to those of FLAG-MurJΔCys-thrombin (Fig. S1). Although some had biogenesis defects, all variants were functional based on their capacity to complement a ΔmurJ chromosomal allele and fully support growth on YT agar (26, 28). Therefore, all FLAG-MurJΔCys-thrombin variants were fully functional and able to complete the lipid II flipping cycle in vivo.

Cross-linking of paired cysteines demonstrates that MurJ adopts the inward-open conformation in vivo

We then used the functional FLAG-MurJΔCys-thrombin double-cysteine variants for our in vivo cysteine cross-linking studies to probe conformational changes that the cavity of MurJ might undergo in cells under physiological conditions. We used maleimide cross-linkers because they are well recognized to be reactive in aqueous environments such as the periplasm and cytoplasm, and they form thioether bonds with sulfhydryl groups of cysteines that are resistant to reversal by reducing agents (39). Specifically, we used 1,6-bis(maleimido)hexane (BMH; which has a flexible 16-Å spacer arm length), 1,2-bis(maleimido)ethane (BMOE; which has a flexible 8-Å spacer arm length), and N,N’-(o-phenylene)dimaleimide (o-PDM; which has a rigid 6-Å spacer arm length) (Fig. S2).

Because E. coli MurJ was crystallized in the inward-open conformation (30), we first tested two cysteine pairs located on the periplasmic side of MurJ that should be cross-linked with BMOE and o-PDM in this state. However, these residues would be expected to be too far away to be cross-linked in the outward-open side conformation according to the in silico model (30). Namely, we tested A34C/A344C, localized in the periplasmic loop (PL) between TMs 1–2 (PL1-2) and PL9-10, respectively, and V43C/T251C, localized in TM2 and TM7, respectively (Fig. 1). As expected, when cells were not treated with cross-linkers, treatment of isolated membranes with thrombin led to the disappearance of full-length FLAG-MurJ (Fig. 3A) and the concomitant appearance of the fragment corresponding to the cleaved FLAG-N lobe (Fig. S3A). In contrast, if cells were first treated with either BMOE and o-PDM and their membranes were then subjected to thrombin treatment, we could still detect a significant portion of full-length FLAG-MurJ (Fig. 3A) but less of the cleaved FLAG-N lobe (Fig. S3A). These results demonstrated that BMOE and o-PDM cross-linked A34C/A344C and V43C/T251C.

To validate that these results were reflecting specific intra-molecular cross-links in biologically relevant conformations, we first demonstrated that the maleimide homobifunctional cross-linkers could not cross-link MurJΔCys-thrombin (WT) and a subset of variants containing single-cysteine substitutions in either the cytoplasmic or periplasmic side of MurJ. Despite treatment with cross-linkers, membrane fractions containing these variants showed complete cleavage by thrombin (Fig. 4A and Fig. S6A). Additionally, we also demonstrated that, as expected, cross-linking did not occur in a double-cysteine variant (A69C/A344C) in which one cysteine is located in the cytoplasmic end of TM2 (A69C) and the other is in PL9-10 (A344C) (Fig. 4A and Fig. S6A). Lastly, if cells producing the aforementioned periplasmic V43C/T251C pair were pretreated with N-ethylmaleimide (NEM), which covalently blocks cysteines, before adding the cross-linking reagents, the ability to cross-link the V43C/T251C variant was lost (Fig. 4B and Fig. S6B). Together, these data supported our conclusion that the ability of BMOE and o-PDM to cross-link the periplasmic A34C/A344C and V43C/T251C pairs indicated that MurJ attains the inward- or cytoplasmic-open conformation in vivo.

Cross-linking of paired cysteines demonstrates that MurJ adopts the outward-open conformation in vivo

Next, we applied the same strategy with cysteines located on the cytoplasmic side of MurJ to determine whether it adopts the outward-open conformation in vivo (Fig. 1). In the MurJ outward-open homology model, the distance between the cytoplasmic ends of TM2 (N lobe) and TM8 (C lobe) is short enough...
cysteine mutant was pretreated with NEM before adding cross-linkers (Fig. 4B and Fig. S6B). Furthermore, our results are consistent with a recent study in which residues in TM2 (Val-65, Pro-66, and Ala-69) were suggested to participate in extensive hydrophobic interactions with residues in TM8 (Leu-288, Pro-289, and Ser-292) in the outward-open conformation (30).

By contrast to the other seven pairs, the K72C/K293C pair was not cross-linked with any reagent (Fig. 3B and Fig. S3B). This was surprising because the Ca distance between these two residues in the MurJ outward-open homology model is well within the anticipated range of the cross-linkers (Table 1). Our results suggest that these cysteine substitutions are oriented in such a way that their cross-linking is prevented. Interestingly, in both the MurJ inward-open structure and the outward-open homology model, the side chains of Lys-72 and Lys-293 point parallel to each other, and they are located near where the cytoplasmic ends of TM2 and TM8 meet in the homology model (Fig. S5). Therefore, if TM2 and TM8 were to cross each other more than expected from the model, cross-linking between Lys-72 and Lys-293 would be impossible because these residues would be separated by the last turn in TM2. Alternatively, residues in the nearby cytoplasmic loop between TM4 and TM5 may be extending between the cysteine pair and thus hampering the cross-linking, or the orientation of their side chains is such that cross-linking is not possible.

We were also interested in investigating the proximity of TM2 and TM9 during the predicted conformation shifts in MurJ. Therefore, we tested the cross-linking pattern of S73C/E302C (TM2 and TM9, respectively) in the presence of all cross-linkers. We observed cross-linking only with BMH (16 Å) but not with the smaller cross-linkers BMOE (8 Å) and o-PDM (6 Å) (Fig. 3B and Fig. S3B). This result suggests that the sulfhydryl groups of these two residues come within 16 Å of each other during the lipid II flipping cycle, supporting the existence of an outward-open conformation. Furthermore, our results also support the idea that TM2 comes closer to TM8 than to TM9 in the outward-open conformation, which is consistent with the predictions of the homology model (30). We further demonstrated the validity of the homology model by showing that the N155C/S290C (cytoplasmic loop (CL) 4-5 and TM8) and E70C/Q378C (TM2 and CL10-11) pairs could be cross-linked with all three cross-linkers, whereas the E70C/Q378C pair was cross-linked with BMH and BMOE but not with the shorter and more rigid o-PDM cross-linker (Fig. 3B and Fig. S3B).

A summary of cross-linking results for all double-cysteine pairs is presented in Table 1. Collectively, those results demonstrate that MurJ exists in both the inward-open and the outward-open conformations in vivo. Therefore, our data support an alternating-access mechanism of MurJ for the translocation of lipid II across the membrane.

Effect of a protonophore on the cross-linking of MurJ paired cysteines variants

The proton motive force is an electrochemical gradient of protons across the bacterial cell membrane, and it consists of two components, a membrane potential (∆φ) and a pH gradient (∆pH) (40). Protonophores like 3,3’,4’,5-tetrachlorosalicylani-
Probing the alternating-access mechanism for MurJ

To protons and abolish both Δψ and ΔpH. Previously, we showed that lipid II accumulated in cells after TCS treatment (17). Specifically, disruption of membrane potential, not the pH gradient, was responsible for the lipid II buildup in protonophore-treated cells. In vivo SCAM analysis also revealed that the solvent exposure of seven residues in the cavity increased upon membrane depolarization by TCS, suggesting that collapsing membrane potential led to a conformational change in MurJ to favor an outward-open state (17). Because in vivo cysteine cross-linking allows us to specifically probe the ability of MurJ to adopt both the inward- and outward-open conformations in cells, we investigated the effect of protonophore treatment on the cross-linking of cysteine pairs on the periplasmic and cytoplasmic sides of MurJ. We chose α-PDM as the cross-linker because of its small 6-Å spacer arm length. Cross-linking of the periplasmic cysteine pairs by α-PDM should only be possible in the MurJ inward-open conformation; conversely, cross-linking of the cytoplasmic cysteine pairs should only occur in the outward-open conformation. We observed that with increasing concentrations of TCS, cross-linking by α-PDM of periplasmic cysteine pairs (V43C/T251C and A34C/A344C) decreased significantly (Fig. 5, A and B, and Fig. S7). In contrast, the ability of α-PDM to cross-link cytoplasmic cysteine pairs (V65C/L288C, A69C/S292C, S73C/A296C, and S73C/S297C) either slightly increased or remained the same with increasing concentrations of TCS (Fig. 5, A and C, and Fig. S7). Together, these results demonstrate that disruption of membrane potential prevents MurJ from adopting the inward-open state, trapping it in the outward-open state.

Discussion

Transporters are dynamic proteins that attain several conformations during their transport cycles. Obtaining high-resolution crystal structures of transporters in their different conformations, with or without substrates, is a challenging task. It is therefore not surprising that translocation of the cell wall precursor lipid II across the cytoplasmic membrane is the most poorly understood essential step in PG biogenesis. Since its discovery in 2008 (12, 13), mechanistic details about how MurJ flips lipid II have been limited. Experimental evidence from crystal structures of MurJ in the inward-open state (29, 30), in vivo structural probing (17, 26, 34), structure-function analyses (26, 28), and in vivo dependence of lipid II translocation on membrane potential (17) suggest that MurJ might function similarly to structurally related MATE exporters, the better-studied members of the MOP exporter superfamily to which MurJ belongs (14, 27, 41). MATE transporters possess a central cavity that undergoes conformational changes to translocate toxic compounds across the cytoplasmic membrane. The current model for their function is an alternating-access mechanism that depends on the electrochemical gradient across the cytoplasmic membrane and substrate binding (27, 41). Membrane potential also plays an important role in other non-MOP secondary transporters that have been extensively studied such as the intestinal Na+/glucose symporter SGLT1, the Na+-dependent sugar importer from Vibrio parahaemolyticus (vSGLT), and the galactoside/H+ symporter LacY (42, 43). In SGLT1, membrane potential mainly affects the conformational change of the substrate-free transporter to the initial outward-facing state, having an additional minor effect on Na+ binding to the...
extracellular binding site (42, 44). Similarly, in vSGLT, the negative membrane potential of the cell drives vSGLT to the outward-facing state to bind sugar and start the transport cycle (43). In LacY, protonation allows substrate binding, which induces a conformational change that opens the cavity to the other side of the membrane, whereas deprotonation after substrate delivery returns LacY to the initial open state on the other side of the membrane (45).

In this study, we utilized a combination of structure-guided in vivo cysteine cross-linking and proteolysis-coupled gel analysis to probe the conformation of MurJ in cells. Our data demonstrate that MurJ exists in the inward- and outward-open conformations in vivo, supporting an alternating-access mechanism for lipid II transport. Furthermore, our data show that collapsing the membrane potential stalls MurJ in the outward-open conformation because it prevents it from adopting the inward-open state.

On the basis of our results and those of others, we propose the following model for lipid II flipping by MurJ (Fig. 6, A and B). In the inward-open conformation, cytoplasmic lipid II binds to MurJ. It has been hypothesized that although the disaccharide pentapeptide interacts with the MurJ cavity, the hydrophobic groove formed by TM13 and TM14 in the C lobe associates with the undecaprenyl tail (29, 30). Once lipid II has associated with the central cavity, the N lobe and C lobe of the flipase undergo the transition from inward-open to outward-open conformation, similar to the proposed mechanism for some members of the MOP superfamily (27, 46). Once the cavity is open to the periplasm, MurJ can release lipid II so that it can be used to build new peptidoglycan cell wall. Notably, it is unknown whether the lipid tail of lipid II remains associated with the hydrophobic core of the membrane. Nevertheless, there is a lateral open slot between TM1 and TM8 that would allow the unimpeded flipping of lipid II whether the undecaprenyl tail stays associated with TM13-14 or free in the membrane (28–30). After MurJ delivers lipid II to the periplasmic side, it uses the electrochemical potential of a counterion to return to the inward-open conformation (17). Thus, like SGLT1, vSGLT, and LacY, MurJ needs the membrane potential to reset. In the absence of this Δψ, a component of proton motive force, which is negative in the cytoplasm relative to the periplasm (40), MurJ would not be able to make the transition from outward-open to inward-open conformation, therefore disrupting the lipid II transport cycle and resulting in accumulation of lipid II in the cytoplasm (17). Based on both the dependence on Δψ and similarity with MATE transporters (27), we propose that MurJ is likely to bind to a cation in the outward-open conformation to release lipid II and/or undergo the transition from the outward-open to the inward-open state during the lipid II transport cycle. We favor the cation antiport mechanism for MurJ based on homology to MATE transporters that use a cation gradient (27), although we cannot rule out other models such as voltage-driven or anion-coupled symporter mechanisms. Although further studies are needed to identify such cation(s) and fully understand the mechanism of MurJ function, our studies provide the first direct biochemical evidence that MurJ uses an alternating-access mechanism of transport and provide a useful tool to probe conformational states of MurJ in vivo.

**Experimental procedures**

**Bacterial strains and growth conditions**

Strains used are listed in Table S1. Cells were grown at 37 °C with aeration in lysogeny broth (LB), and growth was monitored by absorbance at 600 nm (A_{600}). YT (also known as low-osmolarity medium) agar contained 10 g/liter tryptone, 5 g/liter...
yeast extract, and 15 g/liter agar. When necessary, ampicillin (125 μg/ml), 5-bromo-4-chloro-3-indolyl β-D galactopyranoside (X-Gal; 20 μg/ml), and isopropyl β-D-1-thiogalactopyranoside (0.04 mM) were added to the medium.

**Plasmid construction and site-directed mutagenesis**

Primers are listed in Table S2. Plasmids were constructed using restriction enzymes, Phusion polymerase, T4 DNA ligase, and T4 polynucleotide kinase from New England Biolabs and *Pfu* Turbo polymerase from Agilent Technologies. Construction of pRC7KanMurJ was described previously (24). To construct plasmids pET23/42FLAGMurJ and pET23/42FLAGMurJΔCys, the EcoRI-HindIII 1,603-bp fragments containing the *flag-murJ* and *flag-murJΔCys* alleles were excised from the pFLAGMurJ and pFLAGMurJΔCys plasmids (26), respectively, and ligated into the double-digested pET23/42 vector (47). DH5α transformants containing these plasmids were selected on LB agar containing 125 μg/ml ampicillin.

Plasmid pET23/42FLAGMurJΔCys-thrombin, which encodes a *MurJ* variant with a thrombin protease site (LVPGRS) inserted in the cytoplasmic loop between TM6 and TM7 after residue Phe-221, was generated using PCR with primers 5MurJ/221 Thrombin222 and 3FLAG222MurJ. The insertion was made using site-directed mutagenesis (SDM) PCR (95 °C for 2 min followed by 19 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 14 min and a final extension of 72 °C for 12 min using *Pfu* Turbo polymerase). The resulting PCR product was treated with T4 polynucleotide kinase and then ligated with T4 DNA ligase. DH5α transformants harboring these plasmids were selected on LB agar containing 125 μg/ml ampicillin.

To generate pET23/42FLAGMurJΔCys-thrombin derivatives with cysteine substitutions, native codons were changed to cysteine codons using SDM PCR. First, plasmids with single Cys-codon substitutions in *flag-murJ*ΔCys-thrombin were made and subsequently used as templates to introduce the second Cys-codon substitutions. All the substitutions were made by using SDM PCR with either *Pfu* Turbo polymerase (95 °C for 2 min followed by 19 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 14 min and a final extension of 72 °C for 12 min) or Phusion polymerase (98 °C for 2 min followed by 25 cycles of 98 °C for 30 s, 60 or 65 °C for 30 s, and 72 °C for 5 min and a final extension of 72 °C for 5 min) according to the manufacturer’s instructions. The variants made in pET23/42FLAGMurJΔCys-thrombin were electroplated into DH5α, and transformants were selected on LB agar containing 125 μg/ml ampicillin.

**Functionality test of pET23/42FLAGMurJΔCys-thrombin derivatives**

The ability of pET23/42FLAGMurJΔCys-thrombin–derived plasmids to functionally complement the loss of *murJ* was evaluated as described previously (26, 28, 34) using strain NR3267 (NR754 ΔmurJ::frt pRC7KanMurJ) (24). Briefly, we used the single-copy-number plasmid pRC7KanMurJ, which has two important features that enabled us to screen for the functionality of *murJ* alleles. First, pRC7KanMurJ has a partitioning defect, so it does not equally segregate into daughter cells during division. As a result, it is easily lost from the population of cells in the absence of selection imposed by kanamycin. Therefore, in media without kanamycin, pRC7KanMurJ is rapidly lost in *murJ* cells. Daughter cells of a Δ*murJ* (pRC7KanMurJ) strain, such as NR3267, that have lost pRC7KanMurJ do not survive because *murJ* is essential for viability. As a result, pRC7KanMurJ is maintained in populations of NR3267 even in the absence of kanamycin because cells that lose the plasmid die. Second, pRC7KanMurJ encodes β-gal, allowing us to easily determine its loss by blue/white screening in the presence of X-Gal. Taking advantage of these features of pRC7KanMurJ, we transformed strain NR3267 with pET23/42FLAGMurJΔCys-thrombin–derived plasmids. Functional complementation was evaluated by checking for the loss of pRC7KanMurJ. Plasmids encoding functional *murJ* alleles yielded white colonies, whereas those encoding nonfunctional *murJ* alleles yielded stably blue colonies. Strains expressing functional *murJ* alleles were further assessed for growth defects in low-osmolarity medium (YT agar) by checking the relative growth and colony morphology compared with those of the parent strain after overnight growth at 37 °C (26, 28). Strains carrying fully functional alleles should resemble those carrying the WT *murJ* allele.

**In vivo cysteine cross-linking using maleimide homobifunctional cross-linkers**

Strains were grown to an A600 of 1 in LB medium. Cells from 10 ml of culture were pelleted by centrifugation and resuspended in 500 μl of LB for each treatment. Each 500-μl cell suspension was treated either with dimethyl sulfoxide (DMSO; solvent used for dissolving cross-linkers) or with the homobifunctional maleimide cross-linkers BMH (Thermo Scientific Pierce), BMOE (Thermo Scientific Pierce), and o-PDM (Sigma) at a final concentration of either 0.25 mM for cytoplasmic side variants or 1 mM for periplasmic side variants. Samples were incubated on a rotator at room temperature for 5 min in the dark. For the NEM (Santa Cruz Biotechnology) pretreatment experiments, samples were treated with 10 mM NEM and incubated on a rotator at room temperature for 10 min to block the cysteine before the addition of cross-linkers. After the incubation with cross-linkers, L-cysteine (Sigma; 10 mM final concentration) was added to quench unreacted cross-linkers. After 5 min at room temperature, each sample was washed with phosphate-buffered saline (PBS; pH 7.4) and processed for spheroplast and membrane preparation. For spheroplast formation, the samples were resuspended in spheroplast buffer (50 mM Tris-HCl, pH 8.0, 1 mM sucrose, 2 mM EDTA) with 0.125 mg/ml lysozyme. After incubating at room temperature for 15 min, 40 μl of 1 M MgCl2 was added, and spheroplast formation was confirmed through microscopy. Spheroplasts were collected by 2,057 × g at 4 °C and resuspended in 500 μl of 50 mM Tris-HCl, pH 8.0, containing 1 μl of Benzonase (Novagen). Membranes from the spheroplasts were pelleted using ultracentrifugation at 100,000 rpm for 1 h at 4 °C in a Optima-MAX-TL ultracentrifuge (Beckman Coulter) using a TLA120.2 rotor. The membrane pellet was resuspended in thrombin reaction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2, 1% n-dodecyl β-D-maltoside) (38), and samples were centrifuged to remove unsolubilized or aggregated proteins. After centrifugation, 0.4 unit of thrombin protease (Sigma) was added or not.
(i.e. untreated samples) to 50 μl of the solubilized membrane fraction, and the reaction was incubated overnight at room temperature. Then, an equal volume of 2× AB buffer (6.84 mM Na₂HPO₄, 3.16 mM NaH₂PO₄, 50 mM Tris-HCl, pH 6.8, 6 mM urea, 1% β-mercaptoethanol, 3% SDS, 10% glycerol, 0.1% bromphenol blue) (26, 28) was added to the samples, and samples were loaded onto either 10 or 12% SDS-polyacrylamide gels for electrophoresis and detection by immunoblotting.

**Ionophore treatment and subsequent cysteine cross-linking**

Strains were grown to an A₆₀₀ of 1 in LB medium. Then, 10 ml of these cultures was treated with either DMSO or 100 and 200 μM TCS (Acros Organics), respectively. After incubating samples on a rotator at room temperature for 10 min, samples were treated with 1 mM cross-linker (123–136) and processed as described above.

**Immunoblotting for FLAG-MurJ detection**

Samples were either prepared as explained above for the cysteine cross-linking experiments or as described previously (28) with certain modifications as follows. Cells were grown overnight, normalized by dividing 400 by A₆₀₀ values, pelleted by centrifugation, and lysed with 50 μl of BugBuster protein extraction reagent (Novagen) and 1 μl of Benzonase (Novagen). After incubating the samples on a rotator for 30 min at room temperature, 50 μl of 2× AB buffer was added. Samples were loaded onto a 10% SDS-polyacrylamide gel for electrophoresis. Proteins were then transferred from the gel to a polyvinylidene fluoride membrane at 10 V for 2.5 h using a semidy transfer apparatus (Bio-Rad). Polyvinylidene difluoride membranes were probed with anti-FLAG M2 (1:10,000; Sigma-Aldrich) and anti-mouse horseradish peroxidase (1:10,000; GE Healthcare) antibodies. These membranes were also blotted with anti-LptB (1:50,000; our laboratory collection) and anti-rabbit horseradish peroxidase (1:10,000, GE Healthcare) antibodies to check for equal loading. Signal was developed using the Clarity Western ECL substrate according to the manufacturer’s instructions (Bio-Rad) and detected using a ChemiDoc XRS+ system (Bio-Rad).

**Author contributions**—S. K., F. A. R., and N. R. conceptualization; S. K., F. A. R., A. G. M., and N. R. formal analysis; S. K. and A. G. M. investigation; S. K. and N. R. methodology; S. K., F. A. R., A. G. M., and N. R. writing—original draft; S. K., F. A. R., A. G. M., and N. R. writing—review and editing; N. R. supervision; N. R. funding acquisition; N. R. project administration.

**Acknowledgments**—We thank Emily Butler for the construction of pET23/42FLAGMurJ and pET23/42FLAGMurJΔCys and Rebecca M. Davis for the construction of pET23/42FLAGMurJΔCys-thrombin. We also thank the members of the Ruiz and Kahne laboratories for helpful discussions.

**References**

1. Silhavy, T. J., Kahne, D., and Walker, S. (2010) The bacterial cell envelope. *Cold Spring Harb. Perspect. Biol.* 2, a000414 CrossRef Medline
2. Tymap, A., Banzhaf, M., Gross, C. A., and Vollmer, W. (2011) From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nat. Rev. Microbiol.* 10, 123–136 CrossRef Medline
3. Lowering, A. L., Safadi, S. S., and Strynadka, N. C. (2012) Structural perspective of peptidoglycan biosynthesis and assembly. *Annu. Rev. Biochem.* 81, 451–478 CrossRef Medline
4. Barreteau, H., Kovac, A., Boniface, A., Sova, M., Gobec, S., and Blanot, D. (2008) Cytoplasmic steps of peptidoglycan biosynthesis. *FEMS Microbiol. Rev.* 32, 168–207 CrossRef Medline
5. Bouhss, A., Trunkfield, A. E., Bugg, T. D., and Megin-Lecreulx, D. (2008) The biosynthesis of peptidoglycan lipid-linked intermediates. *FEMS Microbiol. Rev.* 32, 208–233 CrossRef Medline
6. Ruiz, N. (2015) Lipid flippases for bacterial peptidoglycan biosynthesis. *Lipid Insights* 8, 21–31 CrossRef Medline
7. Ruiz, N. (2016) Filling holes in peptidoglycan biogenesis of *Escherichia coli*. *Curr. Opin. Microbiol.* 34, 1–6 CrossRef Medline
8. Egan, A. J., Biboj, J., van’t Vee, I., Breukink, E., and Vollmer, W. (2015) Activities and regulation of peptidoglycan syntheses. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370, 20150031 CrossRef Medline
9. Sauvage, E., Kerff, F., Terrak, M., Ayala, J. A., and Charlier, P. (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol. Rev.* 32, 234–258 CrossRef Medline
10. Schmid, J., Sieber, V., and Rehm, B. (2015) Bacterial exopoly saccharides: biosynthesis pathways and engineering strategies. *Front. Microbiol.* 6, 496 CrossRef Medline
11. Dell, A., Galadari, A., Sastre, F., and Hitchen, P. (2010) Similarities and differences in the glycosylation mechanisms in prokaryotes and eukaryotes. *Int. J. Microbiol.* 2010, 148178 CrossRef Medline
12. Ruiz, N. (2008) Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flippase in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15553–15557 CrossRef Medline
13. Inoue, A., Murata, Y., Takahashi, H., Tsuji, N., Fuyuji, S., and Kato, J. (2008) Involvement of an essential gene, mviN in murein synthesis in *Escherichia coli*. *J. Bacteriol.* 190, 7298–7301 CrossRef Medline
14. Hvorup, R. N., Winnen, B., Chang, A. B., Jiang, Y., Zhou, X. F., and Saier, M. H., Jr. (2003) The multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily. *Eur. J. Biochem.* 270, 799–813 CrossRef Medline
15. Islam, S. T., and Lam, J. S. (2014) Synthesis of bacterial polysaccharides via the Wzx/Wzy-dependent pathway. *Can. J. Microbiol.* 60, 697–716 CrossRef Medline
16. Sham, L. T., Butler, E. K., Lebar, M. D., Kahne, D., Bernhardt, T. G., and Ruiz, N. (2014) Bacterial cell wall. MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis. *Science* 345, 220–222 CrossRef Medline
17. Rubino, F. A., Kumar, S., Ruiz, N., Walker, S., and Kahne, D. E. (2018) Membrane potential is required for MurJ function. *J. Am. Chem. Soc.* 140, 4841–4844 CrossRef Medline
18. Qiao, Y., Sriskunimits, V., Rubino, F., Schaefer, K., Ruiz, N., Walker, S., and Kahne, D. (2017) Lipid II overproduction allows direct assay of transpeptidase inhibition by β-lactams. *Nat. Chem. Biol.* 13, 793–798 CrossRef Medline
19. Bolla, J. R., Sauer, J. B., Wu, D., Mehmood, S., Allison, T. M., and Robinson, C. V. (2018) Direct observation of the influence of cardiolipin and antibiotics on lipid II binding to MurJ. *Nat. Chem. Biol.* 10, 363–371 CrossRef Medline
20. Liu, X., Meieresonne, N. Y., Boultos, A., and den Blaauwen, T. (2018) FtsW activity and lipid II synthesis are required for recruitment of MurJ to midcell during cell division in *Escherichia coli*. *Mol. Microbiol.* 109, 855–868 CrossRef Medline
21. Mohamed, Y. F., and Valvano, M. A. (2014) *A Burkholderia cenocepacia* MurJ (MviN) homolog is essential for cell wall peptidoglycan synthesis and bacterial viability. *Glyobiology* 24, 564–576 CrossRef Medline
22. Huber, J., Donald, R. G., Lee, S. H., Jarantow, L. W., Salvatore, M. J., Meng, X., Painter, R., Onishi, R. H., Occi, J., Dorso, K., Young, K., Park, Y. W., Skwis, S., Zymonofka, J. M., Waddell, T. S., et al. (2009) Chemical genetic identification of peptidoglycan inhibitors potentiating carbapenem activity against methicillin-resistant *Staphylococcus aureus*. *Chem. Biol.* 16, 837–848 CrossRef Medline
23. Monteiro, J. M., Pereira, A. R., Reichmann, N. T., Saraiva, B. M., Fernandes, P. B., Veiga, H., Tavares, A. C., Santos, M., Ferreira, M. T., and Cys and Rebecca M. Davis for the construction of pET23/42FLAGMurJ and pET23/42FLAGMurJΔCys-thrombin. We also thank the members of the Ruiz and Kahne laboratories for helpful discussions.
Probing the alternating-access mechanism for MurJ

Macário, V., VanNieuenhze, M. S., Filipe, S. R., and Pinho, M. G. (2018) Peptidoglycan synthesis drives an FtsZ-treadmilling-independent step of cytokinesis. Nature 554, 528–532 CrossRef Medline

Elhenawy, W., Davis, R. M., Fero, J., Salama, N. R., Felman, M. F., and Ruiz, N. (2016) The O-antigen flipase Wak can substitute for MurJ in peptidoglycan synthesis in Helicobacter pylori and Escherichia coli. PLoS One 11, e0161587 CrossRef Medline

Meeseke, A. J., Sham, L. T., Kimsey, H., Koo, B. M., Gross, C. A., Bernhardt, T. G., and Rudner, D. Z. (2015) MurJ and a novel lipid II flipase are required for cell wall biogenesis in Bacillus subtilis. Proc. Natl. Acad. Sci. U.S.A. 112, 6437–6442 CrossRef Medline

Butler, E. K., Davis, R. M., Bari, V., Nicholson, P. A., and Ruiz, N. (2013) Structures of multidrug and toxic compound extrusion transporters and their mechanistic implications. Channels 10, 88–100 CrossRef Medline

Zheng, S., Sham, L. T., Rubino, F. A., Brock, K. P., Robins, W. P., Melkano- nos, J. J., Marks, D. S., Bernhardt, T. G., and Kruse, A. C. (2018) Structure and mutagenic analysis of the lipid II flipase MurJ from Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 115, 6709–6714 CrossRef Medline

He, X., Szewczyk, P., Karyakin, A., Evin, M., Hong, W. X., Zhang, Q., and Chang, G. (2010) Structure of a cation-bound multidrug and toxic compound extrusion transporter. Nature 467, 991–994 CrossRef Medline

Lu, M., Symersky, J., Radchenko, M., Koide, A., Guo, Y., Nie, R., and Koide, S. (2013) Structures of a Na+-coupled, substrate-bound MATE multidrug transporter. Proc. Natl. Acad. Sci. U.S.A. 110, 2099–2104 CrossRef Medline

Tanaka, Y., Hipolito, C. J., Maturana, A. D., Ito, K., Kuroda, T., Higuchi, T., Kato, T., Kato, H. E., Hattori, M., Kumazaki, K., Tsukazaki, K., Ishitani, R., Suga, H., and Nureki, O. (2013) Structural basis for the drug extrusion mechanism by a MATE multidrug transporter. Nature 496, 247–251 CrossRef Medline

Chamakura, K. R., Sham, L. T., Davis, R. M., Min, L., Cho, H., Ruiz, N., Bernhardt, T. G., and Young, R. (2017) A viral protein antibiotic inhibits lipid II flipase activity. Nat. Microbiol. 2, 1480–1484 CrossRef Medline

Sun, J., and Kaback, H. R. (1997) Proximity of periplasmic loops in the lactose permease of Escherichia coli determined by site-directed cross-linking. Biochemistry 36, 11959–11965 CrossRef Medline

Wu, J., Hardy, D., and Kaback, H. R. (1999) Site-directed chemical cross-linking demonstrates that helix IV is close to helices VII and XI in the lactose permease. Biochemistry 38, 1715–1720 CrossRef Medline

Wu, J., and Kaback, H. R. (1996) A general method for determining helix packing in membrane proteins in situ: helices I and II are close to helix VII in the lactose permease of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 93, 14498–14502 CrossRef Medline

Hennon, S. W., and Dalbey, R. E. (2014) Cross-linking-based flexibility and proximity relationships between the TM segments of the Escherichia coli YidC. Biochemistry 53, 3278–3286 CrossRef Medline

Mulligan, C., and Mindell, J. A. (2017) Pinning down the mechanism of transport: probing the structure and function of transporters using cysteine cross-linking and site-specific labeling. Methods Enzymol. 594, 165–202 CrossRef Medline

Kruvilich, T. A., Sachs, G., and Padan, E. (2011) Molecular aspects of bacterial pH sensing and homeostasis. Nat. Rev. Microbiol. 9, 330–343 CrossRef Medline

Kuroda, T., and Tsuchiya, T. (2009) Multidrug efflux transporters in the MATE family. Biochim. Biophys. Acta 1794, 763–768 CrossRef Medline

Forrest, L. R., Krämer, R., and Ziegler, C. (2011) The structural basis of secondary active transport mechanisms. Biochim. Biophys. Acta 1807, 167–188 CrossRef Medline

Paz, A., Claxton, D. P., Kumar, J. P., Kazmier, K., Bisignano, P., Sharma, S., Nolte, S. A., Liwag, T. M., Nayak, V., Wright, E. M., Grabe, M., Mchaourab, H. S., and Abramson, J. (2018) Conformational transitions of the sodium-dependent sugar transporter, vSGLT. Proc. Natl. Acad. Sci. U.S.A. 115, E2742–E2751 CrossRef Medline

Parent, L., Supplisson, S., Loo, D. D., and Wright, E. M. (1992) Electrogenic properties of the cloned Na+/glucose cotransporter: 1. Voltage-clamp studies. J. Membr. Biol. 125, 49–62 Medline

Kaback, H. R. (2015) A chemiosmotic mechanism of symport. Proc. Natl. Acad. Sci. U.S.A. 112, 1259–1264 CrossRef Medline

Sham, L. T., Zheng, S., Yakhnina, A. A., Kruse, A. C., and Bernhardt, T. G. (2018) Loss of specificity variants of WzxC suggest that substrate recognition is coupled with transporter opening in MOP-family flippases. Mol. Microbiol. 109, 633–641 CrossRef Medline

Wu, T., McCandlish, A. C., Gronenberg, L. S., Chng, S. S., Silhavy, T. J., and Kahne, D. (2006) Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 103, 11754–11759 CrossRef Medline