The TolQ–TolR proteins energize TolA and share homologies with the flagellar motor proteins MotA–MotB

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Summary
The Tol–Pal system of Escherichia coli is required for the maintenance of outer membrane stability. Recently, proton motive force (pmf) has been found to be necessary for the co-precipitation of the outer membrane lipoprotein Pal with the inner membrane TolA protein, indicating that the Tol–Pal system forms a transmembrane link in which TolA is energized. In this study, we show that both TolQ and TolR proteins are essential for the TolA–Pal interaction. A point mutation within the third transmembrane (TM) segment of TolQ was found to affect the TolA–Pal interaction strongly, whereas suppressor mutations within the TM segment of TolR restored this interaction. Modifying the Asp residue within the TM region of TolR indicated that an acidic residue was important for the pmf-dependent interaction of TolA with Pal and outer membrane stabilization. Analysis of sequence alignments of TolQ and TolR homologues from numerous Gram-negative bacterial genomes, together with analyses of the different tolQ–tolR mutants, revealed that the TM domains of TolQ and TolR present structural and functional homologies not only to ExbB and ExbD of the TonB system but also with MotA and MotB of the flagellar motor. The function of these three systems, as ion potential-driven molecular motors, is discussed

Introduction
A large number of important biological processes are coupled, directly or indirectly, to transmembrane ion potentials, as originally hypothesized by Mitchell (1976). In bacterial cells, a number of energetic processes are coupled to such potentials directly; the most well known are the $F_1F_0$ ATP-synthetase and electron transport processes. The flagellar motor, some inner membrane transport systems and a few outer membrane transport systems also use this energy source. In this article, we consider three different systems that use transmembrane ion potentials; the different components of these systems are schematically illustrated in Fig. 1.

The TonB and the Tol–Pal systems are both able to couple the cytoplasmic membrane proton gradient to energy-requiring processes (Braun, 1995; Cascales et al., 2000) and thus energize active transport across the outer membrane and maintain cell envelope integrity respectively. The iron siderophore and cobalamin uptake systems involve an outer membrane receptor, such as FhuA, FepA or BtuB, that normally forms a closed channel that can be opened in an energy-dependent process involving interaction between the N-terminal globular domain of the receptor and the energized C-terminal domain of the TonB protein (Skare et al., 1993; Moeck et al., 1997; Cadieux and Kadner, 1999). The function of the TonB protein depends on the proton motive force (pmf) and a complex of three proteins found in the inner membrane, TonB, ExbB and ExbD, which are necessary to drive energy-dependent uptake through the bacterial outer membrane (for reviews, see Klebba and Newton, 1998; Moeck and Coulton, 1998). Analogous to the TonB system, the Tol–Pal system contains three proteins that interact with each other within the cytoplasmic membrane, TolQ, TolR and TolA (Derouiche et al., 1995; Lazzaroni et al., 1995; Germon et al., 1998; Journet et al., 1999). TolQ and TolR are orthologous to ExbB and ExbD, respectively, whereas TolA and TonB are homologous. Absent in the TonB system, the periplasmic TolB protein and the outer membrane-anchored Pal lipoprotein interact with each other (Bouveret et al., 1995). TolB was also found to interact in vivo with the major outer membrane lipoprotein (Lpp) and OmpA in a Pal-dependent manner (Clavel et al., 1998). The TolA protein is involved in a wide variety of interactions, notably with Pal (Cascales et al., 2000) and TolB (A. Walburger, C. Lazdunski and Corda, in preparation), whereas TolA and TolB are both able to interact in vitro with outer membrane porins (Derouiche et al., 1996; Rigal et al., 1997). The Tol–Pal system, which is found in a wide variety of Gram negative bacteria (Sturgis, 2001), is required for the maintenance of outer membrane stability; it is therefore of interest to study the molecular mechanisms that control and energize the interaction of TolA with the outer membrane lipoprotein Pal.

This study demonstrates that both TolQ and TolR proteins are essential for the TolA–Pal interaction. A point mutation within the third TM segment of TolQ was identified that strongly affected this interaction, whereas a suppressor mutation within the TM region of TolR restored it. These results indicate that the TM domain of TolQ is necessary for the pmf-dependent interaction of TolA with Pal and outer membrane stability. Analysis of the TM domain of TolR revealed that an acidic residue is important for this interaction.

Analysis of sequence alignments of TolQ and TolR homologues from numerous Gram-negative bacterial genomes, together with analyses of the different tolQ–tolR mutants, revealed that the TM domains of TolQ and TolR present structural and functional homologies not only to ExbB and ExbD of the TonB system but also with MotA and MotB of the flagellar motor. The function of these three systems, as ion potential-driven molecular motors, is discussed in the introduction.
membrane stability (Bernadac et al., 1998) and appears to be important for the export of cell envelope components (Gaspar et al., 2000). It is noteworthy that both TonB and Tol–Pal systems have been parasitized and are required for the import through the cell envelope of both colicins and phage DNA (Braun, 1995; Lazdunski et al., 1998).

Relatively little is known about the details of energy coupling in either the TonB or the Tol–Pal systems of Escherichia coli, although studies on the TonB system are the most advanced. It appears that conformational changes in TonB are driven by the cytoplasmic membrane pmf, ExbB, and periplasmic interactions with receptors such as FepA (Larsen et al., 1999). The pmf-dependent interaction probably occurs through interactions between TonB and the ExbB transmembrane (TM) segments (Larsen et al., 1994; 1999) and/or the ExbD TM anchors (Higgs et al., 1998). Within ExbD, two residues have been found to be particularly important for function: an N-terminal aspartate (Asp-25) and a C-terminal leucine (Leu-132) (Braun et al., 1996). Upon TolA overproduction, the Tol–Pal system was found to stabilize the outer membrane integrity of cells devoid Lpp through the TolA–Pal interaction. The pmf has been shown to be necessary for the interaction between the C-terminal periplasmic domain of the cytoplasmic membrane-anchored TolA protein and the outer membrane Pal lipoprotein (Cascales et al., 2000). Recently, energy-dependent conformational changes in TolA have been shown to depend on the TM segment of TolA and of TolQ and TolR proteins (Germon et al., 2001).

At first sight, these two systems have little in common with the flagellar motor other than the use of a transmembrane electrochemical gradient as an energy source. The bacterial flagellum is a complex macromolecular assemblage forming a multipartite structure composed of a long helical propeller, a flexible hook region and a rotary motor in the bacterial cytoplasmic membrane (for reviews, see Blair, 1995; DeRosier, 1998). The torque generation depends on the operation of a number of motors composed of the two proteins MotA and MotB. These proteins are believed to form the stator of the motor, arranged around the periphery of the flagellar basal body, which constitutes the rotor of the motor, and interacting specifically with the protein FlIG, one of the subunits that form this basal body (Zhou et al., 1998a). The suggested mechanism is that protons traverse the membrane through a ‘pore’ formed by the MotA and MotB proteins. This ‘pore’ involves, in particular, a conserved aspartate residue of MotB (Asp-32) (Zhou et al., 1998b). Two conserved proline residues of MotA (Pro-173 and Pro-222) (Braun et al., 1999) are also particularly important in torque generation. On the basis of these studies, a model has been proposed (Braun et al., 1999) in which the first proline senses a conformational state and gates proton uptake by the aspartate residue from the periplasm. The proton uptake causes a change that either permits (via a Brownian ratchet) or drives rotation, a step involving the second proline residue. Finally, proton release into the cytoplasm restores the motor to its initial state.

In this study, the role of the TolQ–R proteins was investigated in relation to the pmf-dependent TolA–Pal interaction. An examination of homologues to the TolQ–R proteins found not only the ExbB–D proteins, as was previously well known (Eick-Helmerich and Braun, 1989), but also the MotA–B proteins, as mentioned recently by Chang et al. (2001). The implications of the homologies found between the TM domains of TolQ–R, ExbB–D and MotA–B, together with their estimated stoichiometries, is discussed in the context of a model for the mechanism of the pmf-dependent function of TolQ–R–A.

Results

TolQ and TolR are both necessary to energize TolA: effect of TolQ on TolR stability

In a previous study, using in vivo cross-linking followed by

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TolA immunoprecipitation, the overexpression of TolQ and TolR with TolA was found to improve the co-precipitation levels of Pal (Cascales et al., 2000). Taken together with the sequence similarities found between E. coli TolQ–R and ExbB–D and the implication of ExbB–D in pmf-coupled TonB function (Braun et al., 1996; Larsen et al., 1999), this suggests that the TolQ and TolR proteins might be important for the energy-dependent cross-linking of TolA with Pal. To test this hypothesis, we investigated the in vivo interaction of Pal with TolA in a variety of strains overexpressing TolA. The co-precipitation of Pal by anti-TolA antibodies was investigated after in vivo cross-linking in parental GM1, tolQ (TPS13) and tolQ–exbB (KP1038) strains harbouring the pTolA plasmid (Fig. 2A). It was shown that the tolQ mutation has a strong polar effect on the expression of tolR (Vianney et al., 1996) and, similarly, the exbB::Tn10 insertion affects the expression of exbD (Braun and Herrmann, 1993; Larsen et al., 1999). We clearly observed the pmf-dependent co-precipitation of Pal in GM1, whereas in the presence of CCCP or in the absence of TolQ–R or both protein pairs, TolQ–R and ExbB–D, similar background levels of Pal were detected. The similarity of these levels and the controls of the immunoprecipitation of TolA allowed us to conclude that the chromosomal expression of ExbB–D is unable to drive the TolA–Pal interaction. A tolB strain was also analysed by the same technique and indicated that TolB was not required for the TolA–Pal interaction (not shown). These results indicate that, under chromosomal expression, TolQ–R are able to drive the co-precipitation of Pal with overproduced TolA after in vivo cross-linking, whereas ExbB–D are not. Thus, these results contrast with the cross-talk observed with the TonB system, in which the chromosomal expression of TolQ–R confers TonB activity (Larsen et al., 1999).

Because of the genetic organization of the tol–pal cluster in two operons, we decided to used a K-12 strain deleted for the first operon (C600tolQRA). In preliminary experiments, the plasmid pQRA encoding the TolQ–R–A proteins was shown to restore the outer membrane integrity of this strain, whereas all the phenotypic outer membrane defects were observed with tolQRA (C600tolQRA pT7.5), tolQ (C600tolQRA pRA) and tolR (C600tolQRA pQA) mutants (Table 1). The requirement of both TolQ and TolR proteins for the co-precipitation of Pal with TolA after in vivo cross-linking is clearly demonstrated in Fig. 2B. In the presence of 10 μM CCCP or in the absence of either TolQ or TolR, Pal is not co-precipitated.

![Fig. 2. In vivo co-immunoprecipitation of Pal with TolA requires TolQ and TolR proteins.](image-url)
ToIR was also found to co-precipitate with TolA after in vivo cross-linking, irrespective of the presence or absence of CCCP or ToIQ. Moreover, the ToIQ–R and ToIR–A complexes, immunodetected previously (Derouiche et al., 1995), were still observed when the respective proteins were overproduced in cells treated or not with CCCP (not shown). These results show that the ToIQ and ToIR proteins are both necessary for the pmf-dependent interaction of TolA with Pal. In contrast, the pmf has no effect on the formation of the inner membrane TolA–Q and TolA–R complexes.

We wished to test whether stabilizing effects might occur between the ToIQ–R–A proteins. Thus, Western blot immunodetections of ToIR and TolA were performed with the cells described in Table 1 treated with spectinomycin for long periods (up to 8 h). The main result observed is that ToIR stability requires the presence of ToIQ, as after only 2 h, its level decreases significantly. However, ToIA stability is not influenced by the absence of either ToIQ or ToIR (Fig. 3). Thus, it appears that ToIA–Q and TolA–R interactions do not interfere with TolIA stability, whereas ToIQ–R interactions may stabilize ToIR.

ToIQ–R and ExbB–D show topological and sequence homologies with the MotA–B proteins

During previous analyses of the tol–pal clusters of bacterial genomes (Sturgis, 2001), the homologies between the TM segments of ToIQ–R and ExbB–D were found to extend to the MotA–B proteins. In order to examine and understand better the relationship between the three different pairs of proteins, ToIQ–R, ExbB–D and MotA–B, a comparative analysis of their protein sequences was undertaken. This analysis brought out a number of interesting points. First, the ToIQ–R and ExbB–D proteins as a whole are not well conserved. Within this context of little conservation, there are nevertheless a number of regions that show much stronger conservation, notably the TM helices of the different proteins and the C-terminal regions of ToIQ and ExbD.

Similarities were evident between the first TM helix of ToIQ proteins and those of ExbB proteins, and similarly between the C-terminal regions of ToIR and ExbD. Most importantly, there was a strong similarity between the C-terminal helical hairpins of ToIQ, ExbB and MotA and also between the N-terminal TM helices of ToIR, ExbD and MotB. The relevance of these sequence alignments is difficult to quantify, depending on a large alignment of a relatively large number of proteins from a wide selection of organisms distributed in a rather non-random fashion across the phylogenetic space. Thus, to provide a visual and comprehensible summary of the information, we show a series of consensus sequences for the conserved TM helices derived from these alignments (Fig. 4).

The consensus sequences show that the ToIQ proteins are more strongly conserved than the ExbB proteins. The consensus derived for the C-terminal helical hairpin of ToIQ, ExbB and MotA proteins (Fig. 4B) indicates that this region of the protein is particularly strongly conserved, arguing strongly for an important functional and/or structural role. Interestingly, in the MotA sequences, this region is less conserved, despite its presumed functional importance in the formation of an ion pore. Comparing the N-terminal regions of the ToIQ, ExbD and MotB proteins gives strong consensus sequences and, as in ToIQ hairpin, it is possible to observe a homology between the different groups of sequences, in particular the aspartate present in the overall 90% consensus sequence (Fig. 4C). This residue has previously been found to play a major role in both TonB function (Braun et al., 1996) and cell motility (Zhou et al., 1998).

The Asp residue within the ToIR TM domain has an important role in the TolA pmf-dependent interaction and outer membrane integrity

In order to determine the importance of the Asp-23 residue of ToIR, site-directed mutagenesis was performed to change it to an Ala, Arg or Glu residue. Thus, the pQRD23A plasmids expressing the TolO D23A, D23R or D23E mutations were analysed in C600 tolQR for their effect in the co-precipitations of Pal with TolA after in vivo cross-linking (Fig. 5). Efficient Pal co-purification was observed with the D23E mutation, an intermediate level with the D23A mutation, and no co-purification of Pal was

Table 1. Strain characteristics.

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<thead>
<tr>
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<th>SDS</th>
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<td>&gt;200</td>
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<td>+</td>
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<td>+</td>
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<td>0</td>
<td>1.7</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

a. Amounts of outer membrane vesicles observed by electron microscopy after negative staining of cells grown on agar plate (classified according to Bernadac et al., 1998); ++, many vesicles on all cells; +, some vesicles on most cells; 0, no vesicles on most cells.

b. Rnase I leakage; ++ and + indicate that the clear zone of RNA hydrolysis was larger or lower than the size of the cell colony respectively (0, no leakage).

c. SDS (% w/v) and vancomycin (µg ml⁻¹) values corresponding to a 50% decrease in cell turbidity measured after 3 h of culture (average values from duplicate experiments, ±20%).

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Precipitated with wild-type TolR. As with TolR wt, CCCP abolished the TolA–Pal interaction (not shown). In contrast, each of the TolR-D23 proteins was found to be detectable with the D23R mutation. For the D23A replacement with an alanine residue does not completely abolish the TolA–Pal interaction, gave a phenotype similar to that observed in a tolQ deletion. In contrast, the D23E mutation restored almost completely cellular integrity. In agreement with the intermediate level of Pal co-precipitation, the D23A mutation partially complemented the outer membrane defects. These results indicate that the Asp residue, which was found to be important for the function of the TonB and Mot systems, also has an important role within the Tol–Pal system. Nevertheless, this residue can be replaced by another acidic residue without affecting either the TolA–Pal interaction or outer membrane integrity, but its replacement with an alanine residue does not completely abolish the TolA–Pal interaction.

Possible interactions between the TolQ-II–III–TolR transmembrane helices and their equivalents in the ExbB–D and MotA–B proteins

In order to obtain a picture of the possible ion pore formed by the TM helices 2 and 3 of TolQ and the helix from TolR, or their equivalents from either ExbB–D or MotA–B, we have examined the pattern of sequence conservation of these proteins together with the conserved residues found to be important for the pmf-dependent interaction. The helical wheel diagrams for these TM helices are indicated with three different levels of consensus, within Tol proteins (outer ring), within Tol and Exb-like proteins (middle ring) and, finally, within all three groups (inner ring) (Fig. 6A). The logic behind these levels is that residues conserved among TolQ–R but not elsewhere could be implicated in interactions with either TolA or the first TM segment of TolQ, and sites involved in internal interactions within a TolQ–TolR complex should be the most conserved across the whole group.

Figure 6A shows that there are surfaces of the TolQ TM helices (TolQ-II and TolQ-III) that are strongly conserved even with the MotA helices; these are marked by arcs. These surfaces regroup strongly conserved small residues, notably four glycines and three alanines. This conservation and pair of sequences would seem to be compatible with a tight helix–helix interface within a helical hairpin. Of particular interest in this observation is the occurrence within this conserved pattern of a GxxxG motif, which has been proposed to be important for such interactions (Senes et al., 2000).

In contrast, the pattern of conservation in the TolR helix is relatively homogeneous. There is no patch that is particularly better conserved than another, and there is reasonably strong conservation of the consensus sequence all around the helix. This distribution would tend to suggest that the TolR TM helix is completely surrounded by proteins, and so the conservation of protein–protein interactions affects all positions similarly.

A proposed structural alignment of the TolQ-II–III–TolR helices is shown in Fig. 6B. It has been shown by a suppressor study (Lazzaroni et al., 1995) that there is a possible interaction between the TolQ-III and TolR helices, specifically the Ala residue marked with a solid arrowhead in the TolQ-III helix (A177) and four residues in the TolR helix, three of which are shown in Fig. 6B by solid arrowheads (P20, A36, P37 and, not shown, S42). A similar interaction between a residue of the MotB helix, equivalent to the valine indicated with an open arrowhead in the TolR consensus and a glycine in the fourth TM helix of MotA, equivalent to the alanine marked with an open arrowhead in the TolQ-III consensus sequence has also been described (Garza et al., 1996). It should be remarked that TolQ–R and MotA–B bring out interactions between the same pair of TM helices and their homologues. The suppressor analysis also revealed two interactions:
between the extreme C-terminal end of TolR and the alanine marked within the TolQ-III TM segment, and between the TolQ-I (Leu-19) and the TolQ-III TM segments (Lazzaroni et al., 1995). Besides these interactions, TM interactions between TolA and TolQ-I (Germon et al., 1998), TonB and ExbB-I (Larsen et al., 1994) have also been demonstrated but will not be discussed further here. In order to show some correlation with the pmf-dependent interaction of the Tol–Pal system of E. coli, some of the tolQ–R suppressor mutants were analysed by examining the interaction of TolA with Pal.

Importance of the E. coli TolQ-III and TolR transmembrane helices in coupling pmf to TolA

To confirm the importance of the TM helices of TolQ-III and the TM helix of TolR in the transduction of energy from the pmf to TolA, we investigated the energy-dependent interaction of Pal with TolA in the presence of the TolQ_{A177V} mutant (tolQ2925) and when this mutation is suppressed by two different suppressor mutants: tolR_{203} (TolRS42N) and tolR_{207} (TolRP37L) (Lazzaroni et al., 1995). We observed that the alanine to valine point mutation in the TolQ-III TM helix significantly reduces the Pal co-precipitation by TolA, suggesting that this mutation limits the ability of TolQ to energize TolA. As described previously, using sample dilutions, the levels of Pal co-precipitations were estimated to be about 10–15% of Pal in C600tolQRA pQ925RA cells compared with C600tolQRA cells transformed with pT7.5 as a control and with pQRA plasmids expressing the D23A, D23R or D23E TolR mutations.
C600tolQRA pQRA cells. However, a normal level of co-precipitation is found when each suppressor (either TolR_S42N or TolR_P37L) is co-expressed (Fig. 5). As expected, no co-precipitation occurs with the suppressors in the presence of CCCP (not shown). Thus, the suppressor mutations restore the normal level of pmf-dependent interaction between TolA and Pal. The reduced ability of anti-TolA to co-precipitate Pal when TolQA177V is expressed suggests that pmf-dependent activation of TolA could be limiting for the function of the Tol–Pal apparatus (as also observed with TolR_D23A). Similarly to TolR-D23A mutations, TolQA177V and suppressor mutations do not modify the co-purification levels of TolR or TolR derivatives. It is noteworthy that this analysis suggests that these mutations affect the function but not the structure of the inner membrane complex. Furthermore, the membrane defects observed in tol mutants (Lazzaroni et al., 1995; Table 1) appear to be sensitive indicators of TolA activation and TolA–Pal interaction. These experimental results would thus seem consistent with the idea that the three conserved helices, TolQ-II, TolQ-III and TolR, or their equivalents in the Exb system form a TM ion pore, as has been proposed for the orthologous helices of the MotA–B system (Braun et al., 1999; van Way et al., 2000). These results also suggest a common mechanism of energy transduction in the three systems.

**Discussion**

*Outer membrane integrity is monitored by the TolA–Pal interaction level*

During the analyses of the membrane effects of tolQ–tolR point mutations on the TolA–Pal interaction, we observed different co-precipitation levels of Pal. Our results indicate that the TolA–Pal energy-dependent interaction is a sensitive indicator of the level of outer membrane integrity. The absence of TolA–Pal complex (tol deletion or TolRD23R), the presence of an intermediate level of complex (TolQA177V or TolRD23A) or the normal amount of complex (wild type, TolQA177V suppressor or TolRD23E) gave, respectively, strong, intermediate and no outer membrane defects. Is the function of the Tol–Pal system only required for the cell integrity? This seems not to be the case, as other processes such as the surface expression of O antigen (Gaspar et al., 2000) require the Tol proteins, indicating that the Tol–Pal system may also be involved in driving newly synthesized cell envelope components through the periplasm (Llobès et al., 2001). The interrelation of these different phenotypes is currently under investigation.

**TolQ and TolR transmembrane helices couple pmf to TolA**

In this study, we demonstrated that TolQ and TolR are both involved in the pmf-dependent activation of TolA and that residues within TolQ-III and TolR TM segments are important for this process. Considering the data presented in Figs 2, 5 and 7, both TolQ and TolR have been shown to be necessary for the transmission of energy from the TM anchor to the periplasmic domain of TolA. Our results agree with the observation of the conformational changes in TolA, which are dependent on TolQ, TolR and the TolA TM domain (Germon et al., 2001), and also suggest that TolA activation may drive its C-terminal domain to Pal (Cascales et al., 2000). This transmission can be interrupted by deletion of either TolQ or TolR or by point mutation in the TM region of TolR (D23R) or modified with
TolQ_{A177V} and TolR_{D23A}, which correspond to residues conserved with the MotA–B TM helices. Furthermore, suppressors of the TolQ_{A177V} point mutation located in the TM region of TolR (S42N and P37L) are able to restore energy transmission to the periplasm. These results demonstrate the importance of the TolQ and TolR proteins in energy transmission to TolA. Moreover, the addition of protonophores that inhibit the TolA–Pal interaction were never found to modify the TolA–R and TolA–Q interactions. Additional experiments indicate that the TolB protein of the tol–pal cluster is not required for energy transmission. These experiments are consistent with those reported for the TonB system, in which transport of Fe^{3+} siderophores is dependent on the presence of the ExbB–D proteins (Harle et al., 1995; Braun et al., 1996).

Thus, TolQ–R seem to transduce the pmf to TolA by a mechanism probably homologous to the transduction of pmf to TonB by ExbB–D. Analogy with models of the Mot system (Braun et al., 1999; van Way et al., 2000) and the suppressor studies (Lazzaroni et al., 1995; Garza et al., 1996) would suggest that an ion pore is formed by the last two TM helices of TolQ, the TolR TM helix and possibly the C-terminal domain of TolR, which is conserved between TolR and ExbD proteins. It is interesting that, although there is no sequence homology between the C-terminal domains of TolR and MotB, the latter is thought to play an important role in ion selectivity (Asai et al., 2000). The involvement of this hydrophilic C-terminal domain of TolR within the TM region is hard to envisage, although one possible solution would be a bilayer-penetrating loop, as is seen in the potassium channel structure (Doyle et al., 1998), which is responsible for forming the ion selectivity filter.

On the basis of the proposed interaction between the two TolQ TM helices, it is possible to trace a number of residues at the interface between these helices that might be involved in a potential ion channel (Fig 6). The conserved glutamate and alanine (solid arrowhead) of TolQ-III and the threonine in TolQ-II could form a near-vertical hydrophilic channel along the interface between the two helices. Below this possible channel would be found the strongly conserved proline residues. The alanine of the TolQ-III helix has already shown its importance, as it is this residue that is mutated in the tolQ925 mutant. By analogy with models of the MotAB motor, ion conductance by TolQR and ExbBD would be associated with changes in the relationship between these two proteins. With an important role for the aspartyl residue of the TolR TM helix, as has been inferred from the ExbDD25N (Braun et al., 1996) or MotBD32 (Zhou et al., 1998b) mutant phenotypes, this is hard to reconcile with our results on the TolR_{D23A} mutant and the presence of a threonine in this position in the ExbD sequence of Porphyromonas gingivalis.

**Structural predictions of the TolQRA complex**

The stoichiometry of the different components of the TolQ–R–A, ExbB–D–TonB and MotA–B systems is not well established. In the case of the Tol complex, a ratio of TolQ to TolR of about 2.7:1 has been determined using...

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**Fig. 7.** Suppressor mutations in the TM helix of TolR of the TolQ-III A177V mutation restore the energy-dependent interaction of Pal with TolA. See the legend to Fig. 2 for protocol, using C600tolQRA cells transformed with various pQRA plasmids encoding TolQ_{A177V} (tolQ925 mutant) with or without the TolRS42N (tolR203) or TolRP37L (tolR207) suppressor.

**Fig. 8.** Model for the arrangement of the TolQRA inner membrane complex. The various transmembrane helices and the C-terminus of TolR of the proposed TolQRA complex from the periplasmic side of the cytoplasmic membrane are shown. The four copies of TolQ are in light grey; the two copies of TolR are in medium grey; and TolA is in dark grey. The putative proton pores are shown in black. A possible reaction cycle coupling proton translocation to TolA activation is detailed in the text.

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T7-specific labelling and membrane fractionation (Guirard et al., 1994). Estimates in C600tolQRA cells harbouring pQRA and pGP1-2 plasmids after T7-specific labelling and membrane purification gave ratios of TolA to TolR and TolR to TolQ of around 1:2 and 1:3 respectively. In the absence of both T7 overexpression and specific labelling, the amounts of TolA–Q–R expressed from their natural promotor was estimated in KP1038 pQRA cells. Membranes were purified and proteins analysed by SDS–PAGE and autoradiography. Although it was not possible to distinguish TolA from other labelled proteins, TolQ–R were quantified giving a similar ratio of 3:1 (not shown). It thus seems unlikely that the motor apparatus is composed of a simple heterodimer of one TolQ and one TolR polypeptide, but rather a more complex assemblage is involved.

In the flagellar motor, estimates from E. coli gave a stoichiometry of four MotA/MotB (Wilson and Macnab, 1990), whereas more recent estimates in the PomA–B motor of Vibrio alginolyticus based on co-purification, protein fusion and cross-linking studies argue strongly for a ratio of 2:1 (Sato and Homma, 2000a,b; Yorimitsu et al., 2000). A related problem is the number of identical subunits that go into forming the structural unit. In the case of the MotA–B system, a recent purification of a reconstitutable channel suggests a structure containing at least PomA2PomB2 (Sato and Homma, 2000a). Recent studies in the Tol–Pal (Journet et al., 1999) and Exb–TonB (Higgs et al., 1998) systems have shown that TolR, ExbD and ExbB are able to form dimers and possibly trimers. The diversity of these findings highlights the difficulty in forming a coherent model, with complexes containing two or three TolR proteins and ratios of TolQ/TolR of 2–4 appearing to be in reasonable agreement with the information available. In a recent paper (Chang et al., 2001), a model for the Exb–TonB system was proposed containing one or two TonB and three copies each of ExbB and ExbD. Based on current evidence, we propose a model for the membrane-embedded portion of the complex (Fig. 8) with a TolQ/R ratio of 2 and an inner membrane unit containing TolQ2TolR3TolA1. Although this is the lower limit for a TolQ/R ratio, it results in a simpler model than higher ratios and would appear to be consistent with the results obtained with the Mot complex.

Figure 8 shows the periplasmic view of the TM parts of a TolQ–R proton-driven motor. In the diagram, the four TolQ proteins (pale grey) are arranged to form a ring (with $C_4$ symmetry) surrounding a TolR (medium grey) dimer with $C_2$ symmetry. This TolR–TolQ interaction breaks the symmetry of the larger external ring, resulting in two distinct types of TolQ environment. The four TolQ proteins each provide a putative proton conductance channel (black), shown at the interfaces of the TolQ-II and TolQ-III TM helices. The interaction of TolQ-I with TolQ-III (Lazzaroni et al., 1995) is represented. An interaction between the C-terminal extremity of the TolR protein with the TM region of TolQ-III (Lazzaroni et al., 1995) is illustrated ‘closing’ half the proton channels. As shown, the $C_4$ symmetry of the TolQ ring has been broken by the TolR proteins. This $C_2$ symmetry is in turn broken by the interaction with the TolA TM helix (dark grey), thus rendering each TolQ environment and TM channel different. The TolA helix is shown interacting with the TolQ-I helix, as has been inferred from suppressor studies (Germon et al., 1998) and by analogy with TonB, in which activity has been found to be dependent on the interaction of the TonB helix with ExbB-I (Larsen and Postle, 2001). The distribution of proteins also appears to be consistent with the majority of cross-linking, protein fusion and genetic suppression data (Larsen et al., 1994; Ahmer et al., 1995; Derouiche et al., 1995; Lazzaroni et al., 1995; Braun et al., 1996; Germon et al., 1998; Higgs et al., 1998; Journet et al., 1999; Sato and Homma, 2000a; Yorimitsu et al., 2000).

Such an arrangement poses a number of questions: does a proton traverse each of the symmetrically related channels to produce mechanical energy, or is one channel sufficient, or does the situation vary depending on the speed and force required? If only one channel is used, what is the function of the symmetry? How does the TolQR ring energize TolA?

For the system to function without uselessly dissipating energy, there must exist an energy-rich state capable of delivering energy to TolA but incapable of conducting protons. In a speculative functional model, the catalytic cycle contains six steps. In the first step, a periplasmic proton penetrates one of the ion conductance channels. This permits or drives the second step, which is a conformational change. In this new conformation, the energized TolA protein can be released, which constitutes the third step. The conformational change and release of TolA allows the proton to exit the conductance channel. In the last two steps, the de-energized TolA protein rebinds, and a conformational change returns TolR to its unprotonated environment. It should be remarked that this conformational change could either return to the original configuration or result in processive movement changing the active channel. In this model, there are a few important principles: first, the TolA protein changes its relation to the rest of the complex (binding or dissociation); secondly, the full complex is asymmetric and, thus, it is possible for only one of the potential ion channels to be ‘selected’ for use and, finally, the cycle is necessarily coupled to TolA activation and thus will not depolarize the cell membrane. Clearly, many different functional cycles could be based on the proposed structural model (Fig. 8); however, the most important point is that a reasonable...
model is possible and is consistent with the majority of published data. It is to be hoped that these models will serve to spur future investigations and clarify the mechanisms used in vivo to transduce an ion potential into mechanical energy and to transmit energy relatively long distances between different cellular structures.

### Experimental procedures

#### Bacterial strains and plasmids

The various bacterial strains and plasmids used in this work are listed in Table 2. Plasmids pQRD23A were constructed by site-directed mutagenesis using polymerase chain reaction (PCR) amplification. Primers designed for the recombinant (R)PCR are 5'-CAACCGTGTTAATTGTTATCCGACCTTGAGATCGCGACGAC and 5'-TCCGAAATACACATTGTACC. The overlapping regions are underlined, while all mutagenic codons introduce a Scra restriction site (in bold). RPCR amplifications were performed as described previously (Ansaldi et al., 1996) using Expand High Fidelity polymerases (Boehringer Mannheim). Recombinant plasmids were first verified by Sca1 digestion. The mutagenized DNA fragment was exchanged in pQRA by Nco1–Sph1 digestion and verified further by DNA sequencing.

#### Growth conditions

Routinely, strains were grown aerobically in Luria–Bertani (LB) medium or on agar LB plates. When required, ampicillin (100 μg ml⁻¹), chloramphenicol (30 μg ml⁻¹), rifampicin (200 μg ml⁻¹), spectinomycin (200 μg ml⁻¹) or arabinose (0.5 mg ml⁻¹) were added.

Under these standard growth conditions, protein expression from the pQRA plasmids and derivatives was under the control of the native promoter, and overproduction was the result of gene dosage effects.

### Sequence database and computational methods

The consensus sequences were based on the analysis of a sequence database containing the sequences of the TolQ–R proteins described previously (Sturgis, 2001) and the ExbB–D proteins identified previously and included in completely sequenced genomes (Sturgis, 2001). This database was expanded by the addition of the TolQ–R and ExbB–D sequences from Xylella fastidiosa (Simpson et al., 2000), and a selection of MotA–B protein sequences.

This database was used to construct a multiple alignment based on that produced by CLUSTALW (Thompson et al., 1994), which was used to incorporate the newer sequences and improve the overall alignment. The modifications served to reduce the number of insertions in TM regions at the expense of those in soluble loops and to align the predicted TM helices. The relevant regions of this alignment are available through the laboratory web site (http://lism.cnrs-mrs.fr/Page_JS.html). To obtain consensus sequences derived from this alignment, a distance matrix was generated from the appropriate regions of the sequences, and several subsets of sequences were sampled from the database with a distance between each member sufficient to avoid the most blatant duplications. These subsets were used to produce the consensus sequences shown in this article. This procedure was found to produce robust results and was adopted because it avoided inducing bias as a result of the inhomogeneous phylogenetic distribution of sequences and, additionally, to compensate for differences in representation of the different types of sequence.

### Outer membrane permeability assays

RNase I leakage: cells were streaked on LB agar containing 1.5% Torula yeast RNA (Sigma). Periplasmic RNase I

### Table 2. Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tr>
<td>GM1</td>
<td>Wild type</td>
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</tr>
<tr>
<td>TPS13</td>
<td>GM1 tolQ(R)</td>
<td>Sun and Webster (1986)</td>
</tr>
<tr>
<td>KP1038</td>
<td>GM1 tolQ(R) exbB(D)</td>
<td>Skare and Postle (1991)</td>
</tr>
<tr>
<td>C600tolQRA</td>
<td>C600 ybgC tolQ tolr toIA</td>
<td>J. C. Lazzaroni</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
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<td>pGP1-2</td>
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<td>Tabor and Richardson (1985)</td>
</tr>
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<td>AmpR</td>
<td>Tabor and Richardson (1985)</td>
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<tr>
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<td>pQ205RA with TolR P37L</td>
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<td>pQ205RA with TolR mutations D23A, D23R or D23E</td>
<td>This work</td>
</tr>
</tbody>
</table>
leakage was estimated after overnight incubation by the addition of 10% trichloroacetic acid.

Electron microscopy analyses and tolerance for SDS and vancomycin were checked as described previously (Bernadac et al., 1998; Cascales et al., 2000).

In vivo cross-linking

In vivo cross-linking experiments were performed on exponentially growing cells. Cells were washed and resuspended at 0.5 A600 in 10 mM sodium phosphate buffer (pH 6.3) and incubated further for 10 min with the addition of 1% FA (Deroiche et al., 1995). Cells were solubilized in loading sample buffer for 20 min at 37°C with the addition of Benzonase (Merck) and were analysed further by Western blotting.

Immunoprecipitation experiments and overlay technique

TolA was immunoprecipitated after in vivo cross-linking with formaldehyde as described previously (Cascales et al., 2000). Cells harbouring pTolA plasmid were induced for 30 min before cross-linking. Co-immunoprecipitation of Pal or TolR Cells harbouring pTolA plasmid were induced for 30 min and cross-linked with 0.5% formaldehyde as described previously (Cascales et al., 2000). Co-immunoprecipitation was performed on polyclonal antibodies (Bouveret et al., 1999). TolA was immunoprecipitated after in vivo cross-linking experiments were performed on strain KP1038, J.-C. Lazzaroni for the pQRA plasmids and the C600tolQRA strain, and E. Bouveret for careful reading of the manuscript. This work was supported by the Centre National de la Recherche Scientifique and the MENRT.

Stability of steady-state TolA and TolR proteins

Cultures were grown at 37°C to an A600 of 0.8 in LB, a sample was removed (at time zero) and spectinomycin was added to the remainder in order to stop protein synthesis. Equivalent samples were harvested at 0, 30, 60, 120, 240 and 480 min, resuspended in Laemmli loading buffer, heated for 15 min at 96°C, separated on 12.5% SDS–polyacrylamide gel and analysed by Western blot immunodetections. Bacterial density was measured in order to verify that no growth occurred.

Protein labelling and membrane preparation

Specific expression of proteins was monitored in strains harbouring both pGP1-2 and pQRA derivative plasmids as described previously (Bénédetti et al., 1991). After [35S]methionine labelling (5 min with 15 μCi), cells were treated with a 100-fold molar excess of unlabelled methionine for 5 min. For non-specific labelling, cells were grown in M9 medium to an A600 of 0.6 and then labelled for 5 min with 15 μCi of [35S]methionine before chase. In order to quantify the Tol proteins within membranes, labelled cells were resuspended at 2.5 A600 in 10 mM Tris-HCl, pH 6.8, 30% sucrose, 200 μg ml⁻¹ lysozyme. After 5 min at room temperature, the same volume of 10 mM Tris-HCl, pH 6.8, 1 mM EDTA was added for 10 min on ice. After five cycles of freeze and thaw and the addition of DNase (100 μg ml⁻¹) and MgCl2 (10 mM), the suspension was centrifuged at 100 000 g for 30 min. The membrane pellet was resuspended at 1.2 A600 in 10 mM Tris-HCl, pH 6.8, 8 M urea, incubated with agitation for 20 min and centrifuged at 100 000 g for 45 min. The pellet was washed with the same buffer devoid of urea before analysis.

Miscellaneous

Prestained protein markers (See-Blue; Novex) were used. After transfer, the nitrocellulose membrane was treated with antisera. Secondary antibodies coupled to alkaline phosphatase were revealed using bromo-chloro-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

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References


transduction through the gram negative outer membrane via TonB–ExbB–ExbD dependent receptor proteins.


Eick-Helmerich, K., and Braun, V. (1989) Import of biopolymers into *E. coli*. Nucleotide sequences of the *exbB* and *exbD* genes are homologous to those of *tolQ* and *tolR* genes respectively. **J Bacteriol** 171: 5117–5126.


Author Queries

**JOB NUMBER:** 2673

**JOURNAL:** MMI

Q1 Author: please give initial(s) for Corda.

Q2 Author: Larsen et al. (2001) has been changed to Larsen and Postle (2001) so that this citation matches the list.

Q3 Author: please define FA.

Q4 Author: Lloubès et al. (2001) in press. Any more details?

Q5 Author: Simpson et al. (2000). Please give 6 authors’ names and initials before et al.

Q6 Author: heading for Table 1 OK? If not, please change.