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Emilie Goemaere, Anthony Devert, Roland Llobès, E. Cascales. Movements of the TolR C-terminal Domain Depend on TolQR Ionizable Key Residues and Regulate Activity of the Tol Complex. *Journal of Biological Chemistry*, 2007, 282 (24), pp.17749-17757. 10.1074/jbc.M701002200 . hal-03274820

HAL Id: hal-03274820

<https://amu.hal.science/hal-03274820>

Submitted on 14 Sep 2021

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Movements of the TolR C-terminal Domain Depend on TolQR Ionizable Key Residues and Regulate Activity of the Tol Complex*

Received for publication, February 2, 2007, and in revised form, April 3, 2007. Published, JBC Papers in Press, April 18, 2007, DOI 10.1074/jbc.M701002200

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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 interface. This complex assembles with a minimal TolQ/TolR ratio of 4:2, therefore involving at least 14 transmembrane helices, which may form the ion pathway. The C-terminal periplasmic domain of TolR protein interacts with TolQ and has been proposed to control the TolQR channel activity. Here, we constructed unique cysteine substitutions in the last 27 residues of TolR. Each of the substitutions results in a functional TolR protein. Disulfide cross-linking demonstrates that the TolQR complex is dynamic, involving conformational modifications of TolR C-terminal domain. We monitored these structural changes by cysteine accessibility experiments and showed that the conformation of this domain is responsive to the proton-motive force and on the presence of critical residues of the ion pathway.

The Tol-Pal complex of the *Escherichia coli* cell envelope utilizes the energy of the transmembrane electrochemical gradient of ions or protons to mechanically drive outer membrane stability and probably the late stages of cell division (1, 2). The inner membrane TolA protein interacts with outer membrane-anchored Pal (peptidoglycan-associated lipoprotein) in a proton-motive force (PMF)²-dependent manner (3). TolQ and TolR are two accessory proteins interacting with TolA in the inner membrane. Based on sequence comparisons and effects of amino acids substitutions, TolQR have been proposed to form a proton or ion channel, which converts PMF to mechanical energy (4, 5). TolQ is an integral inner membrane protein, with three transmembrane-spanning helices (TMHs) (6, 7).

TolR possesses one TMH with the largest portion of the protein protruding into the periplasm (7, 8). This large domain, subdivided in central (called TolR-2) and C-terminal (called TolR-3) domains, is required for TolR dimerization (9). TolQ and TolR proteins interact in the membrane through their TMHs in a 4:2 stoichiometry (4, 9, 10, 11, 12, 13). It has been proposed that ion or proton flow at the interface of the TMHs of the TolQR complex regulates structural modifications within this complex and hence results in a conformational change within TolA (3–5). This modification of TolA conformation has been shown by limited protease accessibility to be dependent upon PMF, TolQ, TolR, and a conserved motif within the TolA inner membrane anchor (14). Recently, we demonstrated that residues conserved on specific faces of the TolQ and TolR TMHs constitute a putative ion pathway, probably responsible for binding and releasing ions or protons (5). These residues are conserved within the TolQ or TolR protein families but also in the homologous ExbB or ExbD and the orthologous MotA or MotB proteins (4, 5). ExbBD proteins are auxiliary partners of TonB protein, which is responsible for the energy-dependent uptake of iron-charged siderophores (15). As for TolA, activity of TonB is dependent upon PMF, ExbB, ExbD, and a conserved motif within the TonB inner membrane anchor, regulating conformational modifications (16, 17). MotA and MotB are the two components of the flagellar stator, responsible for coupling PMF with flagella rotation (18). Another similarity between Tol and TonB systems resides in their utilization by bacterial toxins produced and active against *Escherichia coli* strains. Group A colicins use a subset of the TolQRA and the TolB proteins, whereas group B colicins use the ExbBD-TonB proteins to penetrate and kill bacteria. However, group A colicins do not need any energy to enter the cell, whereas most group B colicins do (19). Colicins 5 and 10, two group B colicins, do not need energy but do not require a TonB-dependent active transporter to cross the OM, suggesting that energy dependence of colicin transport is dictated by the difference of requirements to cross the OM.³ This behavior has been used recently to test point mutations within the TolQR helices. Mutations affecting assembly of the complex were shown to be nonfunctional for both energy-dependent OM stability and energy-independent colicin uptake, whereas a novel class of discriminative mutation has been described, affecting only the energy-dependent pro-

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² The abbreviations used are: PMF, proton-motive force; OM, outer membrane; TMH, transmembrane helix; WT, wild-type; DOC, sodium deoxycholate; DTT, dithiothreitol; Cu-oP, dichloro(1,10-phenanthroline)copper(II); NEM, N-ethylmaleimide; MPB, N^ε-(3-maleimidylpropionyl) biocytin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; pAb, polyclonal antibody; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

³ E. L. Goemaere, L. Journet, I. J. Schalk, R. Lloubès, and E. Cascales, manuscript in preparation.

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cess. These residues have been proposed to be involved in ion transit or conformation regulation (5).

In order to function, ion channels, such as the TolQR, ExbBD, or MotAB complex, require control of ion selectivity and flow. In a membrane system in which control of ion selectivity or flow has been described, this generally occurs via a penetrating loop or a domain on the periplasmic side of the complex, allowing opening and closing of the channel entrance. In the TolQRA complex, candidates for such a role are the periplasmic loop of TolQ and the TolR C-terminal domain. This latter domain has been shown to co-fractionate with membranes when produced in the periplasm of wild-type (WT) cells (9). Furthermore, suppressive mutations of nonfunctional TolQ TMH3 mutants have been isolated in this domain (5, 11). In the work reported here, we have used residue substitutions with cysteines, oxidative cross-linking, and cysteine accessibilities to assess the role of the TolR C-terminal domain. We show that a segment of the TolR C-terminal domain forming a putative amphipathic α -helix dimerizes. We further demonstrate that the TolR C-terminal domain undergoes conformational modifications dependent upon PMF and residues within the TolQR ion pathway.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and Chemicals

WT GM1 strain and its *tolQR* derivative TPS13 used in this study are *E. coli* K12 derivatives (20). The *recA* strain DH5 α was used for cloning procedures. Cells were routinely grown aerobically in Luria-Bertani (LB) medium at 37 °C. When required, medium was supplemented with ampicillin (100 μ g/ml).

Plasmid Construction

Standard methods were used for DNA manipulations. Mutants were engineered as previously described (4), using the pTPS304 plasmid (pUC19 derivative expressing *tolQ* and *tolR*) (20) as template. Under laboratory growth conditions, Western blot analyzes showed that *tolR* is expressed from this plasmid at about WT levels (data not shown). Site-directed substitutions were introduced by quick change mutagenesis using complementary pairs of oligonucleotides (sequences available upon request). Recovered plasmids were first verified by restriction analyzes and then by DNA sequencing of the *tolR* gene, using 5'-CCGCGATTCTGCACCGCC as primer.

Assays for Outer Membrane Stability

Solid Growth Assays—Outer membrane permeability was assessed by measuring the level of detergent susceptibility and periplasmic leakage. Detergent susceptibility was estimated on sodium deoxycholate (DOC)-containing plates (1% final concentration), by spotting 2 μ l of normalized serial dilution of the strain to be tested. This concentration of DOC is sufficient to completely inhibit growth of *tol* mutants without affecting WT strains. Periplasmic ribonuclease I leakage was estimated as previously described (3).

Liquid Growth Assays—SDS sensitivity was tested as previously described (3). Briefly, strains to be tested were grown aerobically overnight in LB medium. Strains were then diluted

to an $A_{600} = 0.05$ in LB medium containing either 0, 0.25, 0.5, 1, or 2% SDS in the presence or absence of 2 mM dithiothreitol (DTT; Sigma). The A_{600} was measured after 90, 135, 180, and 225 min.

Colicin Tolerance Assays

Colicin activities were checked by the presence of halos on a lawn of the strain to be tested, as previously described (21). Data are reported as the maximal dilution of the colicin stock (1 mg/ml) sufficient to inhibit growth.

In Vivo Cross-linking and Oxidative Treatments

8×10^8 exponentially growing cells ($A_{600} \sim 0.6$) were harvested and resuspended in 1.5 ml of 20 mM sodium phosphate buffer (NaP_i, pH 6.8). 500 μ l were treated with 0.3 mM dichloro(1,10-phenanthroline) copper(II) (Cu-oP; Sigma) for 20 min at room temperature. Reduced thiol groups were blocked with 2.5 mM *N*-ethylmaleimide (NEM; Sigma) for 10 min. 500 μ l were treated for 10 min with 2.5 mM NEM. After centrifugation, cell pellets were resuspended in Laemmli loading buffer without reducing agent. 500 μ l were centrifuged and resuspended in Laemmli loading buffer containing DTT. Proteins were separated on a 12.5% SDS-PAGE, and TolR was immunodetected by Western blot analyses.

Cysteine-specific Labeling with MPB

3×10^9 exponentially growing cells were resuspended in 500 μ l of HSEN buffer (100 mM Hepes, 250 mM sucrose, 1 mM EDTA, 100 mM NaCl). Cells were then treated with the cysteine-reactive *N* α -(3-maleimidylpropionyl) biocytin probe (MPB; Invitrogen) (100 μ M final concentration) for 20 min at room temperature. For accessibility experiments in energy depletion conditions, cells were first treated with 20 μ M carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma) for 10 min and washed with HSEN buffer prior to MPB labeling. As a negative control, cells were treated with 2.5 mM NEM for 20 min and washed with HSEN buffer prior to MPB labeling. MPB labeling was quenched by the addition of β -mercaptoethanol (20 mM final concentration). After two washes using HSEN buffer, cells were resuspended in TES (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% SDS) supplemented with DNase (100 μ g/ml final concentration) and a protease inhibitors mixture (Complete; Roche Applied Science). Membrane proteins were solubilized at 37 °C for 30 min with vigorous shaking and subjected to immunoprecipitation using anti-TolR polyclonal antibodies (pAbs).

Immunoprecipitation

TES-solubilized cell extracts were diluted 15-fold in TNE (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl) supplemented with 1% Triton X-100. After incubation at room temperature with vigorous shaking, extracts were centrifuged for 15 min at 18,000 $\times g$ to remove unsolubilized material. Supernatants were incubated overnight at 4 °C with antibody coupled to protein A-Sepharose CL-4B beads (Amersham Biosciences). Beads were then washed three times with TNE supplemented with 1% Triton X-100, TNE supplemented with 0.1% Triton X-100, and Tween 0.1% and TNE supplemented with 0.1% Triton X-100, respectively. Immunopre-

TABLE 1
Functional properties of TolR C-terminal domain substitutions

Mutant	OM stability		Colicin ^a uptake					
	DOC ^b	Ribonuclease I ^c	ColE1	ColA	ColN	ColE2 ^d	ColD ^{e,f}	Col5 ^{e,g}
WT	++	+	4	3	2	4	4	3
Empty vector	—	—	3 ^h	R	R	R	R	R
P116C, D118C to I142C	++	+	4	3	2	4	4	3
Y117C	+ ⁱ	—	4	3	2	4	2 ^j	3
Y117F	++	+	4	3	2	4	4	3
Y117W	++	+	4	3	2	4	4	3
D114A	+	—	4	3	2	4	4	3

^a Colicin sensitivity estimated by 10-fold serial spot dilution (R, resistant bacteria; for sensible bacteria, the number indicates the maximal 10-fold dilution for which a growth inhibition occurs).

^b Growth on 1% DOC-containing plates (—, no growth; ++, WT growth; +, intermediate).

^c Release of periplasmic ribonuclease I, estimated on RNA-containing plates. +, no leakage; —, leakage.

^d Similar results were obtained with colicin E9.

^e Group B colicins were tested using KP1038 (*exbBD-tolQR*) as the recipient strain.

^f Similar results were obtained with colicins M and Ia.

^g Similar results were obtained with colicin 10.

^h Turbidity observed due to cross-complementation between *tolQ* and *exbB* genes.

ⁱ Growth was observed accompanied by a lysis phenomenon (see Fig. 3A).

^j Turbidity observed due to the energy dependence of colicin D, Ia, and M uptake.³

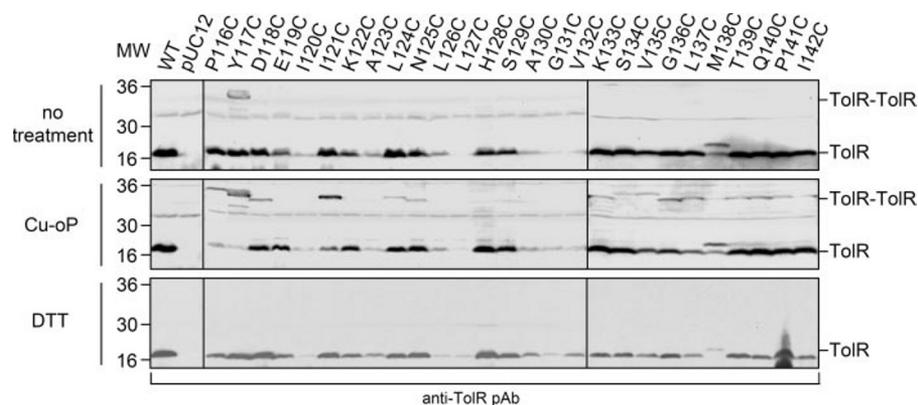


FIGURE 2. TolR residues involved in homodimer formation. 0.2×10^8 cells of the indicated strain were boiled in Laemmli buffer in absence of reducing agent (no treatment; upper panel) in the presence of DTT (lower panel) or after *in vivo* oxidative treatment using Cu-oP (middle panel), loaded onto 12.5% acrylamide SDS-PAGE and immunodetected with anti-TolR pAb. Positions of TolR and TolR dimers are indicated on the right. Molecular weight markers (MW) are indicated on the left.

are shown in Fig. 1B. The Tyr residue and the Ala-Gly tandem are also well conserved within the ExbD C terminus. Widely used computer algorithms predict the presence of an α -helical secondary structure between residues Tyr¹¹⁷ and Gly¹³¹ and of a β -strand between residues Ser¹³⁴ and Thr¹³⁹ (see Fig. 1B).

To gain structural and functional information about the TolR C-terminal domain, we engineered single cysteine substitutions from residue 116 (the proline residue delimitating the central and C-terminal domains) (9) to the C-terminal isoleucine residue (at position 142) (Fig. 1, A and B). Mutations were introduced into the pTPS304 plasmid, which expresses *tolQR* 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 *tolQR* TPS13 strain. Initial studies using the polyclonal anti-TolR antibodies showed that most of the substituted TolR proteins migrated at the same position and accumulated at levels comparable with those of native TolR (Fig. 1C). However, I120C, L126C, L127C, and G131C mutants were expressed at lower levels, and the M138C TolR protein displayed a migration defect.

Phenotypic Characterization of Single Cysteine Mutants— The 27 single substituted TolR mutants were tested for outer

membrane stability, reflected by their capacity to grow on DOC-containing plates as well as the level of periplasmic component release. Unexpectedly, the TolR C-terminal domain was shown to be remarkably tolerant of cysteine substitution, because all but one mutant displayed the WT phenotype (Table 1). The Y117C TolR mutant released ribonuclease I and displayed an intermediate phenotype on DOC plates (see also Fig. 3A). Overnight colonies were translucent, suggesting that lysis occurs.

We then tested the collection of substituted TolR mutants for colicin uptake. All the mutants were susceptible to the different Tol-dependent colicins tested (Table 1). All of the substitutions (except Y117C) were thus classified as functional mutations, whereas the Y117C TolR mutant displays the characteristics of the recently described discriminative substitution (e.g. non-functional for the energy-dependent OM stability but functional for the energy-independent group A colicin uptake) (5). We further tested the substitutions for the uptake of group B colicins after introduction of the mutated pTPS304 plasmids into the *exbBD-tolQR* strain. Group B colicins, except colicins 5 and 10, require energy from the ExbBD-TonB or the TolQR-TonB complex to penetrate the cell (19).³ All of the mutants displayed a WT phenotype, whereas the TolR_{Y117C}-expressing strain presented a WT susceptibility to group B colicins 5 and 10 and an attenuated susceptibility to group B colicins D, M, and Ia (Table 1). This is reminiscent of the discriminative mutations that affect hydrophilic residues that lie within the putative TolQR ionic channel (5) and further suggests that Tyr¹¹⁷ may participate in some way in the TolQR channel activities. Alternatively, we hypothesized that the Y117C TolR mutant might form cysteine-bound homodimers, which would affect the energy-dependent functions of the TolQR complex.

Disulfide Bond Formation between TolR C-terminal Residues—To discriminate between these two hypotheses, we examined the TolR cysteine derivatives by immunoblotting with anti-TolR antibodies in the absence of reducing agent (Fig. 2, *top*). We observed that only the TolR_{Y117C} protein forms homodimers. When treated with the oxidative agent Cu-oP, Y117C as well as P116C, D118C, I121C, L124C, N125C, and K133C to I142C TolR derivatives form homodimers (Fig. 2, *middle*). It is noteworthy that both monomeric and dimeric forms of TolR_{Y117C} are observed, even upon Cu-oP treatment (Fig. 2). However, due to their weak production, dimerization of I120C, L126C, L127C, and M138C could not be determined accurately. Only the monomeric form of TolR was observed when cells were treated with the reducing agent DTT (Fig. 2, *bottom*). These experiments suggest that the phenotype associated with the Y117C mutation is probably due to a dimerization phenomenon.

Y117C Blocks Conformation Modification, Which Results in TolQR Complex Dysfunction—To verify that abolition of the TolR_{Y117C} function is a result of a covalent TolR homodimer formation rather than mutation of a critical residue, we constructed two additional mutants at position Tyr¹¹⁷. Substitutions of Tyr¹¹⁷ by the aromatic phenylalanine or the bulky tryptophan residue were shown to be functional for both OM stability and colicin import (Table 1, Fig. 3A). Besides the high level of Tyr¹¹⁷ conservation within TolR proteins, these results suggest that this residue is not essential for function but that formation of a covalent cysteine-bound TolR dimer abolishes function. To confirm this result, we monitored growth cultures of Y117C, Y117W, and Y117F mutants and control vector expressed into the *tolQR* TPS13 strain in the presence of variable concentrations of SDS and in the absence or presence of the reducing agent DTT (Fig. 3B). As previously shown, *tolQR* cells were sensitive to low concentrations of SDS, whereas growth of complemented cells or cells expressing the Y117W or Y117F TolR mutants was not affected by concentrations of SDS up to 1%. The addition of 2 mM DTT did not affect the growth of any of these strains. However, the Y117C mutant displayed a distinct behavior, since its growth was slowed down, and partial cell lyses occurred after 3 h of culture in the presence of SDS, whereas such a phenomenon was not observed when cells were grown in the presence of DTT (Fig. 3B). To verify that formation of TolR_{Y117C} cross-linked dimers is not toxic for the cell and does not cause a leak of proton or a slow dissipation of PMF, we measured viability and growth rates. Comparison of viability of TolR_{Y117C}-expressing cells with TolR_{WT}-expressing cells in the absence or presence of 0.5% SDS showed that (i) SDS is specifically deleterious for TolR_{Y117C}-expressing cells (comparison of *foreground* and *background* bars) and (ii) viability of TolR_{Y117C}-expressing cells in growth media is similar to the viability of TolR_{WT}-expressing cells (comparison of *background* bars), demonstrating that formation of TolR cross-linked dimers is not toxic for the cell (Fig. 3C). Calculation of generation times in growth media shows no significant difference (31.8 min for TolR_{WT}-expressing cells; 31.2 min for TolR_{Y117C}-expressing cells), suggesting that no proton leak is associated with formation of TolR cross-linked dimers.

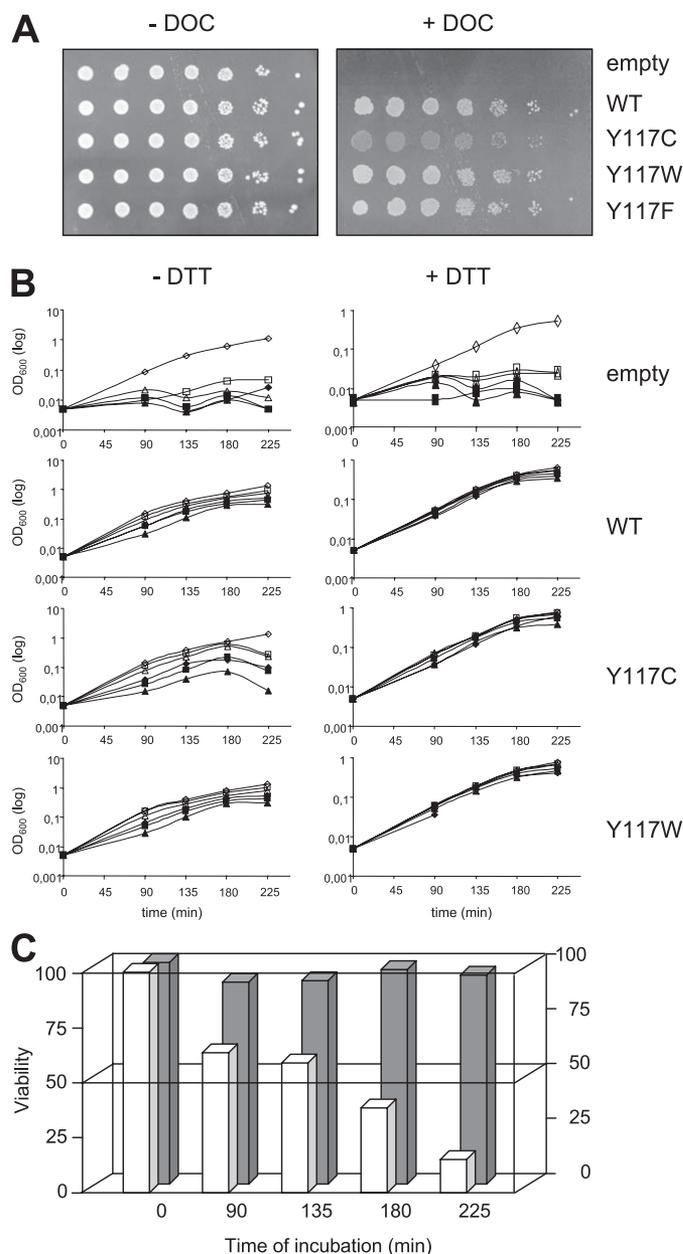


FIGURE 3. Detergent susceptibilities associated with covalent dimerization of the TolR C-terminal domain. A, 2 μ l of 10-fold dilution of normalized cultures were spotted on LB plates or LB plates supplemented with 1% DOC (*tolQR* pUC12 (empty); *tolQR* pTPS304 (WT); or *tolQR* pTPS304 carrying the indicated mutation (Y117C, Y117W, and Y117F)). B, growth curves of the strain indicated in the absence (*left*) or presence (*right*) of DTT, with various concentrations of SDS (\diamond , no SDS; \square , 0.1%; \triangle , 0.25%; \blacklozenge , 0.5%; \blacksquare , 1%; \blacktriangle , 2%). The Y117F mutant is not represented but gave similar results as Y117W. C, viability of TolR_{Y117C}-expressing cells represented as percentages of colony-forming units of TolR_{Y117C}-expressing cells versus colony-forming units of TolR_{WT}-expressing cells after incubation in LB growth medium (*background* dark bars) or in 0.5% SDS-containing LB (*foreground* white bars) during the time indicated.

Overall, these data suggest that the TolR Tyr¹¹⁷ residue is not involved in the TolQR channel activities but rather that the specific Y117C mutation blocks conformational modifications of the TolQR complex. This further suggests that a conformationally dynamic TolQR complex, if essential for OM stability, is not required for colicin translocation.

Accessibility of Cysteine Residues—To gain information about residues buried or accessible to solvent, we treated cells

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with the cysteine-reactive MPB probe. As a control, samples were treated with the nonbiotinylated cysteine-reactive derivative NEM prior to MPB treatment. Fig. 4 shows that TolR residues P116C, D118C, S129C, K133C, Q140C, P141C, and I142C and to a lesser extent E119C, I121C, K122C, N125C, H128C, S134C, G136C, L137C, and M138C are labeled by the biotinylated reagent (*middle*). As expected, NEM-treated samples did not display any labeling (Fig. 4, *top*).

Conformational Changes Depending on Proton-motive Force Monitored by Cysteine Accessibility—Accessibility experiments were repeated after depletion of the cell energy. PMF was collapsed by the addition of the uncoupler carbonyl CCCP prior to labeling using the Cys-reactive MPB probe (Fig. 4, *bottom*).

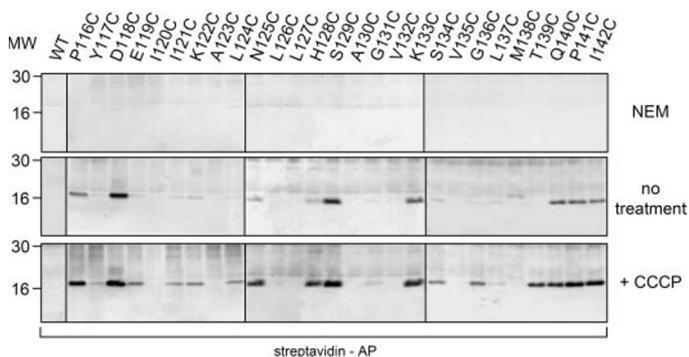


FIGURE 4. Solvent accessibility of introduced cysteine residues is responsive to PMF. Cysteine accessibility was monitored by *in vivo* treatment of 3×10^9 cells with the cysteine-specific biotinylated MPB probe (no treatment; *middle*), after pretreatment with NEM (*top*) or CCCP (*bottom*) as described under "Experimental Procedures." Native and mutant forms of TolR were isolated by immunoprecipitation using anti-TolR pAb and analyzed for MPB labeling by streptavidin-alkaline phosphatase detection. Molecular weight markers (MW) are indicated on the left.

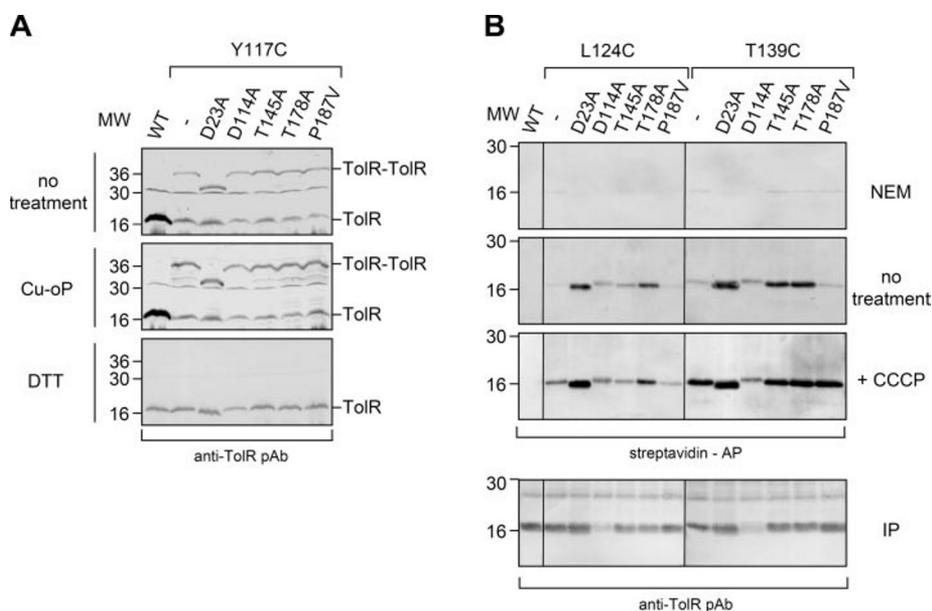


FIGURE 5. Solvent accessibility of TolR L124C and T139C is responsive to ion pathway residues. *A*, effect of mutation of ion pathway residues for TolR Y117C dimer formation. Cells expressing the TolR Y117C mutant in combination with the TolR D23A or D114A or the TolQ T145A, T178A, or P187V substitutions were treated as described in the legend to Fig. 2. MW, molecular weight. *B*, effect of mutation of ion pathway residues for TolR L124C and T139C solvent accessibility. Cells expressing TolR L124C or T139C mutant in combination with the TolR D23A or D114A or the TolQ T145A, T178A, or P187V substitutions were treated as described in the legend to Fig. 4. Anti-TolR detection of immunoprecipitated material (IP) is shown in the lower panel.

TolR L124C, N125C, H128C, G136C, and T139C mutants showed a higher level of labeling in the absence of PMF, whereas a slight increase was observed in the case of TolR E119C, I121C, K122C, and S134C proteins. Overall, this suggests that the TolR C-terminal sequence conformation is responsive to PMF and that solvent accessibility increase occurs for some residues when cells are depleted of energy.

Conformational Changes Depending on Ion Channel Residues Monitored by Cysteine Accessibility—To determine whether this conformational change is depending upon ion transit through the putative TolQR ion channel, we constructed double mutants affected in one residue of the ion pathway and with a cysteine residue at position Tyr¹¹⁷, Leu¹²⁴, or Thr¹³⁹. Four mutations within TolQ and TolR TMHs have been described to affect ion transit (TolR Asp²³, TolQ TMH2 Thr¹⁴⁵, TolQ TMH3 Thr¹⁷⁸ and Pro¹⁸⁷) (5). The aspartate residue located at the beginning of the TolR C terminus (Asp¹¹⁴) and conserved among TolR and ExbD proteins (see Fig. 1, *A* and *B*) has also been proposed to be specifically involved in the energy-dependent TolQR channel activity, since its substitution for an alanine residue results in a TolR protein nonfunctional for OM stability but still functional for colicin uptake (see Table 1). The different combinations were tested for dimerization (Y117C mutants) or accessibility to solvent (L124C and T139C). We showed that none of the secondary mutations affected the dimerization of Y117C TolR proteins (Fig. 5*A*). Mutation of the TolR Asp²³ and Asp¹¹⁴ and TolQ Thr¹⁴⁵ and Thr¹⁷⁸ residues affected the accessibility of L124C and T139C (Fig. 5*B*). The accessibility observed was similar to the accessibility obtained in energy-depleted cells, suggesting that ion binding to these residues regulates the conformation of the TolR C-terminal domain in response to membrane potential. Mutation of the TolQ Pro¹⁸⁷ residue has no effect on the accessibility of the TolR L124C or T139C mutants.

DISCUSSION

Molecular motors using proton or ion-motive force for their activities must be regulated at different levels, such as (i) the selectivity (*e.g.* the choice of the ion to be transported) and (ii) the ion flow (*e.g.* the controlled entry of the ion in the pathway). By controlling the ion flow, this checkpoint prevents the ion channel from being converted as a diffusion channel, which will depolarize the membrane and kill the cell. This type of mechanism has been well characterized for the voltage-dependent K⁺ channel Kv1.6 (22). In this case, one of the extracellular domains forms a penetrating loop going deep into the channel and regulating both ion flow and selectivity (23, 24). In the TolQR complex, a candidate for such a

function is the C-terminal domain of the TolR protein, which was studied in this work using cysteine cross-linking and accessibility. First, this domain is essential for TolR function, since a construct expressing TolR deleted of this domain (TolR1-2) does not complement a *tolR* null mutant (9). Second, fractionation experiments showed that the periplasmic domains (TolR2-3) as well as the TolR C-terminal (TolR3) but not the central (TolR2) domain produced in the periplasm of WT cells associate peripherally with the membrane (9). Although an α -helix, which may be responsible for this membrane association, is predicted in this domain (see Fig. 1B), it does not form a TMH, as demonstrated using topology (7, 8) and cysteine accessibility (residues located at the C terminus of this α -helix are labeled with MPB, a probe unable to cross the inner membrane (this work)) experiments. Third, suppressive mutations of TolQ TMH3 residues Ala¹⁷⁷ and Thr¹⁷⁸ mutants have been identified in this domain (Thr¹³⁹ and Tyr¹¹⁷, respectively), suggesting that the C-terminal domain of TolR is in close contact with TolQ TMH3 (5, 11). Finally, it has been shown that the nonfunctional ExbD D25N mutation might be rescued by the deletion of the ExbD C terminus (25). Recently, Hosking *et al.* (26) demonstrated that a periplasmic segment of the MotB protein is probably involved in blocking access to the MotAB ion channel, before the incorporation of the MotAB complex into the flagellar stator. This sequence is not conserved within the TolR or ExbD proteins, suggesting a specific role during flagella assembly. However, if the TolR C-terminal domain is responsible for selectivity or ion flow regulation, one can hypothesize that it will undergo structural transitions. Abergel *et al.* (27) obtained diffracting crystals of the periplasmic region of TolR. However, this structure has not yet been reported probably due to conformational flexibilities. To gain information about the TolR C-terminal periplasmic domain and its hypothesized conformational modifications, we performed a cysteine-scanning study. This work provides significant advances for structural information, such as dimer formation or conformational modification.

First, we used a biotinylated maleimide probe, which reacts with solvent-accessible sulfhydryl groups. Labeled cysteines within the putative α -helix are periodically spaced, suggesting that one side of the helix might face the solvent. This side of the α -helix is composed of hydrophilic or polar residues in all TolR proteins, whereas the inaccessible residues locate on the opposite "hydrophobic" face (see Figs. 1B and 6B). This suggests that this side of the α -helix structure is either buried in a hydrophobic core or in close contact with the membrane (Fig. 6, B and C).

In the extreme C-terminal segment of TolR, accessibility does not seem to follow a periodical pattern. However, positions of hydrophilic or neutral residues are labeled, whereas positions of hydrophobic residues are not accessible (see Fig. 6A). This and secondary structure predictions suggest that this C-terminal segment may form a globular structure, with hydrophilic residues at the surface and hydrophobic residues buried in the three-dimensional structure. This structure may form a plug into the putative TolQR ion channel, since it is protected from the action of carboxypeptidase (11), and suppressive mutation of the TolQ TMH3 A177V mutant has been previously mapped into this segment (T139M, respectively) (11). In

this region, many positions displayed a different pattern of labeling in response to proton-motive force and ion pathway residues (see below), suggesting that this segment undergoes structural modifications during the energy-converting process. It is tempting to hypothesize that this putative plug might control opening and/or closing of the TolQR channel. Pairwise combinations with TolQ TMH cysteine substitutions are in progress to determine the localization of this segment into the TolQR complex.

Second, our results suggest that the extreme C terminus of TolR (from Lys¹³³ to Ile¹⁴²) and three residues at the beginning of the putative helical secondary structure (Tyr¹¹⁷, Ile¹²¹, and Leu¹²⁴) are determinants of TolR-TolR interaction. We observed that the Tyr¹¹⁷ residue is a strong determinant of dimerization, since its replacement by a cysteine residue allows formation of a cysteine-bound homodimer. The other combinations allow formation of Cys-bound homodimers upon oxidation with the catalyst copper(II) *ortho*-phenanthroline. Although the property of the C-terminal domain of TolR to dimerize has been previously demonstrated (9), our study showed that the amphipathic α -helix is involved in this process. These positions are found periodically, suggesting that one face of the α -helix is involved in dimerization. Because the dimerization interface is situated between the hydrophilic and the hydrophobic sides of this helix, it suggests that both α -helices associate as a head-to-tail dimer (see Fig. 6, B and C). However, because of the pattern of oxidative cross-linking was restricted to the N terminus of the helix, we further propose that the two Tyr¹¹⁷ residues face each other, and, as a consequence, dimer formation involves only the N-terminal segments of both helices (see Fig. 6C).

Maleimide accessibilities in energized and de-energized conditions demonstrated different labeling patterns, showing that the TolR C-terminal domain undergoes structural modifications. This is dependent upon proton-motive force and residues within the ion pathway, except TolQ Pro¹⁸⁷. One can hypothesize that binding or release of ions or protons from these specific residues controls conformational modifications in the TolR C-terminal domain, hence resulting in distinct localization of the putative plug. One good candidate for this sensing function is the TolR Asp¹¹⁴ residue. This study showed that Asp¹¹⁴ is a critical residue for ion or proton conduction. We suggest that it can bind ions or protons at the entrance of the TolQR TM channel, its ionization state modulating TolR C-terminal conformation. The role of the TolQ Pro¹⁸⁷ residue might be to regulate TolQ conformation in response to ion or proton binding. However, this TolR molecular motion is probably responsible for dimerization of Y117C mutant, since its formation is time-dependent. We propose that TolR alternates in two different conformations, one of which allows close contacts of the two Tyr¹¹⁷ residues. Because mutations within the ion pathway do not alter formation of TolR_{Y117C} dimers, one can suggest that dimer formation occurs in the nonenergized form of the complex. Phenotypically, formation of the TolR_{Y117C} covalent dimer abolishes OM stability, whereas it has no effect on colicin import. Because of the phenotype of the Y117C (class III) and Y117W and Y117F (class I) substitutions, we concluded that the TolR C-terminal conformational flexibility is necessary

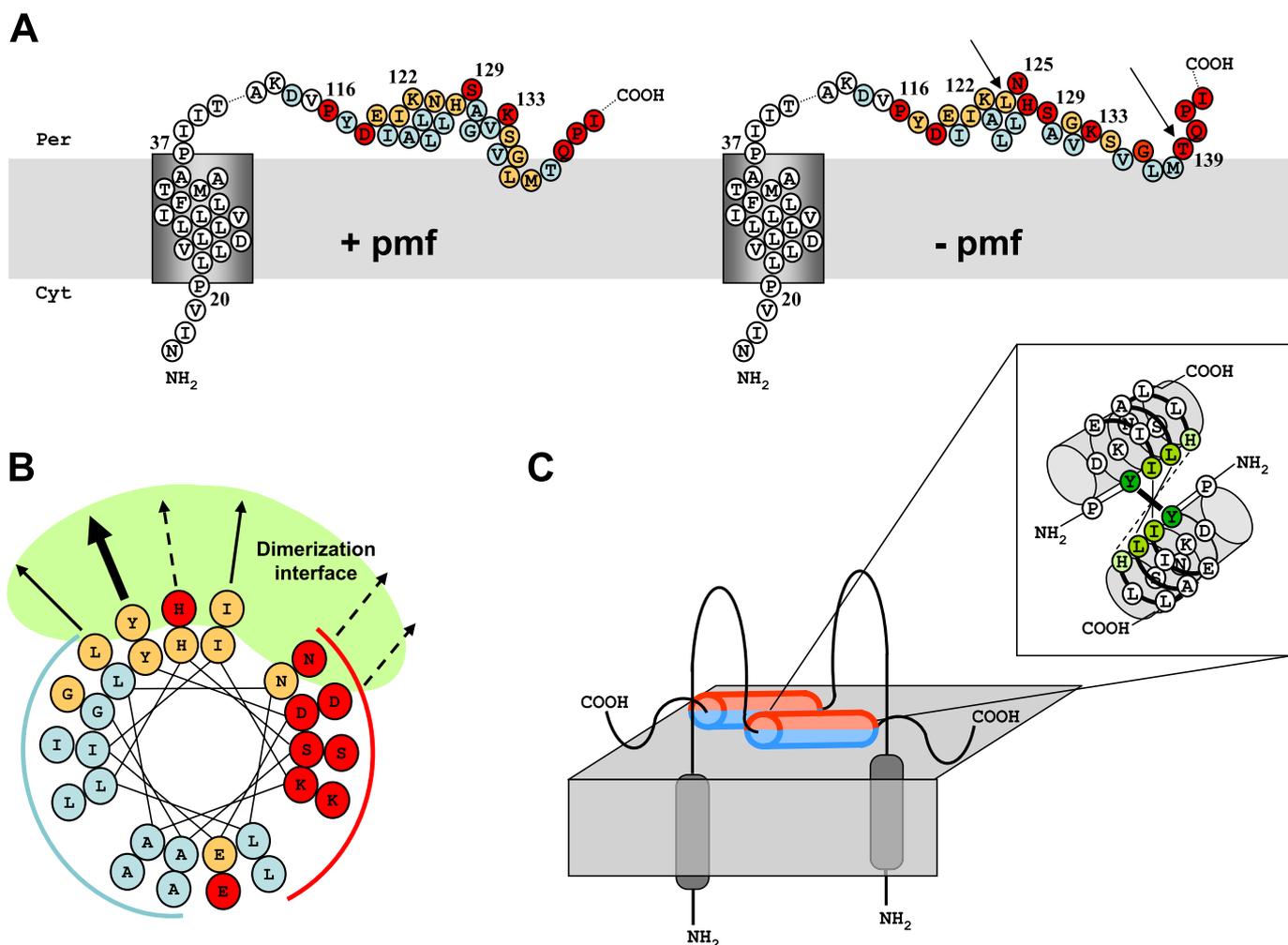


FIGURE 6. Models for TolR C-terminal domain. *A*, lateral views of TolR domains I (TMH) and III (the central domain II is represented by a *dashed line*) in the presence (*left representation*) and absence (*right representation*) of proton-motive force (*pmf*). This model is deduced from the cysteine accessibility analyses and predictions of secondary structures using computer algorithms. Cysteine substitution mutations highly labeled (*red circles*), faintly labeled (*orange circles*), or unlabeled (*light blue circles*) by *in vivo* treatment of whole cells with MPB are represented. The two main accessibility modifications observed are indicated by *arrows*. *Cyt*, cytoplasmic side; *Per*, periplasmic side. *B*, helical wheel projection of the predicted amphipathic α -helix of the TolR C terminus (residues Tyr¹¹⁷–Gly¹³¹) with a summary of cysteine accessibility experiments in the presence (*inner ring*) or absence (*outer ring*) of proton-motive force. The *color code* is identical to that of *A*. The hydrophobic (*light blue arc*) and hydrophilic (*red arc*) faces of the helix and residues involved in TolR C terminus homodimerization are indicated (*thick arrow*, dimerization in normal growth conditions; *regular arrow*, dimerization induced by oxidative treatment; *dashed arrow*, weak dimerization in the presence of oxidative treatment). The dimerization interface is represented by the *light green area*. *C*, model for organization of the TolR dimer in the membrane, representing the C-terminal helices as a head-to-tail dimer, with the hydrophobic face (*light blue*) in contact with the membrane and the hydrophilic side (*red*) facing the periplasm. The dimerization results obtained in this study are indicated in the *inset*, showing that the head-to-tail α -helices interact through their N terminus (*dark green circles* and *thick line*; residue dimerizing in normal conditions; *green circles* and *regular lines*, residues dimerizing in oxidative conditions; *light green circles* and *dashed line*, residue faintly dimerizing in oxidative conditions; *white circles*, no dimerization observed in normal or oxidative conditions).

for the energy-dependent outer membrane stability process, whereas it is not required for the energy-independent group A colicin import. In a time behavior manner, we hypothesized that the TolQR_{Y117C} population stably maintains OM organization until the population of dynamically active TolR_{Y117C} proteins is not sufficient to assume its function. This was confirmed by growth cultures of WT and mutant cells in the presence of detergent. In the absence of reducing agent, TolR_{Y117C}-expressing cells were grown until late exponential phase and then partially lysed, whereas this is not observed when cells were grown in the presence of DTT. None of these phenotypes have been observed with TolR_{WT}, TolR_{Y117F}, or TolR_{Y117W}-expressing cells, demonstrating the correlation between TolR dimer formation and TolQR complex dysfunction. However,

monomeric forms of TolR_{Y117C} are still present in untreated cells (see Fig. 2), a result that may explain the partial cell lyses and the intermediate level of the (energy-dependent) group B colicin uptake (see Table 1). On the other hand, TolR dimer formation had no effect on group A colicin import, suggesting that a dynamic TolQR complex is not a requisite for group A colicin transport. This correlates with the fact that colicins A and E1 do not require proton-motive force to enter and kill the bacterial cell (19).

All of the data reported herein demonstrate that the TolR C-terminal domain is a dynamic structure, responsive to the proton-motive force and the transit of ion or protons within the TolQR channel and further suggest a role of the TolR C-terminal domain in regulating activity of the TolQR channel. This

differs from the role of the periplasmic segment of MotB, called plug, which has been proposed to block the channel before its incorporation within the flagella supramolecular structure (26). However, a conserved amphipathic α -helix present within the MotB periplasmic domain (residues 170–182) is located between the plug (residues 53–64) and the peptidoglycan binding motif (residues 210–225) and shares residue conservation with the TolR or ExbD C-terminal α -helix. Interestingly, suppressive mutations of MotA TMH4 (homologous to the TolQ or ExbB TMH3) have been isolated in the MotB amphipathic α -helix (28, 29). Whether the TolR C-terminal domain forms a penetrating loop, a plug, or a surface structure at the periplasmic side of the TolQR complex, regulating ion flow or opening/closing of the channel, is currently under consideration in our laboratory.

Acknowledgments—We thank Denis Duché, Laure Journet, and Stéphanie Pommier for critical reading of the manuscript and Aurélie Barnéoud-Arnoulet and Rémi Thomé for corrections. We thank Colin Kleanthous, Dominique Mengin-Lecreulx, and Denis Duché for purified colicins E9, M and Ia, and D, respectively; Valérie Prima and Martine Chartier for DNA sequencing; and Marthe Gavioli and Olivier Uderso for technical assistance.

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