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Minireview

TolC – the bacterial exit duct for proteins and drugs

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Abstract The TolC structure has unveiled a common mechanism for the movement of molecules, large and small, from the bacterial cell cytosol, across two membranes and the intervening periplasm, into the environment. Trimeric TolC is a remarkable cell exit duct that differs radically from other membrane proteins, comprising a 100-Å long α-barrel that projects across the periplasmic space, anchored by a 40-Å long β-barrel spanning the outer membrane. The periplasmic entrance of TolC is closed until recruitment by substrate-specific translocases in the inner membrane triggers its transition to the open state, achieved by an iris-like 'untwisting' of the tunnel α-helices. TolC-dependent machineries present ubiquitous exit routes for virulence proteins and antibacterial drugs, and their conserved structure, specifically the electronegative TolC entrance constriction, may present a target for inhibitors of multidrug-resistant pathogens.

Key words: Protein export; Membrane protein; Multidrug resistance; Drug efflux

1. TolC: a role in bacterial protein export and the efflux of small toxic molecules

The envelope protein TolC was named many years ago when its loss by mutation was seen to confer tolerance to specific colicins and bacteriophage [1–3], and to 'pleiotropically' increase bacterial sensitivity to environmental stresses like detergents, bile salts and organic solvents [4–7]. While TolC is exploited 'passively' as a bacterial cell entry receptor by the phage and colicins, it plays an active and central role in the expulsion of a plethora of molecules from Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* [5,8,9].

It was first found that TolC is needed for the atypical export of large proteins, including toxins and enzymes directed at mammalian hosts during infection [10–13]. Gram-negative bacteria have two cell membranes, cytoplasmic (inner) and outer, separated by an intervening periplasmic space [14]. Proteins destined for the cell surface or the external medium, for example during the assembly of adhesion pili [15] or flagella [16], or delivery of virulence effector proteins into mammalian cells, are typically secreted by large multiprotein assemblies that either span the periplasm or establish two-step mechanisms employing periplasmic intermediates [15–17]. TolC-dependent ('type I') export bypasses the periplasm and yet requires only outer membrane TolC acting with an inner membrane translocase of two proteins, a traffic ATPase and an 'adaptor' protein [10,12,18]. Our work has focussed on type I export of the 110-kDa *E. coli* hemolysin (HlyA) [19], but the same process exports many toxins, proteases and lipases [20] from pathogens of humans, animals and plants, and also substrates like glycanases from plant-nodulating bacteria [21,22]. The export signal in these proteins is uncleaved and located at the extreme C-terminus [23,24].

TolC was subsequently also shown to have an active part in the efflux of small noxious molecules like detergents, but in the last few years it has achieved wider prominence as its efflux substrates were found to include a wide range of antibacterial drugs [25,26]. This established TolC as a key player in the growing problem of multidrug resistance in pathogenic bacteria. As in protein export, the efflux of antibacterial drugs and other small inhibitors involves TolC cooperating with an inner membrane translocase, and this too comprises an adaptor protein and a protein providing energy, in this case usually from proton antiport [25]. Bacteria typically have many efflux pumps with broad specificities. For example, while *E. coli* AcrAB-TolC determines resistance to many antibiotics, dyes, detergents, fatty acids, bile salts and organic solvents [5–7,26], EmrAB-TolC expels hydrophobic uncouplers of oxidative phosphorylation, organomercurials and antibacterial drugs like nalidixic acid and thiolactomycin [27].

2. TolC – the key to understanding the protein export and drug efflux mechanisms

From biochemical studies we knew that protein export was effected by recruitment of TolC by substrate-laden inner membrane translocases [28] (see later). Nevertheless, it was a mystery how events at the inner membrane were coupled, without periplasmic intermediates, to passage through what was imagined to be a simple outer membrane porin-like channel. Electron microscopy of two-dimensional crystals [12] intimated that trimeric TolC might have a novel single transmembrane pore and perhaps an additional domain that could contribute to a periplasmic bypass. But it was the high-resolution crystal structure which revealed vividly how TolC could allow 'direct' passage of proteins and drugs from the cytosol out of the cell [29].

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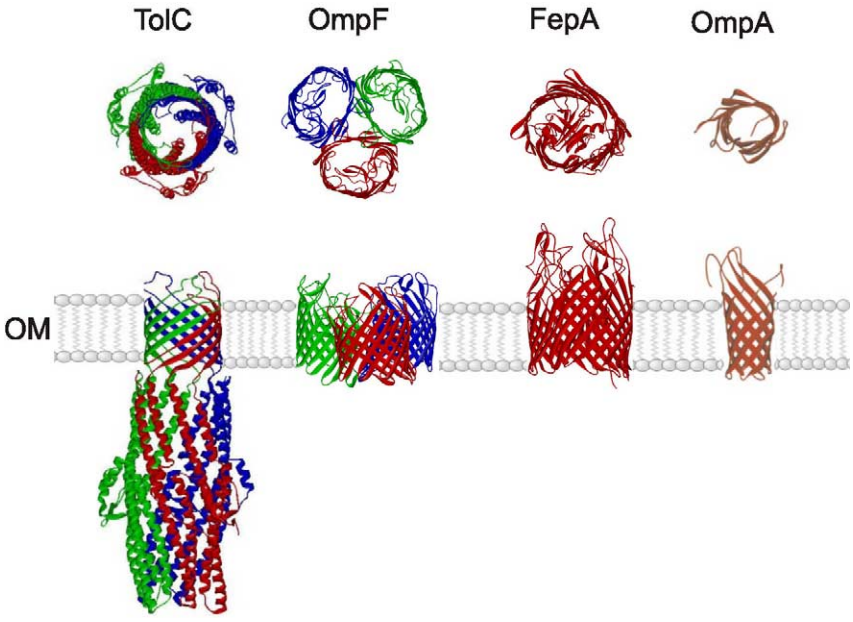

Fig. 1. The structures of TolC and other *E. coli* outer membrane (OM) proteins. Each outer membrane protein is viewed from above the lipid bilayer (upper) and through the plane of the membrane (lower). Colours indicate protomers. The monomeric OmpA forms the smallest β-barrel of eight β-strands [61], the large β-barrels of the iron siderophore transporters FhuA and FepA [33,34] comprise 22 β-strands, while in trimeric porins each monomer forms a β-barrel of 16 (e.g. OmpF) or 18 (e.g. LamB) β-strands [59,60].

3. A periplasmic α-barrel anchored by a β-barrel spanning the outer membrane

X-ray crystallography [29] at 2.1-Å resolution revealed the TolC homotrimer as a 140-Å long cylinder which is made up of a 100-Å long α-helical barrel (the 'tunnel domain') projecting through the periplasmic space and anchored in the outer membrane by a 40-Å long β-barrel channel (Fig. 1). This TolC 'channel-tunnel' presents a hollow conduit extending from near the inner membrane to the cell exterior. Throughout the outer membrane channel and most of the contiguous tunnel the accessible interior diameter of the single pore is 19.8 Å average. Three TolC protomers each contribute four β-strands to the single 12-strand outer membrane β-barrel, an architecture unknown in other bacterial membrane [29] proteins (Table 1). This channel is constitutively open to the surrounding medium, and it does not have an inward folded extracellular loop that typically constricts channel-forming proteins [31,32], or a plug domain like that of the large β-barrels of FhuA and FepA [33,34].

The periplasmic α-barrel comprises 12 α-helices (four from each monomer) that follow a left-handed superhelical twist and pack in an antiparallel arrangement [29]. These tend to be underwound in the upper half compared to helices in a conventional two-stranded coiled coil, enabling the helices to lie on the surface of a cylinder. The 12 helices pack laterally side-by-side and form two separate interfaces, stabilised by an intermeshing of side-chain ('knobs-into-holes' packing [35]). In the lower half of the α-barrel neighbouring helices form six pairs of regular two-stranded coiled coils, but one from each monomer folds inwards at the end of the tunnel (Fig. 1). This constricts the entrance to establish a resting closed state, with an effective diameter of approximately 3.9 Å [29,30]. This constriction has to be opened for substrate access.

Table 1
Structural properties of TolC and other outer membrane proteins

Properties	TolC	OmpF	FepA	OmpA
Length (Å)	140	35	70	57
Radius (Å)	17.5	15.5	19.9	13
Constriction diameter (Å)	3.9	11	n/a	n/a
Number of pores	1	3	n/a	1
Number of monomers	3	3	1	1
β strands per monomer	4	16	22	8
Conductance (pS)	80	840	n/a	n/a

Conductances in picoSiemens are measured in 1 M KCl or NaCl.

TolC homologues are ubiquitous among Gram-negative bacteria, and already nearly a hundred have been identified [36–38]. *E. coli* TolC can act in both protein export and inhibitor efflux, but typically bacteria have multiple system-specific homologues [38]. Although these homologues can vary in length by over 100 amino acids [37,38], this is due primarily to extensions at the periplasmic N- and C-termini and gaps or insertions either in the equatorial domain outside the α/β barrel core structure, or the extracellular loops. The α-helices and β-strands of the channel-tunnel structure do not vary substantially in length, and deletions or insertions are poorly tolerated [39]. Sequence divergence is evident, correlating with function in export or efflux, and few amino acids are well conserved, but these conserved residues occur at structurally significant points like the entrance sequence, and highlight conservation of the basic fold [37,38].

4. Assembly of the TolC-dependent machineries

In both the protein export and drug efflux machineries, TolC interacts with an inner membrane translocase of two inner membrane proteins, an energy source and an adaptor protein (Fig. 2). The translocase is assembled constitutively [28,40], i.e. in the absence of substrate and TolC, and in each system it provides substrate specificity and energy. TolC is an integral part of the machinery [10,12]; when it is

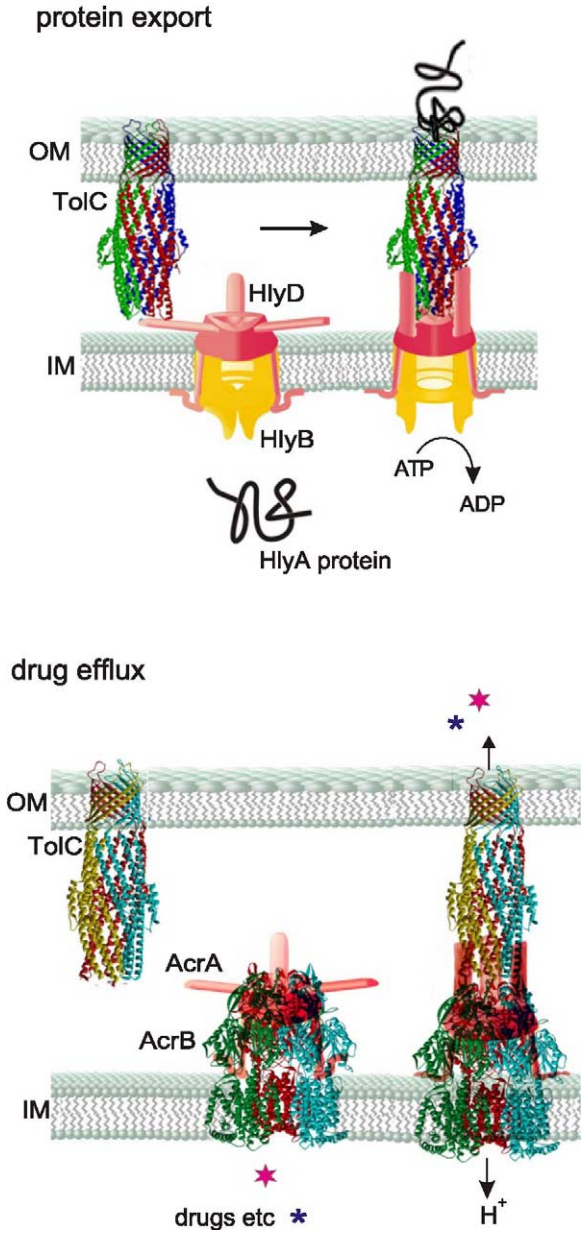

Fig. 2. Assembly of TolC-dependent machineries. Models of protein export (top) and drug efflux (bottom) achieved by reversible interaction of TolC or a homologue with substrate-specific inner membrane (IM) complexes (translocases). These contain a common adaptor protein and an energy-providing protein, in export a traffic ATPase and in drug efflux most commonly a proton antiporter (the figure shows AcrB, which has a substantial periplasmic domain). In this way a contiguous exit route is assembled from the cytosol to the outside environment. In each case it is proposed that a key event is opening the TolC entrance. The assembly process may in both cases require the cell proton-motive force, but substrate translocation is driven by the specific activities of the translocase, whether from ATP or proton movement.

deleted from the hemolysin export system there is no residual substrate passage to the periplasm or from spheroplasts [12,25,28]. TolC must be recruited by each substrate-laden translocase.

What are the translocase components? In type I protein export, the translocase contains a membrane traffic ATPase [41–43], exemplified by the homodimeric 707-residue hemolysin B (HlyB), which is predicted to have an N-terminal domain encompassing six transmembrane helices, fused to a c. 250 residue C-terminal ATPase domain in the cytosol [18,44]. The efflux systems have one of several types of energy-providing protein [5,8,19], either a proton antiporter or ATP-binding protein. The 3.5-Å structure of the *E. coli* AcrB [45] has revealed that, unlike the protein export ATPases, the trimeric proton antiporter has a large periplasmic domain, of length 70 Å and diameter 80 Å, which comprises two large hydrophilic loops from each monomer. From the top, AcrB resembles a funnel, with the protomers (each 1049 residues) appearing interlocked by three hairpin structures protruding from one protomer into the next. The transmembrane region, length 50 Å and diameter 100 Å, comprises 36 α-helices, 12 from each protomer. Interactions between protomers are restricted to single α-helices from each neighbour so that the transmembrane domain appears as a chamber with an opening at the 'cytoplasmic' side of the membrane [45]. Protein export and drug efflux systems have a common adaptor protein (Fig. 2). Adaptor proteins have a similar size and hydropathy profile [36,37], and biophysical and electron microscopy studies of AcrA predict an extended oligomeric structure [46]. The 478-residue export adaptor HlyD is predicted to have a small N-terminal cytosolic domain (residues 1–59) connected by a single transmembrane helix to a large periplasmic coiled-coil domain (residues 81–478) [36,37]. Some drug efflux adaptors are also predicted to be anchored in the inner membrane by a transmembrane helix, e.g. EmrA, but others, e.g. AcrA, are not, and membrane interaction may be favoured by an N-terminal lipid modification.

Cross-linking shows that both the translocase components HlyB and HlyD can interact independently with the HlyA substrate in vivo [28], and evidence supports the possibility that the initial engagement involves the export signal [47]. Recruitment of TolC by these translocases is clearly a central event in the mechanism and in vivo cross-linking shows that during protein export it is mediated by the adaptor (HlyD) periplasmic domain [28], putatively by coiled-coil interactions. This periplasmic interaction is triggered by substrate binding at the cytoplasmic face of the translocase, but the initial engagement, the 'signal recognition' is not sufficient for export. A distinct subsequent step requires transduction of the binding signal to trigger recruitment of TolC, specifically involving the small (38-residue) N-terminal cytosolic domain of the adaptor [48]. This domain comprises a conserved potential amphipathic helix followed by a group of charged residues. When this charged cluster alone is deleted, the substrate is still engaged by the adaptor (and traffic ATPase) but export and TolC recruitment are disabled [48,49]. These data suggest that interaction by protein substrates at the cytosolic face of the export machinery involves initial engagement at the cytosolic ATPase domain, with interaction extending to the adaptor cytosolic domain, possibly augmenting the initial engagement and also mediating transduction of the substrate-binding signal to the large coiled coil of the TolC-recruiting adaptor periplasmic domain.

The AcrB structure [45] indicates that the top of the AcrB periplasmic domain might touch the TolC α-barrel domain, six vertical hairpins, at the apex of the AcrB trimer could dock with the six α-helix turn α-helix structures at the bottom of TolC, especially as the diameters are about the same. Such a direct interaction between TolC and the energy component

might be stable or not, and might not be possible in the protein export pathway as the traffic ATPases are not predicted to have the periplasmic loops. This is also true for the MFS and ATP-binding cassette (ABC) pumps, as they too lack a periplasmic domain, and may indicate a difference in emphasis in adaptor function, with the adaptor protein AcrA having a principal role in stabilising the AcrB-TolC docking rather than overt recruitment, as has been proposed for the export adaptor HlyD [28,49]. This seems to be supported by our current analyses of the AcrAB/TolC efflux system, which shows that while the AcrA adaptor has micromolar affinity with each of the AcrB antiporter and TolC, no binding is detected between these last two proteins in vitro (Koronakis and colleagues, unpublished), possibly reflecting that their interaction needs to be stabilised by AcrA. At this stage a better understanding awaits further biochemical investigation and the structure of an adaptor component.

5. Translocation of substrates by the export and efflux pumps

The use of chemical uncouplers has suggested that an early stage requiring the electrochemical potential reflects assembly of the complete substrate-bound export complex [50], and that in the subsequent (post-assembly) stage of export translocation is driven using ATP hydrolysis by the export ATPase [50]. In support of this view, ATPase mutations preventing hydrolysis of bound ATP abolish protein export but not the assembly of the substrate-engaged HlyB/HlyD/TolC complex [28,51,52]. It is possible that the large substrate passes through the translocase in a partially unfolded state, resulting in 'ratcheted' translocation driven by ATP hydrolysis, but this is speculation, especially as no contacts have been defined between internal regions of the substrate and any of the membrane exporter components. What is known is that once the protein substrate passes out of the cell the inner and outer membrane components revert to their resting state, i.e. the active export complex is transient [28]. The machinery therefore seems to export large proteins with a substrate-responsive pump action (Fig. 2).

While it seems evident that the myriad of small efflux substrates could enter through the opening of the AcrB antiporter at the cytosolic face of the inner membrane, it has been suggested that substrates located in or at the membrane might enter AcrB by other routes after association with the inner membrane lipid bilayer [45]. The crystal structure of the AcrB antiporter liganded with structurally diverse substrates [53] revealed them bound at non-identical positions in the central cavity of the transmembrane domain, but this is likely to be only a snap-shot of an undetermined point in the efflux process, especially as domain swapping has indicated that the periplasmic domain plays a major role in substrate specificity [54,55]. What seems clear is that, like proteins, drugs will be channelled to the TolC entrance, which must be opened.

6. Twist to open – access to TolC by an iris-like movement of entrance helices

Opening the periplasmic tunnel entrance is clearly key to the function of TolC and the assembled export and efflux machineries. An allosteric mechanism has been proposed for opening, based on the observation that the three inner coiled coils (comprising helices H7 and H8) differ from the outer

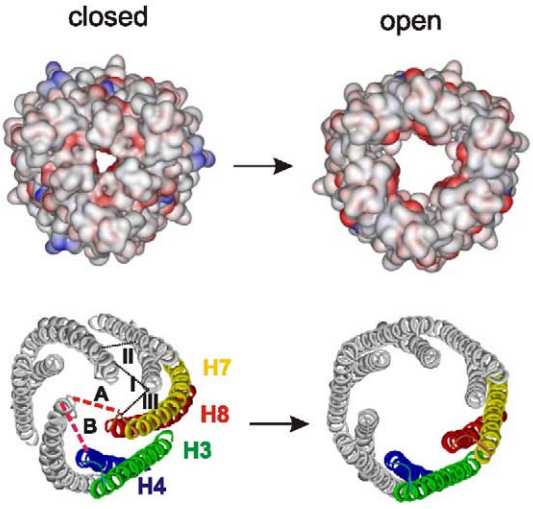

Fig. 3. The closed and modelled open states of TolC. Space-filled (upper) and ribbon (lower) depictions of the closed and modelled open states of the tunnel entrance, viewed from the periplasm. The coiled coils of one protomer are coloured (H3/4 and H7/H8, helix numbering taken from [29]) and show the constraining intramonomer (I and II) and intermonomer (III) links.

coiled coils (H3/H4) only by small changes in superhelical twist [29]. It envisages that transition to the open state is achieved by the inner coils realigning with the outer coils, thereby enlarging the aperture diameter (Fig. 3). Comparison of the resting closed state and modelled open state of the entrance identifies three inter- and intra-molecular links that constrain the three inner coils in the closed conformation [29,30]. Links I and II connect each inner coiled coil to the outer coil of the same monomer by hydrogen bonds, while link III connects each inner coiled coil to the outer coil of the adjacent monomer by a salt bridge and a hydrogen bond. These links must be disrupted for the inner coiled coils to move outwards and enlarge the entrance diameter.

We have supported this model by in vivo and in vitro experiments. Formation of critical salt bridges and hydrogen bonds was prevented by substituting the participating residues, and as the periplasmic entrance is the sole constriction of TolC, change in the diameter of the entrance aperture was monitored as the conductance of purified TolC proteins in black lipid bilayers [30]. Elimination of individual connections (I, II or III) caused incremental weakening of the circular network, but when both components of link III were disrupted simultaneously with the intra-monomer link I there was a synergistic effect, dictating a six- to 10-fold increase over wild-type conductance. This would be compatible with an opening aperture of 16 Å, similar to the *Staphylococcus aureus* toxin [56]. The results support the model in which transition to the open state of TolC is achieved by an iris-like realignment of the tunnel entrance helices generating an aperture that corresponds to the modelled open state (Fig. 3). Complementary in vivo evidence for this opening mechanism and its essential role in protein export was obtained by introducing disulphide bonds, directly or via a chemical cross-linker, to constrain entrance coiled coils [57]. Protein export from *E. coli* carrying the locked TolC variants was assessed in parallel by monitoring the size of the entrance aperture in artificial lipid membranes. TolC-dependent export was completely abolished when the coiled coils were cross-linked at the nar-

rowest point of the entrance constriction, either between adjacent monomers (link A), or by connecting the inner coil of each monomer to the outer coiled coil of its adjacent monomer (link B) [57]. Although hemolysin was not exported, it was still engaged by the type I inner membrane translocase, and triggered recruitment of the locked TolC. Untwisting the entrance helices is therefore essential for TolC function, and acts specifically to open the entrance to allow passage of the substrate engaged at the inner membrane complex.

7. Pumps as drug targets – is it possible to block the TolC entrance?

Knowledge of the multidrug efflux proteins will not only further understanding of the mechanisms of export and efflux but may facilitate design of potential antibacterial agents for the treatment of multidrug-resistant infections. The obvious target site is the periplasmic entrance of TolC. This is the sole constriction in the single pore with a minimum effective diameter of 3.9 Å, and it is lined by a ring of six aspartate residues, D^{371} and D^{374} from each monomer [29,58]. TolC function in artificial lipid bilayers is severely inhibited by cations entering the channel-tunnel from the channel ('extracellular') side. Divalent and trivalent cations block the transmembrane ion flux, with hexamminecobalt binding at nanomolar concentrations. When either or both of the entrance aspartates are substituted by alanines, high-affinity binding is abolished [58] and blocking of the membrane pore is alleviated, indicating that the inhibitor binds to the electronegative aspartate ring. This is supported by a crystal structure of the TolC–$Co(NH_3)_6^{3+}$ (ligand) complex, which indicates a ligand molecule bound at the entrance constriction (our unpublished results). This first biochemical and structural characterisation of an in vitro inhibitor of TolC may suggest a strategy to develop bioactive molecules, especially as the electronegative entrance is widely conserved throughout the TolC family of Gram-negative bacteria.

8. The future?

Elucidating the structures of all the components of TolC-dependent systems and defining the multilateral interactions between them and their substrates will explain how these membrane nanomachines work. This will explain how virulence protein export and multidrug efflux are achieved, and perhaps suggest ways in which they might be combatted.

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