The structure and mechanism of the TolC outer membrane transport protein

Luca Federici, Fabien Walas and Ben Luisi*

Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge CB2 1GA, UK

Gram-negative bacteria have evolved specialized multi-component systems that transport molecules from the cytoplasm to the extracellular environment in energy-dependent processes. Central to one of these systems is the TolC family of outer membrane proteins. TolC of *Escherichia coli* is a very versatile channel that can interact with a wide range of inner membrane, energy-driven pumps to export compounds ranging from small antibiotic molecules to large toxic proteins. Thus TolC and its associated partner proteins confer invasive virulence and drug resistance to Gram-negative bacterial pathogens. However TolC is also a source of vulnerability, as it is a conduit for the uptake of bactericidal proteins known as colicins. Recently the crystal structures have been reported of TolC and its inner-membrane partner protein AcrB, a proton antiporter. The mechanisms of colicin uptake via TolC have also been extensively studied and the structures of some of the implicated protein components have been determined. In this review we focus on the current understanding of the structure and function relationships in TolC-mediated transport systems.

THE Gram-negative bacteria are characterized by a distinctive two-layered membrane system. The protective outer membrane, which is exposed to the external environment and contains special lipid components including lipopolysaccharide, contains many different kinds of molecular pores that allow the free diffusion of water and ions. The inner membrane defines the cytoplasmic boundary, and the intervening space between the inner and outer membranes, known as the periplasm, is densely packed with peptidoglycan and other complex molecules. The physical separation of inner and outer membranes may vary according to physiological conditions, and it is thought that these membranes may come closer together whenever proteins are transported from the cell interior to the exterior[1,2].

Although the double-membrane system confers protection and other benefits to the bacterium, the movement of molecules across the double-membrane enclosure presents certain strategic problems to the cell. The periplasm contains no ATPase or other energy-providing processes, because this portion of the bacterium may be in equilibrium with the external environment. However, the energy required to do the work of moving molecules – either across a concentration gradient in the case of nutrients, or across a diffusion barrier in the case of secreted proteins – can be provided by inner membrane proteins that serve as engines fueled by ATP or proton electrochemical gradients.

A number of distinctive transmembrane transport processes have evolved in the Gram-negative bacteria to move proteins and other molecules across the membrane. In *Escherichia coli*, five different systems have been characterized to date[3–9]. The system we shall focus on here is known as Type I transport, which employs a three-component pump that utilizes the free energy of ATP binding and hydrolysis to move proteins vectorially across both inner and outer membranes in an apparently single-step process that requires no intermediates within the periplasm[10,11]. One component of this pump is represented by the TolC outer membrane protein. In the transport process, the TolC interacts with a periplasmic protein, known often as a membrane fusion protein, and an inner membrane ATPase. The best characterized example of this system is the transporter for the invasive toxin hemolysin[12]. The transport process is thought to proceed by the recruitment of the nascent hemolysin polypeptide by the inner membrane protein HylB, which may pre-exist in complex with the membrane fusion protein HlyD (Figure 1 *a*). On binding ATP, the TolC is engaged, and the protein passes through the assembly. The association of the components is transient and reversible, and after the substrate molecule has been transported, the components dissociate.

The inner membrane protein HlyB belongs to the wide family of ATP-binding cassette proteins, or 'ABC' proteins, which use the free energy of ATP binding and hydrolysis to translocate molecules. These proteins are widely distributed in nature and are found in all three domains of life: archaebacteria, eubacteria and the eukaryotes[13]. In HlyB, the transmembrane and ATPase domains are contained within a single polypeptide chain, but in other systems, these domains are encoded in separate subunits[14]. An example of a multisubunit ABC ATPase is the vitamin B_{12} receptor, BtuCD, which is a heterotetramer. The oligomeric construction of the ATPase suggests that it functions through allosteric transition, as might be expected for a mechanical pump. The crystal structure of the

*For correspondence. (e-mail: ben@cryst.bioc.cam.ac.uk)

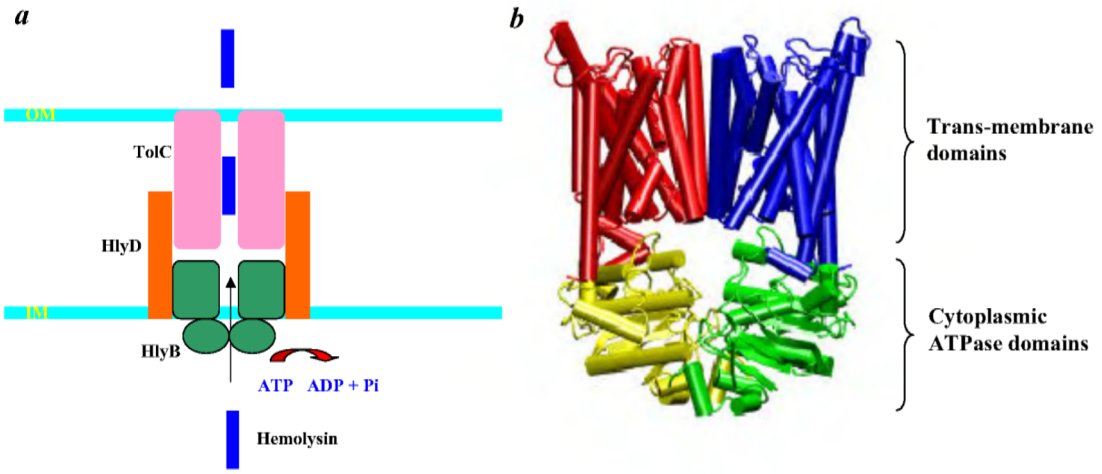

Figure 1. *a*, A schematic for the proposed transport of hemolysin through a type I ternary assembly involving an inner membrane ABC transporter (HlyB), a membrane fusion protein (HlyD) and the outer membrane protein TolC. *b*, The crystal structure of a representative bacterial ABC transporter: the BtuCD heterotetramer from *E. coli*. This protein functions in the energy-dependent uptake of vitamin B_{12}. The 2-fold molecular symmetry axis is oriented vertically.

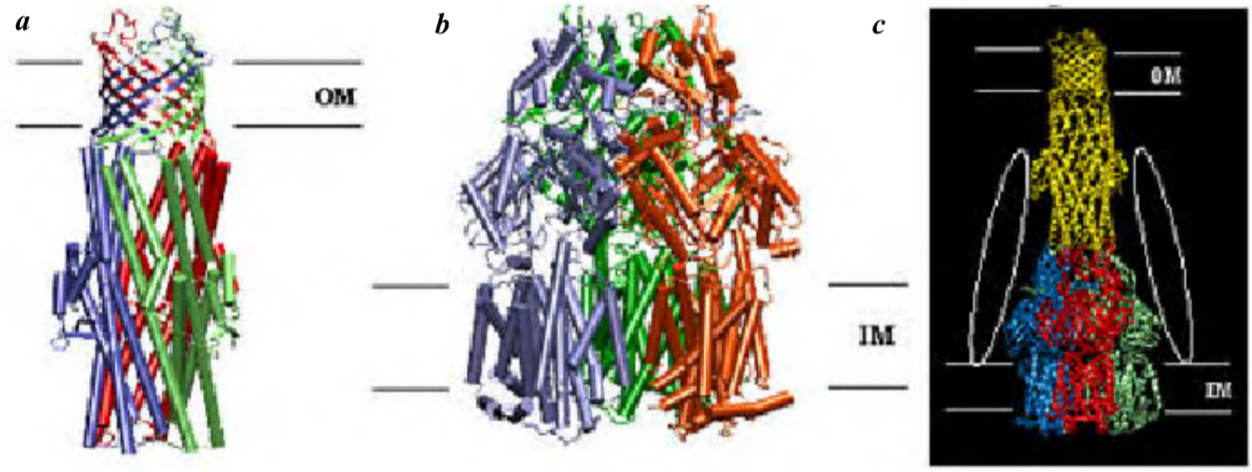

Figure 2. The crystal structures of the outer membrane protein TolC (***a***) and the inner membrane protein AcrB (***b***). Both proteins are from *E. coli* and their 3-fold axes are oriented vertically (***c***). The docking of the two structures into a hypothetical complex. The structure of the third component, AcrA, is not known and is shown schematically by the elongated ovals to bind both the inner and the outer membrane proteins.

E. coli BtuCD has been solved by Douglas Rees and colleagues, and is shown in Figure 1 *b* (ref. 15). The green and yellow subunits are the ATPase domains, while the red and blue helical domains are the transmembrane components.

TolC is very versatile, and will interact with a number of different inner membrane protein/membrane fusion protein (MFP) partners to transport different proteins, drugs, bile salts and other molecules. Like the protein secretion pumps, the drug efflux pumps involving TolC or its homologs is three-component, comprising another MFP and an inner membrane proton antiporter. The antiporters may belong to the 'RND' (resistance, nodulation and division) family – so-called for the phenotypic effects of their mutations – or to the Major Facilitator Superfamily (MFS)[16]. The specificity for the transport substrate appears to be due to recognition by inner-membrane protein/MFP, with TolC perhaps playing the role of a non-discriminating channel.

Insight into the mechanism of these efflux pumps has come from the crystal structures of the TolC protein[17], and of the inner-membrane proton antiporter AcrB[18]. Figure 2 shows the two component structures and their hypothetical complex. The membrane fusion component, i.e. AcrA, is missing from the model, but is shown schematically as the body that links the two proteins.

Both the TolC and AcrB are homo-trimeric, and the model of their complex aligns the three-fold axes. The portion of the TolC that is embedded in the outer membrane is a self-closing beta-barrel that is similar to the fold observed in the porins, which are outer membrane channels. TolC represents the first porin-like architecture in which the barrel domain is made from oligomeric association rather than a single polypeptide chain. The periplasmic portion is composed almost entirely of α-helices, and some of these are nearly 100 Å long. Near the porin-portion of the trimer, the helices form a cylindrical architecture which we have referred to as an α-helical barrel[19]. The distal end of TolC, at the farthest point from the inner membrane, is sealed almost hermetically by the inward curvature of coiled-coil helices. The narrowest point of the seal is only 3 Å in diameter, which is only sufficiently large for the smallest counterion, and the constriction here accounts for the small conductive permeability of the TolC[17]. It is clear on examining this crystal structure that the molecule must undergo an allosteric transition to open this barrier. We have suggested that this transition involves the lateral breaking of contacts between adjacent coiled-coil pairs[20].

The trimeric AcrB resembles a jellyfish, with twelve transmembrane α-helices per monomer. The topology is consistent with functional studies, and this defines the orientation of the structure in the membrane. The large headpiece region of the protein spans 70 Å from the periplasmic side of the membrane. There is a central chamber that is accessible both from the periplasm and the cytoplasm. Three entrances from the periplasm, referred to as vestibules, are formed from the inter-subunit interfaces. Both the cytoplasmic and periplasmic entrances are likely to be pathways for transported drugs, and their existence explains nicely how the protein confers resistance to different classes of drugs that are active in the cytoplasm, such as the macrolides, or in the periplasm, where the beta-lactam antibiotics function[18]. The central chamber is connected to the periplasm by a funnel that is closed in the AcrB crystal structure. It has been postulated that the funnel is opened through a conformational change in the periplasmic headpiece caused by proton translocation[21].

To date, there is no experimental structure available for a MFP. Solution hydrodynamic data indicate that AcrA has an extended conformation capable of spanning the entire periplasmic space[22]. A similar observation has been reported for another MFP partner of TolC, EmrA[23]. Johnson and Church[24] have proposed that the MFPs are organized as a split domain, where two halves of a lipoyl/biotinyl domain are separated by a coiled-coil domain. Those authors have suggested that the coiled-coil undergoes a structural transition from an elongated state, where the helices associate linearly, to a second, more condensed state where the helices bend in the middle to form a self-complementary interaction. According to this model, the structural transition of the MFP protein would pinch together the inner and outer membrane, which may be consistent with observation from electron microscopy studies. A second, more simple, possibility is that MFPs facilitate the docking of the inner and outer membrane proteins. In fact the periplasmic extensions of TolC and AcrB taken together already account for ~170 Å which is within the estimates for the periplasmic space separation[21].

We have generated a model for the open state of TolC based on the structural repeat within the molecule[20]. According to this model, the coiled-coils change their superhelical curvature while maintaining the knobs-into-holes interfacial packing. However, the contacts between adjacent sets of coiled-coils are changed upon the transition to the open state. One consequence of this changed packing is that a groove is created between adjacent coiled-coils. We noted that this groove could serve as binding site for a helical domain of the MFP. If the helix of the MFP were to engage here, it could pack to form a structure that resembles an anti-parallel four-helix bundle, whereby three helices of TolC are contacted by a single helix provided by the MFP. A hypothetical model for this interaction is shown in Figure 3.

Role of TolC and other Tol proteins in the entry of colicin into the cell

Colicins are bactericidal peptides made by one strain or species of bacteria as an offensive weapon against other bacteria. Nearly 40 years ago, it was discovered by Salvador Luria and colleagues that mutations of certain genes confer tolerance to the bactericidal effects of colicins[25]. Accordingly, the gene products were labelled TolA, TolB and TolC, which is the principal subject of this review. Thus, it is clear that while TolC provides selective advantages to pathogenic organisms, through the secretion of invasive toxins such as hemolysin or the protection against noxious agents and antibiotics, it is also a source of vulnerability in the competition for ecological niches.

There are many different classes of colicins, some that act by puncturing the inner membrane and thereby killing the cells by dissipating its energy sources, and others that function by cleaving the ribosome and starving the cell of nascent proteins[26,27]. The colicin E1 protein is of the former class, and it enters the bacterium through TolC, facilitated by the TolA and TolB proteins[28]. The details of the mechanism are not known, but it is thought that the initial step is the binding of colicin E1 to the vitamin B_{12} receptor BtuB (see Figure 4 *a*). A crystal structure of the recognition (R) domain of the colicin E3 in complex with the receptor BtuB has been recently determined[29] and provides a possible model for the first stage of colicin E1 entry. A partial unfolding at the termini of the colicin E3 coiled-coil is observed, which could be propagated to the translocation (T) domain. In the case of the colicin E1,

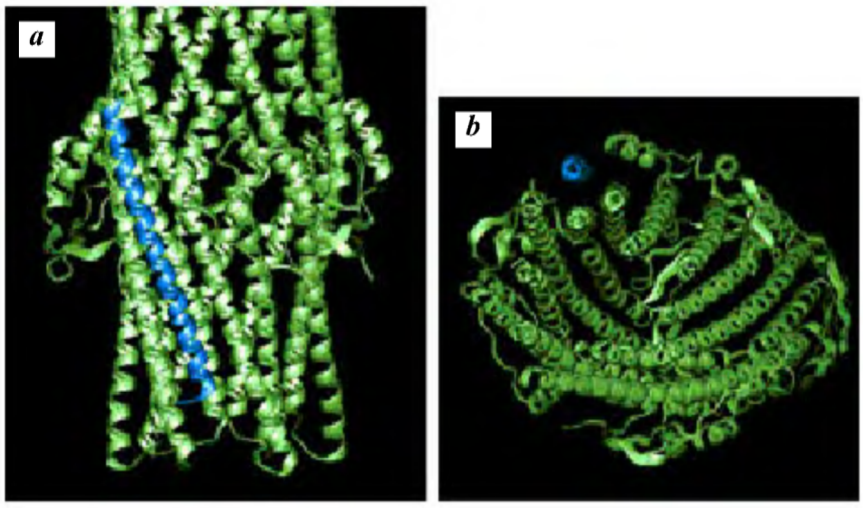

Figure 3. A speculation on the interaction between the TolC (green) and an α-helix of a membrane fusion protein (blue). TolC is shown in the modelled 'open state'. ***a***, Front view. TolC molecular 3-fold axis is vertical. Only one helix of the membrane fusion protein is shown for clarity, but the fully engaged complex model has as many as six helices bound; ***b***, View along the axis of the MFP α-helix. The 3-fold axis of TolC is inclined about 30° with respect to the normal to the plane of the paper.

the partially unfolded protein is then thought to be threaded through the TolC channel, through its translocation domain. At this stage the TolC channel would open or be actively opened, permitting the entry of the toxin into the periplasmic space where it engages the TolA protein. The interaction between TolA and colicin E1 has been recently investigated by FRET (fluorescence resonance energy transfer) using the related colicin N translocation (T) domain. The T domain appears to be partly unfolded but not extended and a concerted refolding has been detected upon TolA binding[30]. TolA is an inner-membrane protein with an elongated periplasmic portion, identified as domains III and II; domain I is a transmembrane helix that associates with TolQ and TolR inner membrane proteins[31]. We have recently determined the crystal structure of TolA domain III and investigated the complete periplasmic fraction of the protein (domains II+III) by small angle X-ray scattering (Figure 4 *c*)[32]. The molecule is monomeric and its elongated stalk shape can accommodate domain III and a helical bundle corresponding to the portion occupied by domain II. The three-component inner-membrane assembly formed by TolA, TolQ and TolR is probably a mechanical device that is driven by proton-motive force[33].

We have recently investigated the structure of a fragment of the colicin E1 toxin in complex with TolC, and we observe some broken electron density within the chamber that appears to be α-helices from the toxin (C. Fanutti, L. Federici, W. Cramer *et al.*, unpublished results). The representative density, revealing the α-helical fragments, is shown in Figure 4 *b*. We propose that the complex is a partially unfolded state of colicin E1 translocation domain that is an intermediate in the uptake of the toxin.

Interestingly, there might be a mechanistic connection between the uptake of the vitamin B_{12} and the uptake of colicin E1. We speculate that colicin E1 import, like vitamin B_{12} uptake, is dependent on proton-motive force. We base this speculation on the similarity of the TolA protein with the TonB energy-dependent import system[32], which is required for the uptake of the vitamin[34]. Perhaps the opening of the closed TolC channel is energized by the interactions of the TolA protein with the colicin E1 as it extruded from the TolC channel, so that the colicin is threaded into the periplasm. This hypothesis awaits further testing.

Other TolC homologues

The distinctive architecture of the TolC homotrimer, in which β-barrel and α-barrel supersecondary structures meet, requires special patterns of amino acid residues, and these can be used to identify remote homologues[20]. To date, homologues have only been found in Gram-negative bacteria, and none have been identified in eukaryotic mitochondria or chloroplasts.

Recently, Jun Nishi, and colleagues have identified a remote homologue of TolC, called AatA, from a pathogenic strain of *E. coli* that is associated with severe diarrhoea in children. Although this protein shares less than 20% sequence identity with TolC, it is clearly a structural homologue based on the pattern of conserved residues that define the key structural features of the TolC architecture[35]. This protein is encoded on a plasmid along with an inner-membrane permease (AatP), an ATPase subunit (AatC), and two other proteins that localize to the perip-

lasm (AatB and AatD). All of these proteins are required for the export of the protein known as dispersin (Aap), which facilitates the spread of the bacteria on the intestinal epithelium. The transport mechanism is thought to be different from the three-component pumps described earlier. The dispersin protein is first transported to the periplasm in an Aat-independent step, most likely via the Sec secretion pathway that removes a signal peptide during this process. Then somehow the Aat system engages and transports the dispersin protein, through the TolC homologue, from the periplasm. It seems likely that the closed channel is opened by the activation of the AT-Pase. The details of the mechanism await elucidation (Figure 5).

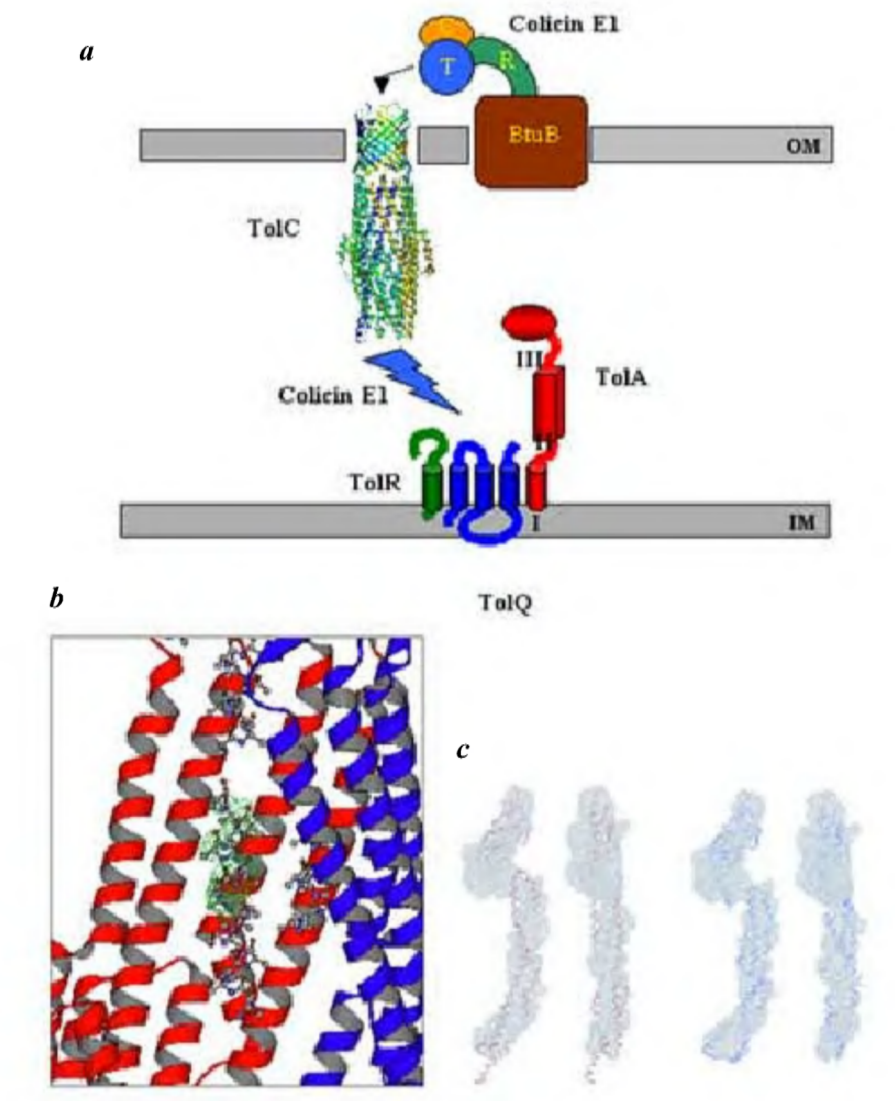

Figure 4. The entry of the bactericidal colicin E1 into a bacterium. ***a***, A cartoon of the components and the postulated pathway. The vitamin B_{12} receptor may activate the threading of the colicin E1, which passes through the TolC channel. The protein may then be received by the TolA protein. The TolA/TolQ/TolR inner membrane assembly is likely to be an inner-membrane proton-dependent motor. ***b***, An electron density map and preliminary model for a fragment of *E. coli* colicin E1 engaged in the interior channel of TolC. For clarity, one of the subunits of the TolC trimer has been removed to reveal the channel. ***c***, A model for the TolA protein, based on X-ray crystallography and X-ray solution scattering[32]. The molecular shape has been interpreted by fitting TolA domain III and a helical bundle resembling domain II. Two ribbon models are shown, one using the spectrin repeat of actinin (red), the second using two repeats of the alanine-rich helical bundle from the enzyme $IIA^{lactose}$ (blue).

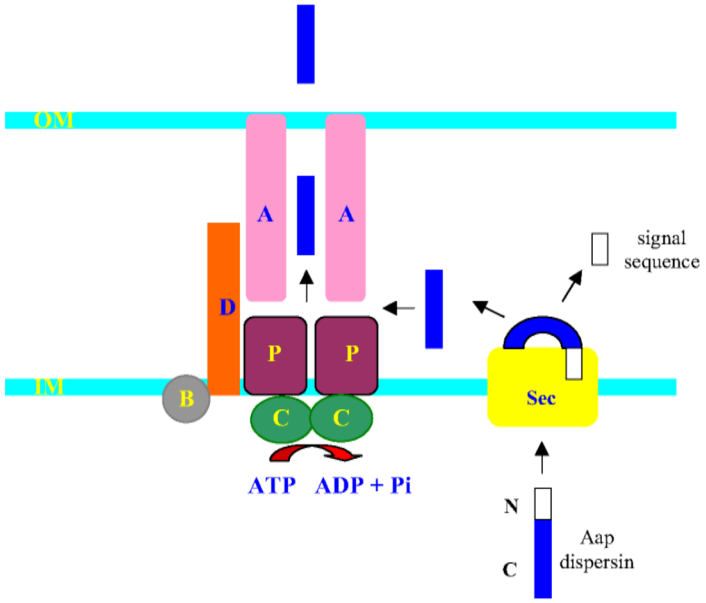

Figure 5. A schematic of the proposed transport of the dispersin (Aap) protein through a homologue of TolC. Five proteins in the Aat operon are involved. AatA is a TolC homologue, AatP is an inner membrane permease, AatC an ATPase while AatB and AatD have unknown function. The figure was adapted from ref. 35.

Conclusions and perspectives

As far as we can tell, the TolC system is unique to Gram-negative bacteria. We have used our structural templates for the α-helical barrel to search for remote homologues in other organisms, including the mitochondria and plastids of eukaryotes, but we could not find any related molecule. Other mechanisms have evolved for protein transport, for example the Tim/Tom complex of mitochondria, and the Gram-negative bacteria themselves have many other different mechanisms for the movement of proteins and drugs across the membrane. However, the TolC-mediated mechanism is important in the virulence and drug-resistance of pathogenic Gram-negative bacteria and is of interest as a therapeutic target. We anticipate that further structural insights will shed light on this mechanism and lead to the development of useful drugs to inhibit bacterial virulence and toxic shock.

Note added in proof: The crystal structure of the membrane fusion protein MexA of *Pseudomonas aeruginosa* has been solved recently (pdb code 1VF7). The structure confirms predictions[24] that the MFP contains a coiled-coil domain and a lipoyl/biotinyl domain.

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ACKNOWLEDGEMENTS. Work in our laboratory is supported by the Wellcome Trust (UK). L.F. is the recipient of a European Community 'Marie Curie' fellowship.