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*ROTATION OF THE TolR TRANSMEMBRANE HELIX**

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The TolQRA proteins of *Escherichia coli* form an inner membrane complex involved in the maintenance of the outer membrane stability and in the late stages of cell division. The TolQR complex uses the proton motive force to regulate TolA conformation and its interaction with the outer membrane Pal lipoprotein. It has been proposed that an ion channel forms at the TolQR transmembrane helix (TMH) interface. This complex assembles with a minimal TolQ:TolR ratio of 4–6:2 and therefore involves 14–20 TMHs. To define the organization of the transmembrane helices in the membrane within the TolQR complex, we initiated a cysteine scanning study. In this study, we report results for the systematic replacement of each residue of the TolR TMH. Phenotypic analyses first showed that most of the mutants are functional. Three mutants, TolR L22C, D23C, and V24C, were shown to affect TolQR functioning. Disulfide bond complex formation further showed that two TolR anchors are close enough to interact. Two substitutions, L22C and V24C, form high level of dimers, suggesting that the TolR helix rotates as molecular gears between these two positions and that disulfide bond formation between these residues blocked the rotary motion. Mutations of critical residues located within the TolQ TMH2 and TMH3 and the TolR TMH and proposed to form the ion pathway prevent rotation between these two residues. TolR anchors may form molecular gears that oscillate in response to proton motive force to regulate channel activity.

The Tol proteins form a complex involved in the maintenance of cell envelope integrity (1, 2) and in the late stage of the cell division process (3). Three of these proteins, TolQ, TolR, and TolA interact through their transmembrane (TM)[2] segments in the inner membrane (4–8). In the periplasm, TolB, the outer membrane-anchored peptidoglycan-associated lipoprotein Pal, and the C-terminal globular domain of TolA bind to each other to form a network linking the inner and outer membranes and the peptidoglycan layer (9–11). Previous studies showed that the TolQ, TolR, and TolA proteins form a complex with a 4–6:2:1 stoichiometry. The proton motive force (PMF), the TolQ and TolR proteins, and the TMH of TolA regulate the conformation of the periplasmic region of TolA and probably its subsequent interaction with Pal (10, 12, 13). The TolQ-R complex thus appears to form a molecular motor that converts chemical energy derived from the PMF to mechanical movements (12). This appears possible through the passage of ions in an aqueous lumen formed by the single TMH of TolR and two helices (TMH2 and TMH3) of TolQ (12, 14). Several residues, including the critical Asp^{23} residue that lies within the TolR TM segment, have been shown to be necessary for the TolQR complex to function as a molecular motor. Because of the hydrophilic behavior of these residues, it has been suggested that they probably form the aqueous ion pathway. The proline residue at position 187 of TolQ (TMH3) is also essential and has been proposed to regulate conformational modifications of the TolQR complex in response to ion transit through the aqueous channel (14). Recently, a cysteine scanning study demonstrated that the C-terminal periplasmic domain of TolR forms head-to-tail homo-dimers (15), a result that has been confirmed by the NMR structure of the *Haemophilus influenzae* TolR periplasmic domain (16). Cysteine accessibility indicates that the same domain changes conformation in response to PMF and to critical residues suspected to form the ion pathway. Consequently, it has been proposed that the C-terminal domain of TolR may regulate ion entrance or ion flows through the TolQ-R TM channel (15).

The TolQ-TolR complex is homologous to the ExbB-ExbD complex that functions in membrane transport and to the MotA-MotB complex that forms the stator of the flagellar motor (12, 17, 18). Biochemical analyses on the homologous TolR and ExbD proteins using chemical cross-linkers indicated that TolR forms homo-dimers (8), whereas ExbD forms dimers and trimers (19). Determinants for TolR dimer formation mainly localize in the periplasmic domain of the protein. The three-dimensional structure of the *H. influenzae* TolR periplasmic domain confirmed the formation of homodimers with a head-to-tail conformation (16), whereas the three-dimensional structure of ExbD obtained under acid pH conditions corresponds to a monomer (20). No structural information is avail-

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[2] The abbreviations used are: TM, transmembrane; TMH, transmembrane helix; PMF, proton motive force; WT, wild type; DTT, dithithreitol; HA, hemagglutinin; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

able for MotB; however, extensive mutagenesis has been performed using targeted disulfide cross-linking and evidenced that the TMHs from two MotB molecules are close enough to interact (21).

Using the whole batch of available data, an arrangement of four molecules of MotA and two MotB has been proposed in the flagellar motor (22). Based on the stoichiometry of one TolA molecule, a model for the arrangement of the TolQRA complex containing four to six TolQ and two TolR molecules has been proposed (12). Similar ratios were measured for the ExbB-D-TonB complex (23). This will form a minimal complex composed of 15–21 helices. To understand how the TolQRA TM segments interact in the membrane and how they mechanically respond to PMF or ion transit, we initiated a cysteine scanning approach. Herein, we performed a cysteine substitution mutagenesis of TolR TMH residues. The phenotypic consequences of residue replacements and the patterns of disulfide cross-linking obtained are consistent with an organization of two TolR TM segments in close contact through two distinct faces, suggesting helix rotation. Further experiments support the model in which ion transit at the TolQR TM helices interface induce a rotary motion of the TolR TM segment between the two faces.

EXPERIMENTAL PROCEDURES

Strain, Plasmid Construction, and Growth Conditions—GM1 *Escherichia coli* K12 strain (WT), and its derivatives, TPS300 and TPS13 (*tolR*::Cm and *tolQR*, respectively) (24), were used in this study. pOK-QR was constructed by insertion of a EcoRI-BamHI fragment from pTPS304 (a ColE1 pUC19 derivatives encoding *tolQ-tolR*) (24) into the P15A derivative pOK12 vector (25). pUC-R and pOK-R constructs were obtained by deletion of the *tolQ* gene from plasmid pTPS304 and pOK-QR, respectively, using recombinant PCR (26) and oligonucleotides 5′-TTAAACTCCGCGACAATAGACTTGGGAAGCGCACGAGGCTT and 5′-GTCTATTGTCGCGGAGTTTAAGCAATGGCCAGAGCGCGTGGACGA. In these constructs, the *tolQ* Shine Dalgarno is followed by *tolR* gene. *tolR* mutants were engineered as described previously (12), using the pUC-R or the pOK-R plasmids as templates. Site-directed substitutions were introduced by QuikChange mutagenesis PCR using complementary pairs of oligonucleotides (sequences available upon request). Plasmid pOK-Q_{HA}, encoding a functional TolQ protein carrying a C-terminal hemagglutinin (HA) tag and compatible with the pUC-R derivatives, will be described elsewhere. pOK-Q_{HA} carrying point mutations (T145A, T178A, and P187V TolQ mutant proteins) were constructed by QuikChange mutagenesis. All of the constructs were verified by restriction analyses and further DNA sequencing. Routinely, cells were grown aerobically in LB medium at 37 °C supplemented with ampicillin (100 μg/ml; pUC derivatives), kanamycin (50 μg/ml; pOK derivatives), or chloramphenicol (40 μg/ml).

Assays for Outer Membrane Stability—Outer membrane permeability was assessed by measuring the level of detergent susceptibility, as well as the level of periplasmic RNase I release in the extracellular medium. Detergent susceptibility was measured by the level of survival of the strain on 1% deoxycholate supplemented LB plates or on LB liquid medium containing various concentrations of SDS (as described previously) (27). In the latter case, survival is reported as the concentration of SDS sufficient to inhibit 50% of the cell growth after 3 h of cultures (LD_{50}). RNase I leakage was measured on RNA-containing plates, as described previously (27).

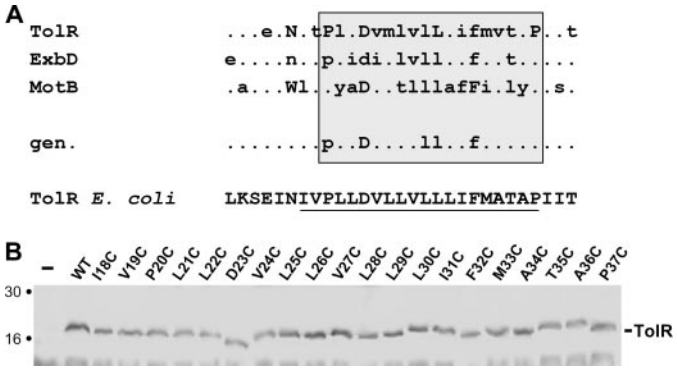

FIGURE 1. **Conservation of TolR anchor.** *A*, alignment of TolR, ExbD, and MotB N-terminal consensus sequences (from Ref. 12) (*uppercase letter*, residue conserved in >90% of the sequences; *lowercase letter*, residue conserved in >60% of the sequences; *dot*, not conserved residue). The *E. coli* TolR N-terminal sequence is indicated (*bottom row*). The frame represents the position of the TM domain. The residues mutated in this study are *underlined* in the *E. coli* TolR sequence. *B*, steady state levels of WT and mutants TolR proteins used in this study. 0.2×10^8 cells were boiled in Laemmli buffer supplemented with β-mercaptoethanol, loaded onto 12.5% acrylamide Tricine SDS-PAGE, and immunodetected with anti-TolR polyclonal antibody. The molecular weight markers are indicated on the *left*.

In Vivo Disulfide Bond Formation and Immunodetection—*In vivo* disulfide bond formation was essentially carried out as described previously (15) with slight modifications. 8×10^8 exponentially growing cells were harvested and resuspended in 1 ml of 20 mM sodium phosphate buffer, pH 6.8, and then treated for 10 min with 2.5 mM *N*-ethylmaleimide (Sigma) to block reduced thiol groups. When required, the cells were treated with the oxidative catalyst copper (II) orthophenanthroline 0.3 mM (CuoP; Sigma) prior washing in sodium phosphate buffer and blocking with *N*-ethylmaleimide. After centrifugation, the cell pellets were resuspended in Laemmli loading buffer in the presence or absence of the reducing agent β-mercaptoethanol. *In vivo* formaldehyde cross-linking was performed as described previously (29). Proteins were separated on a 15% SDS-PAGE, and immunodetections were performed using the anti-TolR polyclonal antibody (8), secondary goat antibodies coupled to alkaline phosphatase and 5-bromo-4-chloro-3-indolylphosphate (Sigma), and nitroblue tetrazolium (Sigma).

RESULTS

Sequence Conservation—The TolR, ExbD, and MotB proteins share similarities at the sequence level and probably at the structural and functional levels. Fig. 1*A* shows a sequence conservation consensus of all TolR, ExbD, and MotB TM anchors available on the protein data base. The general consensus obtained from the TolR, ExbD, and MotB consensuses shows

that the aspartate residue at position 23, two leucine residues at positions 28 and 29, and a phenylalanine residue at position 32 are conserved, whereas many other positions present residue with similar hydrophobic properties (positions relative to the *E. coli* TolR sequence; Fig. 1*A*). However, because of the nature of a transmembrane helix and the general hydrophobicity of the residues, these residues probably act as structural determinants. The functional role of the Asp^{23} residue has been documented in the TolR protein, as well as in the ExbD and MotB homologues. It has been proposed that this aspartate plays a central role in the PMF-dependent process and may couple ion or proton transport to mechanical movement within the TolQR-ExbBD-MotAB or PomAB complexes (12, 14, 30–33). A recent accessibility study showed that it is indeed the case because TolR C-terminal residues display different solvent accessibilities depending on PMF and residues important for channel formation, including the TolR Asp^{23} residue (15). To understand how transmembrane helices organize together in the membrane and how PMF and hydrophilic ion channel residues regulate structural transitions, we initiated a cysteine scanning approach using site-directed mutagenesis.

Cysteine Scanning: Mutagenesis and Expression Levels of Cys Substitutions—To gain information about the structural organization and dynamic features of the TolR transmembrane anchor, each amino acid residue of the helix (residues 18–37) (34, 35) was individually replaced with cysteine. Mutations were introduced into the pUC-R plasmid, which expresses *tolR* at WT levels, by a recombinant PCR technique. Steady state levels of cysteine substituted TolR proteins were determined after introduction of the mutated plasmids into the *tolR* TPS300 strain. Initial studies using the polyclonal anti-TolR antibodies showed that the substituted TolR proteins migrate at the same position and accumulate at comparable levels as native TolR (Fig. 1*B*). However, as shown previously (5, 12), mutations of the Asp^{23} or the Pro^{187} residue led to migration defects on denaturing gel electrophoresis.

Phenotypic Characterization of Single Cysteine Mutants—The 20 singly substituted TolR mutants were tested for outer membrane stability, reflected by their capacity to grow on deoxycholate-containing plates as well as the level of periplasmic components release. Like most transmembrane helices, the TolR transmembrane segment was shown to be remarkably tolerant to cysteine substitution because all but three mutants displayed WT phenotype (Table 1). As expected, the strain producing the D23C TolR protein released RNase I in the medium and did not grow in presence of deoxycholate. Similar phenotypes were observed when the L22C and V24C *tolR* alleles were expressed. The TolR protein is also parasitized by bacterial toxins, called colicins, to penetrate and kill the target cell (for a review, see Ref. 36). We then tested the collection of substituted TolR mutants for group A colicin uptake, a process independent of PMF (36). All the strain producing the TolR mutants, except D23C, were killed by the Tol-dependent colicins (Table 1). We further tested the *tolR* substitutions for the uptake of group B colicins after introduction of the mutated plasmids into the *exbBD-tolQR* strain expressing *tolQ* (from the pOK-Q_{HA} plasmid). In contrast to group A colicins, group B colicins, except colicins 5 and 10, require energy from the ExbBD-TonB or the chimera TolQR-TonB complex (a phenomenon known as "cross-complementation"), to penetrate the cell (36).[3] Production of TolQ and the $TolR_{D23C}$ protein in the *exbBD-tolQR* strain renders cells resistant to all group B colicins tested (B, D, Ia, M, 5, and 10), whereas the L22C and V24C mutations prevented cell killing by group B colicins except colicins 5 and 10 (data not shown). These results confirmed that the D23C mutation abolishes all TolR functions (outer membrane stability and colicin import), whereas the L22C and V24C mutations specifically blocked the PMF-dependent TolR function (outer membrane stability) but not the energy-independent colicin import process. Taken together, the phenotype analysis suggests that the TolR Cys substitutions lie within the three previously characterized classes (Ref. 14 and Fig. 2): functional mutants (class I; all except L22C, D23C, and V24C), nonfunctional mutants (class II: D23C), and discriminative mutants (class III: L22C and V24C). This latter category includes mutations affecting TolQR channel activities, either at the level of channel formation (14) or prevention of conformational changes through disulfide bond formation between cysteine residues (15). Because of the nature of these substitutions, we suspected the L22C and V24C TolR proteins to dimerize. However, as seen on the helical wheel projection (Fig. 2), the Leu^{22} and Val^{24} residues are located on opposite faces, suggesting that if the TolR TMH dimerizes, helix rotation should occur. Alternatively, these

TABLE 1

Phenotypes of strains producing Cys-substituted TolR proteins

TPS300 (*tolR*) cells expressing WT *tolR* or *tolR* cysteine mutants were tested for level of import of group A colicins A, N, and E2 (*tolR*-dependent colicins) and group B colicin D (*tol*-independent), susceptibility to deoxycholate (DOC), and leakage of the periplasmic RNase I. Colicin sensitivity was tested on bacterial lawns by the spot dilution assay. The number indicated represents the maximal 10-fold dilution of the colicin stock (1 mg/ml) still able to kill the strain tested (R, resistant; S, sensitive; 4, sensitive to 10^4 dilution). Deoxycholate resistance was tested by 100-fold dilutions of the strain on 1% DOC LB plates. The number indicated represents the maximal dilution for which the strain still grows. RNase I leakage was estimated on RNA containing LB plates (−, no leakage; +, low levels of leakage; ++, high levels of leakage). Identical results were obtained using *tolR* cysteine mutants expressed from the pOK-R plasmid (data not shown).

	Colicin				DOC	RNase
	A	N	E2	D		
None	R	R	R	S	S	++
WT	4	4	4	S	4	−
I18C	3	3	3	S	4	−
V19C	3	3	3	S	4	−
P20C	3	3	4	S	4	−
L21C	3	3	4	S	4	−
L22C	3	3	3	S	S	++
D23C	R	R	R	S	S	++
V24C	3	3	3	S	S	++
L25C	4	4	4	S	4	−
L26C	4	4	4	S	4	−
V27C	3	3	4	S	4	−
L28C	4	3	4	S	2	−
L29C	3	3	4	S	4	−
L30C	3	3	4	S	2	+
I31C	3	3	4	S	4	−
F32C	3	3	4	S	4	−
M33C	4	3	4	S	4	−
A34C	3	3	4	S	3	−
T35C	3	3	4	S	3	−
A36C	3	3	4	S	4	−
P37C	3	3	4	S	3	−

[3] E. L. Goemaere, L. Journet, I. Schalk, R. Lloubes, and E. Cascales, unpublished results.

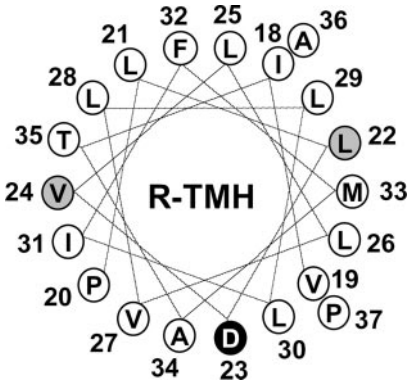

FIGURE 2. **Summary of TolR cysteine substitutions phenotypes.** The TolR anchor residues are represented on a helical wheel projection. The color code corresponds to the phenotype, as classified in Ref. 14. *White*, functional cysteine mutants (class I); *black*, nonfunctional mutants (class II; affected in TolQR complex assembly); *gray*, discriminative mutants (class III, nonfunctional for the PMF-dependent outer membrane integrity, functional for the PMF-independent colicin import).

TABLE 2

Survival of TolR mutant strains to the detergent SDS

tolR cells expressing none (empty vector pUC12), *tolR* (pUC-R), or *tolR* mutants L22C, D23C, or V24C were grown in liquid LB medium supplemented with different concentrations of SDS, ranging from 0.1 to 2%, in the presence or absence of the reducing agent DTT. A_{600} measured after 3 h of growth was used to calculate the percentage of survival of each strain for each concentration of SDS. This results were reported on a graph, and the LD_{50} was calculated.

Strain	LD_{50}	
	−DTT	+DTT
None	<0.1	<0.1
WT	0.9	>2
L22C	<0.1	1.5
D23C	<0.1	<0.1
V24C	<0.1	>2

positions may be on the same face if the TolR TMH is subjected to important physical constraints such as strong helix torsion.

To test whether the phenotypes observed for the TolR L22C and V24C mutants were generated through disulfide bond formation, we performed detergent susceptibility experiments in liquid medium in the presence or absence of the reducing agent dithiothreitol (DTT). Previous studies showed that the growth of *E. coli* cells is not affected by DTT concentrations up to 5 mM (37), and indeed we observed that the presence of DTT in the medium increase SDS resistance of WT strain by a factor of ~2 (Table 2). Survival of the $TolR_{L22C}$- or $TolR_{V24C}$-producing cells was affected by low concentrations of SDS, whereas resistance to SDS was restored to WT levels (increased resistance factor >15–20-fold) when DTT was added in the medium, suggesting that disulfide bonds reduction by DTT restores TolR function (Table 2). As a control, we showed that DTT has no effect on survival of the $TolR_{D23C}$-producing strain. These results suggest that the discriminative phenotype (class III) observed for the L22C and V24C TolR mutants is likely engendered by formation of disulfide bonds and not by lateral chain constraints.

Disulfide Bond Formation between TolR Helix Residues—Journet *et al.* (8) showed that TolR dimerizes, mainly through the central periplasmic domain. To define whether and how the helices of the TolR dimer interact in the membrane, we performed immunodetection of the TolR cysteine derivatives in absence of reducing agent. As shown on Fig. 3*A*, the two mutants L22C and V24C gave a high level of dimers (**, *lower panel*), whereas a number of other mutants, including I18C, L21C, L25C, L28C, L29C, form dimers at lower levels (*, *lower panel*). Interestingly, all of these positions are located on one side of the TolR helix and comprise between the L22C and V24C positions (Fig. 3*B*). The intensity of dimer formation is enhanced for all these mutants when living cells are treated with the oxidative agent copper (II) *ortho*-phenanthroline, whereas low levels of dimers of V19C, L26C, V27C, F32C, M33C, T35C, A36C, and P37C TolR mutants appear (data not shown). All of these mutants, including $TolR_{D23C}$, are likely to assemble correctly because they dimerize and interact with TolQ after *in vivo para*-formaldehyde cross-linking (data not shown). A helical wheel projection summarizing dimer formation is shown on Fig. 3*B*. Because of the level of covalently bound $TolR_{L22C}$ and $TolR_{V24C}$ dimers, the phenotype of cells expressing these mutants on outer membrane stability is likely due to prevention of TolR conformational modifications, as shown for the $TolR_{Y117C}$ mutant situated in the C-terminal domain (15).

To confirm that disulfide bond formation between L22C or V24C TolR proteins prevents functioning of the TolQR complex, the growth curves of these cells were monitored in the absence or presence of 0.25% SDS. $TolR_{L22C}$- and $TolR_{V24C}$-producing cells grew in LB medium, but their growths were affected in presence of SDS. After 90 min, DTT was added to the final concentration of 5 mM in the culture medium. The $TolR_{L22C}$- and $TolR_{V24C}$-producing cells then started growing with a generation time similar to WT cells, demonstrating that reversion of the cross-link by the addition of a reducing agent allows growth to occur in presence of SDS (Fig. 4). In contrast, the addition of DTT has no effect on the growth of the $TolR_{D23C}$-producing cells, for which TolR function is not altered by disulfide bond formation.

Dimerization of TolR-Cys Mutants Affected in Ionizable Key Residues—TolR C-terminal domain has been shown to change conformation relative to the PMF and the presence of key ionizable residues within TolQ and TolR TM helices (15). If TolR transmembrane segments rotate similarly to ratchet gears between Leu^{22} and Val^{24}, one may hypothesize that this rotation mechanism would be transmitted to the C-terminal domain, leading to conformational modifications. We thus constructed double mutants, including a single cysteine substitution with a high (L22C and V24C) or low (L25C, L28C, L29C) level of dimer formation, in combination with a mutation of the TolR Asp^{23} (D23A) or TolQ Thr^{145} (T145A), Thr^{178} (T178A), or Pro^{187} (P187V) residues, four residues critical for the TolQR ion pathway (14). These double substitutions mutants were tested for their ability to form dimers *in vivo*. We showed that mutations within the key ionizable residues Asp^{23}, Thr^{145}, and Thr^{178} do not affect formation of $TolR_{L22C}$ or $TolR_{V24C}$ dimers, whereas formation of L25C, L28C, or L29C TolR dimers was abolished, even in the presence of dichloro(1,10-phenanthroline) copper(II) (Fig. 5). However, as seen in the case of the formation of the TolR C-terminal Y117C dimer, mutation of the Pro^{187} residue does not abolish dimer formation. Overall, this suggests that the two Leu^{22} or the two Val^{24} residues are in close contact during TolR dimer assembly in the membrane

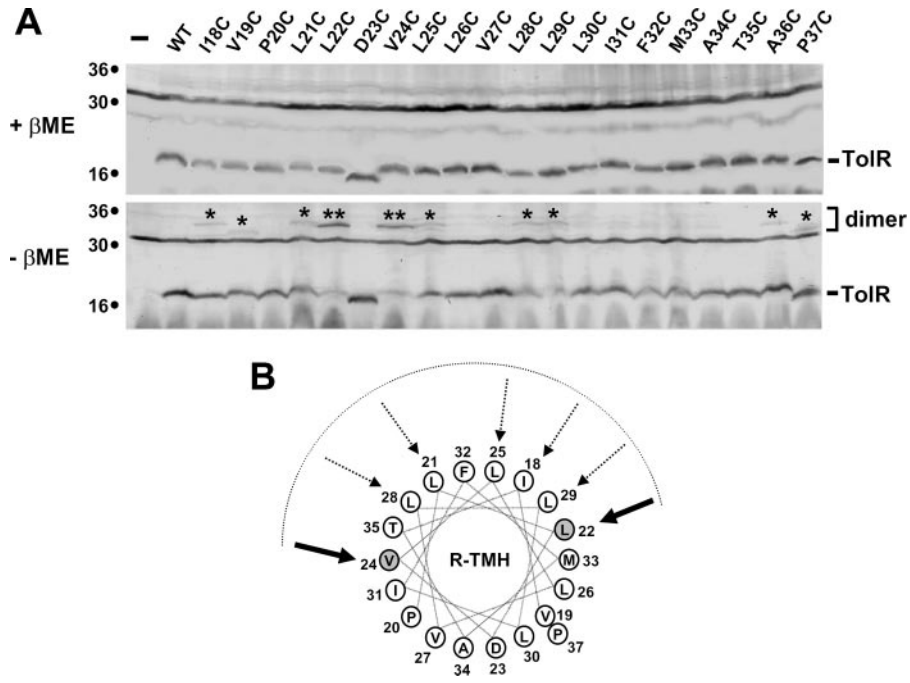

FIGURE 3. **TolR residues involved in homodimer formation.** *A*, 0.2 × 10^8 cells of the indicated strain were boiled in Laemmli buffer in presence of β-mercaptoethanol (+*βME*, *upper panel*) or in absence of reducing agent (−*βME*, *lower panel*), loaded onto 12.5% acrylamide SDS-PAGE, and immunodetected with anti-TolR polyclonal antibody. The positions of TolR and TolR dimers are indicated on the *right*. *, low levels of dimerization; **, high levels of dimerization. The molecular weight markers are indicated on the *left*. *B*, representation of results from *A* on a helical wheel projection. The interface of dimerization is indicated by the *dotted lane*. *Arrows* indicate positions of substitutions that form dimers (solid *arrows*, high levels; *dotted arrows*, low levels).

and that rotation between the two helices is induced by torque generation by the TolQR ion channel.

DISCUSSION

The TolQ, TolR, and TolA proteins form a complex at the inner membrane, interacting through their transmembrane segments, as evidenced using chemical cross-linking or isolation of allele-specific suppressive mutations (5–8, 12, 14). These led to the conclusion that the transmembrane helix of TolR is at the proximity of the helices II and III of TolQ, whereas TolQ transmembrane III contacts the transmembrane I, which is in interaction with the transmembrane anchor of the TolA protein. Stoichiometry analyses showed that the ratio of the TolQ-TolR-TolA complex is 4–6:2:1 (4, 12), adding further complexity in helix organization. However, trying to reconcile all the results and the faces of transmembrane segments in interaction has been shown to be difficult, raising the possibility that structural modifications and movements of helices during functioning may occur. The idea that the TolQRA complex undergoes conformational changes was first suggested by the observation that TolA interacts with the outer membrane-anchored peptidoglycan-associated lipoprotein Pal in a PMF- and TolQR-dependent manner (10, 12). Structural modifications of TolA dependent on PMF and on the TolQ and TolR proteins were demonstrated using protease accessibilities (13). Recently, we demonstrated using cysteine accessibilities that the C-terminal periplasmic domain of the TolR protein is subjected to structural modifications (15).

The observation that the TolQ and TolR helices share similarities, not only with the homologous ExbB and ExbD proteins but also with the flagella stator components MotA and MotB, led to the idea that these three couples of proteins may share a common mechanism of energy transfer (12, 17, 18). This was exemplified by the presence of a conserved aspartate residue with TolR, ExbD, and MotB, which is critical for function (12, 30–32, 38). Following these studies, it has been proposed that the three helices (TolR, TolQ-II, and TolQ-III) may delimit a channel by which ions transit and that a "signal" is then transmitted to the TolA protein via the TolQ-I segment. An analysis of TolQ transmembrane residues further demonstrated that short length side chain residues are involved in helix packing, whereas hydrophilic residues may participate in the energy transduction mechanism (14). In the current model, which is actively tested in our laboratory, a dimer of TolR anchors is surrounded by four to six TolQ molecules, thus allowing the formation of four to six ion pathways, for which channel activity may be regulated by mechanical movements.

To determine how the TolQRA helices interact and how they undergo structural modifications, we performed a systematic cysteine scanning of the TolQRA helices. Here, we reported results obtained for the TolR TM helix. Our results clearly showed that cysteines from two distinct monomers are close enough to efficiently form a disulfide bond dimer without the aid of an oxidative agent. Similar results were obtained in a systematic cysteine scanning study of the flagellar MotB protein TMH (21). In the case of the TolR protein, this is clearly shown for the L22C and V24C mutants. Disulfide bond formation blocks TolR dimers, which prevents function of the Tol system and growth in presence of detergent. Interestingly, this phenotype is reversed by the addition of reducing agent in the medium, as shown for cysteine mutations occluding the SecY protein channel (39). On a perfect helical wheel projection of the TolR TMH, the Leu^{22} and Val^{24} residues are separated by an angle of ~160°. In between, most substitutions form dimers, albeit at lower levels. These results raise the hypotheses (i) that the TolR transmembrane helix rotates between the two "extreme" positions (Leu^{22} and Val^{24}) using the "intermediate" positions such as molecular ratchet gears or cogwheels or (ii) that the transmembrane helix is subject to physical torsions or constraints in such a way that all these residues (intermediate and extreme) are located on a reduced, unique face. The first model has been proposed in light of similar results using the MotB transmembrane anchor as model system (21). In the case of MotB, projection on a similar wheel than the one used in Fig. 2 shows that the angle between the two extreme residues is

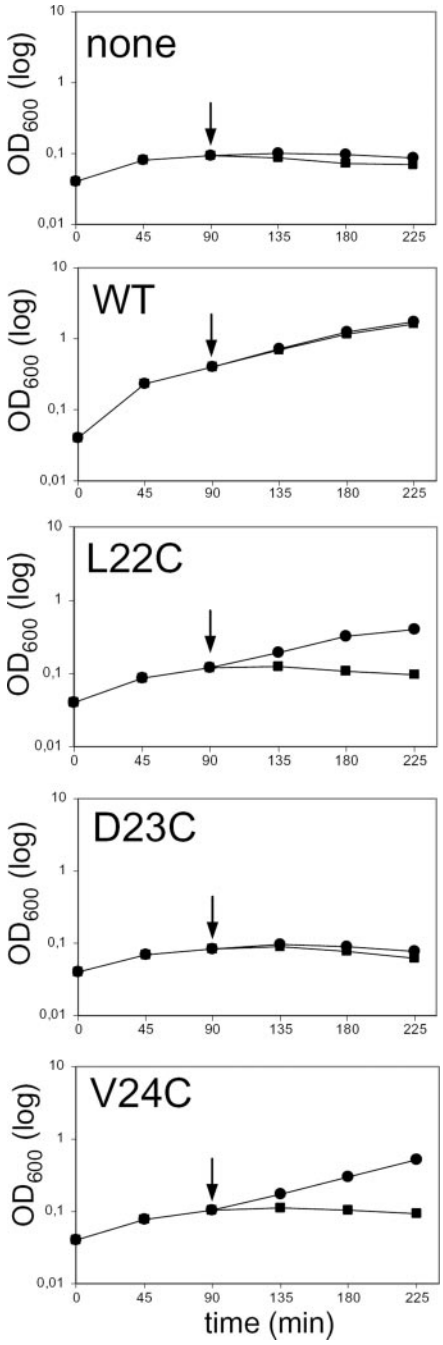

FIGURE 4. **TolR functioning is specifically prevented by disulfide bond formation.** 10^9 bacteria of the indicated strain were inoculated in 10 ml of medium in presence of 0.25% SDS, and growth was monitored by measuring absorbance at 600 nm every 45 min. After 90 min of culture, DTT was added (*closed circles*) or not (*closed squares*) to the final concentration of 5 mM (*arrow*).

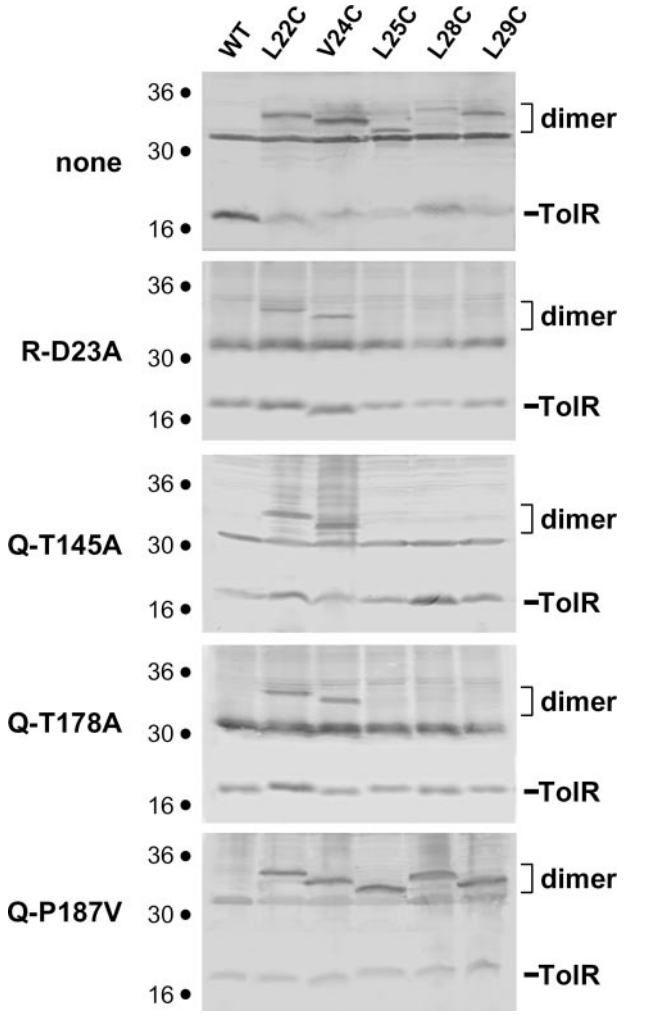

FIGURE 5. **TolR anchor rotations depend on key residues of the ion pathway.** 0.2×10^8 cells producing the indicated TolR Cys substitution (*lanes*) in combination with the TolR or TolQ mutations (*panels*), treated with the oxidative agent copper (II) orthophenanthroline to increase dimer formation, were boiled in Laemmli buffer in absence of reducing agent, loaded onto 12.5% acrylamide SDS-PAGE, and immunodetected with anti-TolR polyclonal antibody. The positions of TolR and TolR dimers are indicated on the *right*. The molecular weight markers are indicated on the *left*. The mutant names on the *top* and on the *left* indicate positions of primary and secondary mutations, respectively.

~110°. However, these results cannot discriminate between the two hypotheses. One may hypothesize that in the absence of movement, the TolR dimer rotation will be stopped in both of the extreme positions (hypothesis (i) above), whereas all extreme and intermediate substitutions will still promote helix dimerization (hypothesis (ii) above). Our results using mutations that affect residues that regulate ion transit favor the first hypothesis. It seems thus likely that the two TolR helices from a dimer oscillate between two extreme conformations (Fig. 6). Alternatively, because the 4–6:2 stoichiometry of the TolQR complex represents the "minimal complex," one may hypothesize that TolR form tetramers. In this case, both L22C and V24C substitutions may form disulfide bonds independently of helix rotation. However, this hypothesis can be ruled out because the L22C/V24C TolR double mutant only forms dimers as single substitutions do (data not shown).

The Asp^{23} residue, which has been proposed to be critical for ion conduction, is located ~100° from either Leu^{22} or Val^{24} on a helical wheel projection (Fig. 2). Using the rotary motion, the Asp^{23} residue may thus alternate between two different ion channels, as suggested for MotB. Such a rotation may thus place the Thr^{35} residue at the position previously occupied by the Asp^{23} residue in the channel. The presence of a hydrophilic residue at that position may thus stabilize the putative aqueous channel. The TolR T35C mutant displayed a WT phenotype, but mutation of the Thr^{35} residue by an alanine confers cell sensitivity toward deoxycholate, but cells remain sensitive to

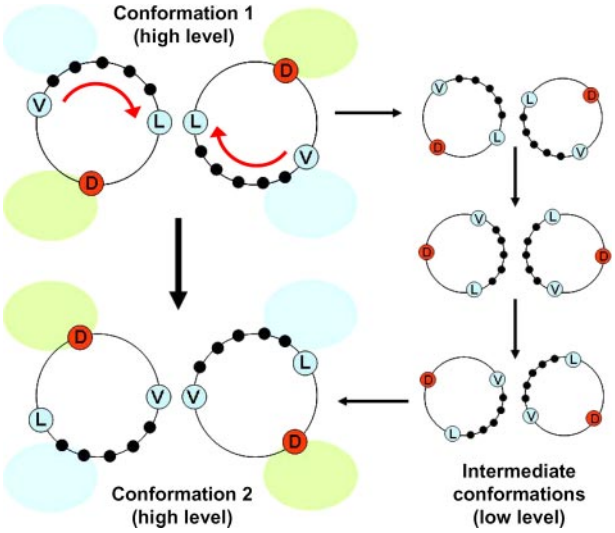

FIGURE 6. **A model for the TolR anchor rotary motion.** The TolR anchors from two distinct TolR molecules are represented at the extreme positions of the dimerization interface (corresponding to high levels of dimerization) and thus oscillate between conformations 1 and 2 (*red arcs*). This may involve passage through a number of intermediate conformations (corresponding to low levels of dimerization) such as molecular gears. The aspartate residue then oscillates between two channels, regulating opening (*green areas*, open or active channels) or closure (*blue areas*, closed or inactive channels).

colicins (data not shown), classifying such a mutation as discriminative (class III), suggesting a functional role of this residue in response to PMF. This implies that rotation of the TolR anchor is functionally important, which is confirmed by the fact that disulfide bond formation with TolR L22C or V24C lead to cells that are impaired in membrane integrity but still sensitive for colicin import (Tables 1 and 2).

Because the level of dimerization of the TolR L22C and V24C is higher than for the intermediate position, one may hypothesize that the time necessary to reach one extreme position from the other is fast and that the rotary motion stops transiently at the extreme positions. However, the TolR F32C and T35C mutant do not dimerize, even if these residues are located between the two extreme positions. This may suggest that the two TolR helices from a dimer are not close together on the whole length but rather are closer at the cytoplasmic side than at the periplasmic side of the inner membrane. This may account for the strong dimerization of Leu^{22} and Val^{24} residues, which are situated at the cytoplasmic side of the membrane; however, the Ile^{18} and Leu^{21} residues, which are located at the cytoplasmic side of the TolR helix, have intermediate dimerization properties. Our results showed that, similarly to TolR C-terminal domain movement (15), TolR helix rotation is regulated by three channel residues, the TolR Asp^{23} residue and the Thr^{145} and Thr^{178} residues located on helices II and III of TolQ, respectively. In the case of the MotAB complex, it has been shown using protease accessibilities that MotA conformation is regulated by the MotB Asp residue (17). The discriminative mutation P187V does not abolish rotation, a result consistent with the observation that this residue does not regulate TolR C-terminal conformational modification (15). The TolQ Pro^{187} residue has been shown to be required for the Tol complex functioning and has been suggested to couple ion transit to conformational changes (14). Our results suggest that TolR anchors rotate independently of Pro^{187}, which thus may regulate conformational modifications of TolQ.

Overall, our results showed that two helices from two different TolR molecules are close to each other in the TolQR complex. These two TolR TMHs rotate as molecular gears between two positions, L22C and V24C. This rotation is functionally important for activity of the TolQR ion channel.

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