

STRUCTURE AND FUNCTION OF TolC: The Bacterial Exit Duct for Proteins and Drugs

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■ **Abstract** The bacterial TolC protein plays a common role in the expulsion of diverse molecules, which include protein toxins and antibacterial drugs, from the cell. TolC is a trimeric 12-stranded α/β barrel, comprising an α -helical *trans*-periplasmic tunnel embedded in the outer membrane by a contiguous β -barrel channel. This structure establishes a 140 Å long single pore fundamentally different to other membrane proteins and presents an exit duct to substrates, large and small, engaged at specific inner membrane translocases. TolC is open to the outside medium but is closed at its periplasmic entrance. When TolC is recruited by a substrate-laden translocase, the entrance is opened to allow substrate passage through a contiguous machinery spanning the entire cell envelope, from the cytosol to the external environment. Transition to the transient open state is achieved by an iris-like mechanism in which entrance α -helices undergo an untwisting realignment, thought to be stabilized by interaction with periplasmic helices of the translocase. TolC family proteins are ubiquitous among gram-negative bacteria, and the conserved entrance aperture presents a possible chemotherapeutic target in multidrug-resistant pathogens.

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INTRODUCTION

It has been known for some time that mutants of *Escherichia coli* lacking the protein TolC exhibit pleiotropic phenotypes, which include tolerance to colicins and bacteriophage (1–3) and heightened sensitivity to environmental stresses [such as detergents, bile salts, and organic solvents (4–7)]. Although tolerance is due to the exploitation of TolC as a cell surface receptor, it is now known that the other phenotypes reflect the role of TolC in the expulsion of a wide range of molecules from gram-negative bacteria. TolC family proteins are central to the export of many large proteins, which include several that aid bacterial survival in mammalian hosts (8–11), and also the efflux of a plethora of small noxious molecules that are inhibitory to the bacteria (10, 12, 13). These efflux substrates have recently been shown to include antibacterial drugs. TolC is therefore a key player in the increasing problem of multidrug resistance and is important to the survival of pathogens during infections (7, 14–19). This review will discuss the structure of TolC and its common function in protein export and multidrug efflux.

TOLC-DEPENDENT PROTEIN EXPORT AND DRUG EFFLUX

Proteins destined for the cell surface or the surrounding medium of gram-negative bacteria, such as *E. coli*, must cross both the inner (cytoplasmic) and outer membranes and the intervening periplasmic space (20), which is believed to measure at least 130Å across. Most are secreted by large multiprotein assemblies that either span the periplasm or establish two-step mechanisms employing periplasmic intermediates (21–23). TolC-dependent type I protein export contrasts with these pathways because it bypasses the periplasm but requires only the outer membrane TolC, acting with an inner membrane translocase containing a traffic ATPase and an accessory or adaptor protein (11, 17, 24–27). Our work has focused on type I export of the 110 kDa hemolysin toxin (HlyA) (26) common among uropathogenic and enterohemorrhagic *E. coli*, but the same mechanism exports many other proteins, which include toxins, proteases, and lipases from pathogens of humans, animals, and plants (17, 28, 29) (Table 1). Genes encoding the protein substrates are usually located in operons coding for the corresponding export proteins. Export substrates are not subject to N-terminal processing by the signal peptidase; their secretion signal is typically uncleaved, about 50–60 residues long, and located at the extreme C terminus (30–32). There is little primary sequence identity among the signals, but interchangeability of type I export genes suggests that they may contain common higher order structures, such as an amphipathic α -helix (33, 34). C-terminal polypeptides are exported, and they direct export of heterologous proteins through the type I system, albeit weakly (32, 35).

TABLE 1 Substrates of TolC-dependent type I protein export

Substrates ^a	Protein export systems (inner membrane/ outer membrane) ^b	Bacterium
HlyA hemolysin	HlyBD/TolC	<i>Escherichia coli</i>
LktA leukotoxin	LktBD/—	<i>Pasteurella haemolytica</i>
AaltA leukotoxin	AaltBD/—	<i>Actinobacillus actinomycetemcomitans</i>
ApxI/II/III hemolysin	ApxBD/—	<i>Actinobacillus pleuropneumoniae</i>
CyaA adenylate cyclase	CyaBD/CyaE	<i>Bordetella pertussis</i>
PrtC protease	PrtDE/PrtF	<i>Erwinia chrysanthemi</i>
HasA metalloprotease	HasDE/HasF	<i>Serratia marcescens</i>
LipA lipase	LipBC/LipD	<i>Serratia marcescens</i>
Colicin V	CvaBA/TolC	<i>Escherichia coli</i>
Apr alkaline protease	AprDE/AprF	<i>Pseudomonas aeruginosa</i>
TliA thermostable lipase	TliDE/TliF	<i>Pseudomonas fluorescens</i>
PlyA exopolysaccharide glycanase	PrsDE/—	<i>Rhizobium leguminosarum</i>
SapA S-layer protein	SapDE/SapF	<i>Campylobacter fetus</i>
RasA S-layer protein	RsaDE/—	<i>Caulobacter crescentus</i>

^aAll substrates have a C-terminal uncleaved export signal, except the hemoprotein HasA, which has a cleaved C-terminal signal, and Colicin V, which has an N-terminal signal with similarity to the leader peptides of lantibiotics of gram-positive bacteria.

^bNo data is shown by —.

As in type I protein export, the TolC-dependent efflux of antibacterial drugs and other small inhibitory molecules involves TolC interacting with a translocase/pump of two inner membrane proteins (6, 15, 16). These also comprise a protein of the adaptor family and an energy-providing protein, which is sometimes an ATP-binding cassette (ABC) protein, but more often it is a proton antiporter of either the resistance nodulation division (RND) or major facilitator superfamily (MFS) class (10, 36, 37). Cells typically have several TolC homologues that act in a number of parallel efflux pumps, which typically have broad and sometimes overlapping substrate specificities (Table 2). For example, the *E. coli* genome encodes TolC, three homologues, and about 30 inner membrane translocases of the ABC, MFS (e.g., *E. coli* EmrAB), and RND (e.g., *E. coli* AcrAB) families (38), whereas the opportunistic pathogen *Pseudomonas aeruginosa*, in which multidrug resistance (MDR) poses a particular and growing clinical problem, has four major efflux (Mex) systems containing an RND proton antiporter and one of the three TolC homologues, OprM, OprJ, and OprN (39, 40).

TABLE 2 TolC-dependent drug efflux systems of *E. coli* and *P. aeruginosa*

Substrates ^a	Efflux systems (inner membrane/ outer membrane)
<i>E. coli</i>	
AC, AZ, BL, BS, CH, CM, CV, CP, DOC, EB, ER, FA, FQ, FU, NAL, NV, RF, TC, SDS, TX	AcrAB (RND)/TolC AcrEF (RND)/TolC
LC, CCCP, NAL	EmrAB (MFS)/TolC
ML	MacAB (ABC)/TolC
<i>P. aeruginosa</i>	
AC, AH, BL, CL, CM, CV, EB, FQ, ER, NV, SM, SDS, TL, TP	MexAB (RND)/OprM
AC, AH, CL, CM, CV, EB, ER, FQ, NV, SDS, TC, TP, TS	MexCD (RND)/OprJ
AH, CM, ER, FQ, TP, TS	MexEF (RND)/OprN
AG, ER, FQ, TC	MexXY (RND)/OprN

^aSubstrate abbreviations: AC, acriflavin; AG, aminoglycoside; AH, aromatic hydrocarbons; AZ, azithromycin; BL, β -lactams; BS, bile salts; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CH, cholate; CL, cerulenin; CM, chloramphenicol; CP, ciprofloxacin; CV, crystal violet; DOC, deoxycholate; EB, ethidium bromide; ER, erythromycin; FA, fatty acids; FQ, fluoroquinolones; FU, fusidic acid; LC, lipophilic cations; ML, macrolides; NAL, nalidixic acid; NV, novobiocin; RF, rifampicin; SDS, sodium dodecyl sulfate; SM, sulphonamides; TC, tetracycline; TL, thiolactomycin; TS, triclosan; TP, trimethoprim; TX, Triton X-100.

In addition to its role in these three-component protein export and drug efflux machineries, *E. coli* TolC is also utilized for the exit of low-molecular-weight peptides, such as the heat-stable enterotoxin, cationic antimicrobial peptides, and microcins, which are transported to the periplasm either by the housekeeping Sec system or other designated inner membrane transport systems (41–43). Little is known of how TolC is accessed by these periplasmic peptides.

THE TolC STRUCTURE: THE KEY TO A COMMON MECHANISM OF EXPORT AND EFFLUX

Biochemical studies have shown that TolC-dependent protein export requires direct contact between outer membrane TolC and substrate-laden translocases in the inner membrane (44). However, it was not evident how substrate engagement at the inner membrane could be coupled, without periplasmic intermediates, to substrate exit through what was imagined to be a simple outer membrane porin-like channel. Reconstitution of purified TolC into phospholipid bilayers

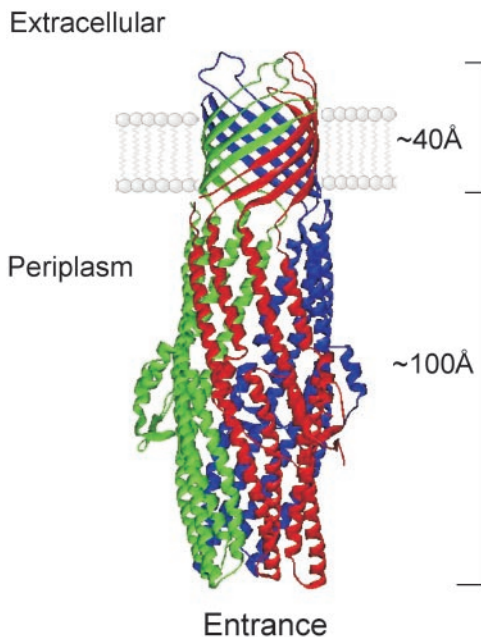
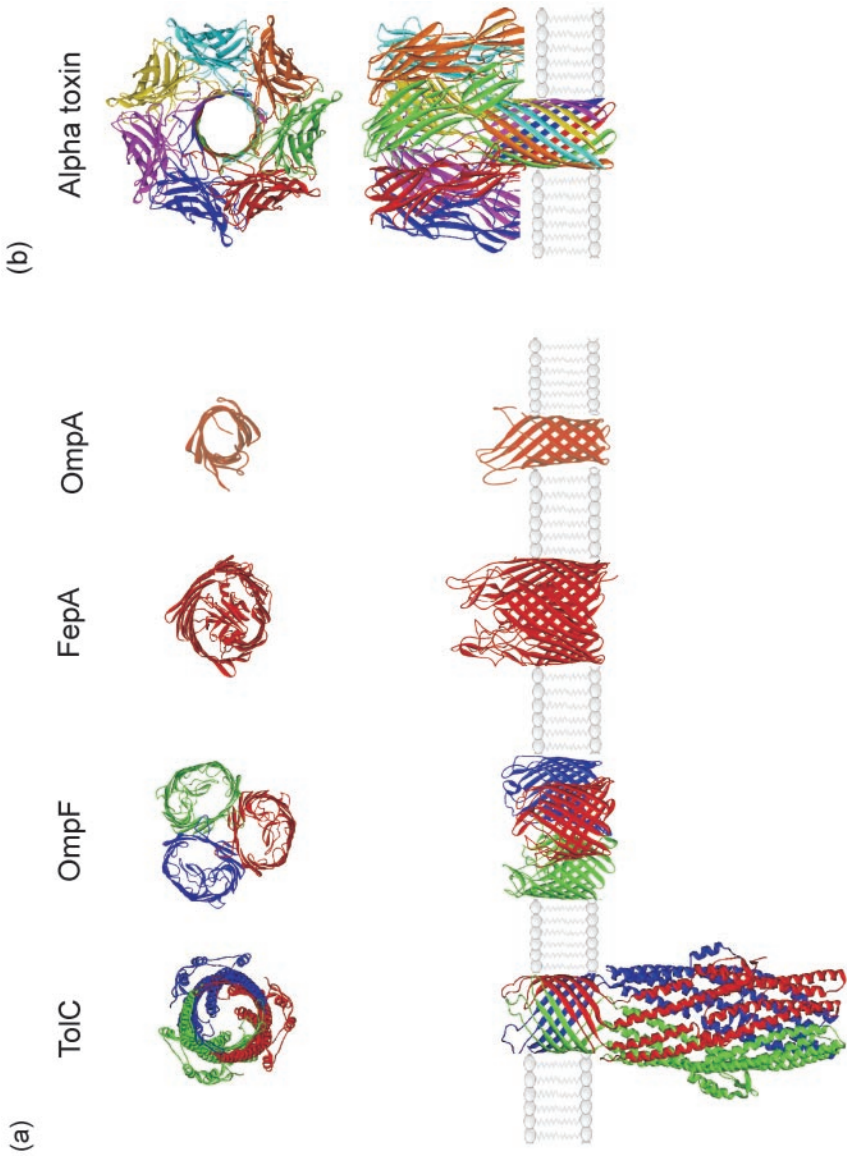


Figure 1 The structure of TolC. C α trace of the trimer (47). The individual protomers are colored blue, red, and green. The lipid bilayer represents the bacterial outer membrane. The molecular threefold rotation axis is aligned vertically and is assumed to be normal to the plane of the outer membrane. The outer membrane embedded β barrel is open to the extracellular medium, but the coiled coils taper to close the periplasmic entrance of the α -helical barrel.

allowed electron microscopy of two-dimensional crystals of 13Å resolution (11), establishing that the 471 amino acid TolC is trimeric and indicating that it had a novel single pore and possibly an additional domain that could contribute to a periplasmic bypass. Nevertheless, to establish how TolC could mediate export and efflux, a high-resolution structure was needed. Crystallization of TolC was a complex process. Initially, crystals of the intact membrane protein had high mosaicity, and crystallization was further complicated by the nature of TolC, as it turned out to be an atypical membrane protein with a lipid-embedded domain fused to a large extramembranous domain stable in an aqueous environment. Satisfactory crystal packing was only achieved when the flexible C-terminal 43 residues were removed, a truncation that did not attenuate TolC function in *E. coli*. Collection of multiple wavelength anomalous dispersion (MAD) data using selenomethionine (SeMet) derivatives was finally possible when heterogeneity in the oxidation state of selenium was overcome by oxidation of the SeMet residues (45). The resulting TolC crystals were loosely packed with a relatively high solvent content of 70% (46). It is noteworthy that the charged residues (in the



equatorial domain and extracellular loops, see below) that dictated intermolecular contact in the TolC crystal lattice are variable throughout TolC homologues, and therefore they might not be crystallized by the same protocol.

TolC: A TRANS-PERIPLASMIC CHANNEL TUNNEL

X-ray crystallography at 2.1Å resolution (47) revealed that TolC is fundamentally different to known outer membrane proteins. The TolC homotrimer is a tapered hollow cylinder 140Å in length; it comprises a 40Å long outer membrane β barrel (the channel domain) that anchors a contiguous 100Å long α -helical barrel projecting across the periplasmic space (the tunnel domain) (Figure 1). A third domain, with a mixed α/β structure, forms a belt around the equator of the tunnel. TolC thus provides a water-filled exit duct with a volume of 43,000Å³. The average accessible interior diameter of the single TolC channel-tunnel pore is 19.8Å (30Å backbone to backbone) throughout the outer membrane channel and most of the tunnel. TolC is a 12-stranded barrel. Each of the three monomers contributes four antiparallel β -strands and four antiparallel α -helical strands (two continuous long helices and two pairs of shorter helices) to form the channel and the tunnel domains, respectively.

Although β barrels are typical of outer membrane proteins (48–50), the TolC channel domain is distinct because the trimer forms a single β barrel (Figure 2a, Table 3). In TolC, the protomers each contribute four β -strands to the 12-strand β barrel, an architecture distinct from other membrane proteins (51, 52), which form one barrel per monomer (Figure 2a). It is to some degree comparable to that of the *Staphylococcus aureus* alpha-toxin in which seven subunits assemble a single barrel in mammalian target membranes (53) (Figure 2b). The TolC outer membrane β barrel is constitutively open to the external medium; it lacks the inward folded loop that constricts the β barrels of channel-forming proteins like OmpA (54, 55) and does not have a plug domain, such as the one that closes the β barrels of FhuA and FepA (56, 57) (Figure 2a). The small extracellular loops of TolC are the sites of colicin and bacteriophage attachment (3). Notwithstanding these singularities of the TolC β barrel, the most distinctive feature of TolC is the 100Å-long periplasmic tunnel (Figure 1). The TolC α barrel comprises 12

Figure 2 TolC and other pore-forming proteins. Viewed from above the lipid bilayer (*upper*) and through the plane of the membrane (*lower*), (a) *E. coli* outer membrane proteins TolC, OmpF, FepA, and OmpA. The porin OmpF is trimeric, but each monomer forms a barrel of 16 β -strands (51). Monomeric OmpA forms the smallest known barrel of 8 β -strands (52), while the large β barrels of the iron siderophore transporters FhuA and FepA comprise 22 β -strands (56, 57). (b) The pore-forming *S. aureus* alpha-toxin inserted in a eukaryotic membrane. Seven subunits contribute 14 β -strands to a single barrel (53).

TABLE 3 Structural properties of TolC and other membrane proteins

Properties	<i>E. coli</i> TolC	<i>E. coli</i> OmpF	<i>E. coli</i> FepA	<i>E. coli</i> OmpA	<i>S. aureus</i> alpha-toxin
Length (Å)	140	35	70	57	100
Radius (Å)	17.5	15.5	19.9	13	8
Constriction diameter (Å)	3.9	11	n/a	n/a	14
Number of pores	1	3	1	1	1
Number of monomers	3	3	1	1	7
β -strands per monomer	4	16	22	8	2
Conductance ^a (pS)	80	840	n/a	n/a	1000

^aConductances are measured at 1M KCl or NaCl.

α -helices (4 from each monomer) packing in an antiparallel arrangement. The structural principles underlying how helices are constrained in the α barrel have been described (46, 47, 58). The barrel is assembled by each of the 12 helices packed laterally with two neighboring helices. This is stabilized by intermeshing of side chains, known as “knobs-into-holes” packing. Throughout the α barrel the helices follow a left-handed superhelical twist, but they are underwound in the upper (β -barrel-proximal) half compared to helices in a conventional two-stranded coiled coil (Figure 3). This enables them to lie on a cylindrical surface, possibly further facilitated by bulkier side chains that tend to partition to the outside of the barrel. Assembly of the tunnel is additionally supported by hydrogen bonds within and between the helices, and salt bridges formed at the interface of the monomers may play a role in the trimerization. (It is not known how TolC trimers are assembled in vivo nor whether this is aided by cellular proteins, such as chaperones or enzymes that degrade the peptidoglycan.) In the lower (β -barrel-distal) half of the α barrel, neighboring helices form six pairs of regular two-stranded coiled coils, and at the periplasmic end, one coil from each monomer folds inward. This constricts the periplasmic entrance to a resting closed state, with an effective diameter of $\sim 3.9\text{\AA}$ (47), which is consistent with the small (c.80pS) conductance of TolC in planar lipid bilayers (59, 60). The periplasmic entrance is the only constriction in the TolC pore. The structure indicated vividly the means by which TolC allows the exit of a wide range of substrates from the cell, by presenting a common exit duct for substrates engaged by inner membrane translocases.

TOLC IS CONSERVED THROUGHOUT GRAM-NEGATIVE BACTERIA

TolC homologues are seemingly ubiquitous among gram-negative bacteria, as nearly 100 family members have been identified in over 30 bacterial species (10, 61). Primary sequence similarity correlates with the function of the homologues

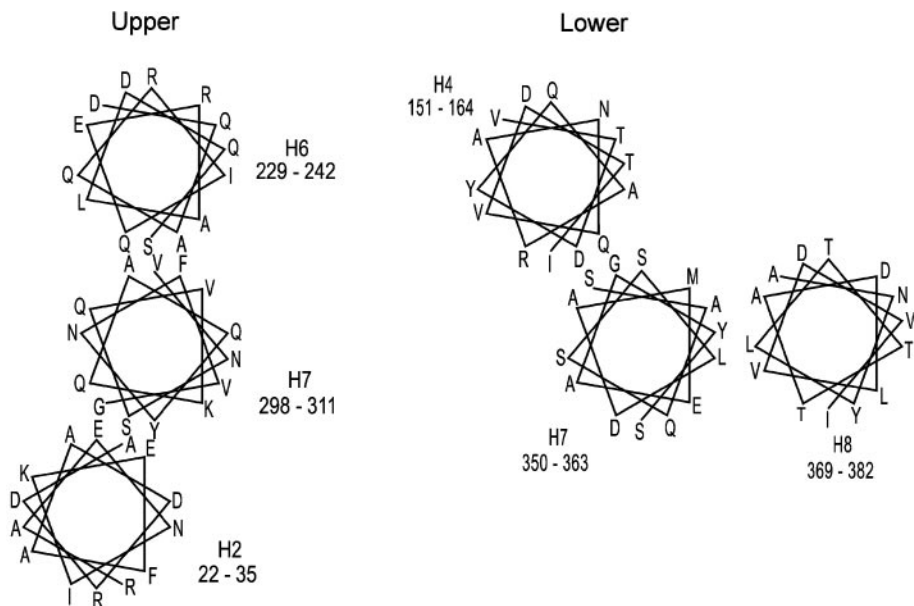


Figure 3 Helix interactions in the TolC periplasmic α barrel. Helical wheel representations summarizing the inter-helical contacts in the upper part (*left*) and the lower part (*right*) of the α -helical barrel.

in protein export, cation efflux, or multidrug efflux (61, 62) (Figure 4). The sequences and structures of the N- and C-terminal halves of the TolC monomer are similar to each other (47), and this internal duplication is evident throughout the family. The strongest intramolecular identity is seen in *Bordetella pertussis* CyaE (61) (Table 1), and CyaE is also nearest to the root of the tree, suggesting that it is closest to the family progenitor.

Although homologues vary in sequence length, this is due primarily to extensions at the periplasmic N and C termini (61). Significant sequence gaps or insertions occur only in the equatorial domain outside the α/β -barrel structure and in the extracellular loops. Sequences encoding the α/β barrels do not vary substantially in length, and experimental deletions or insertions in the barrel domains are poorly tolerated (65, 66). Few amino acids are conserved among the TolC homologues, but those that are seem structurally significant. In particular, transition from the left-twisted α barrel of the periplasmic domain to the right-twisted β barrel of the outer membrane channel domain is accommodated by conserved linkers containing proline and glycine. Glycines facilitate a tight turn between the helices forming the periplasmic tunnel entrance, and small residues, such as alanine and serine, at the interface of tunnel-forming helices allow the dense packing that determines tapering and entrance closure. At the entrance aperture, an electronegative inner surface is a conserved feature, most commonly

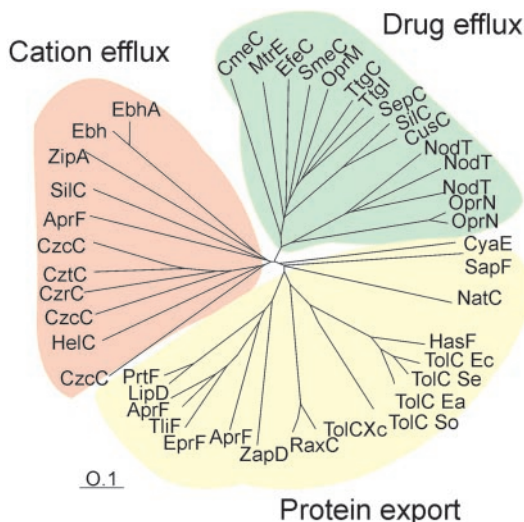


Figure 4 The TolC family. The phylogenetic tree of homologues is derived from primary amino acid sequence alignment (63) and sorted by Treeview (64). TolC homologue function, either known or strongly implicated by the coding gene context, is indicated as cation efflux, drug efflux, or protein export (homologues of unknown function are not included). Scale 0.1 indicates 0.1 nucleotide substitutions.

due to a ring of aspartic acid residues (47) (in *E. coli* TolC Asp³⁷¹ and Asp³⁷⁴). Conserved aromatic residues face outward to form a ring around the channel domain at the base of the β -strands, delimiting the inner edge of the lipid bilayer. This seems to be a universal feature in OM protein structures and possibly performs an anchor function. Conservation of the principle structural elements suggests that the functions of TolC are common to the homologues (61). This is compatible with genetic data indicating that at least some TolC homologues are interchangeable (67–69).

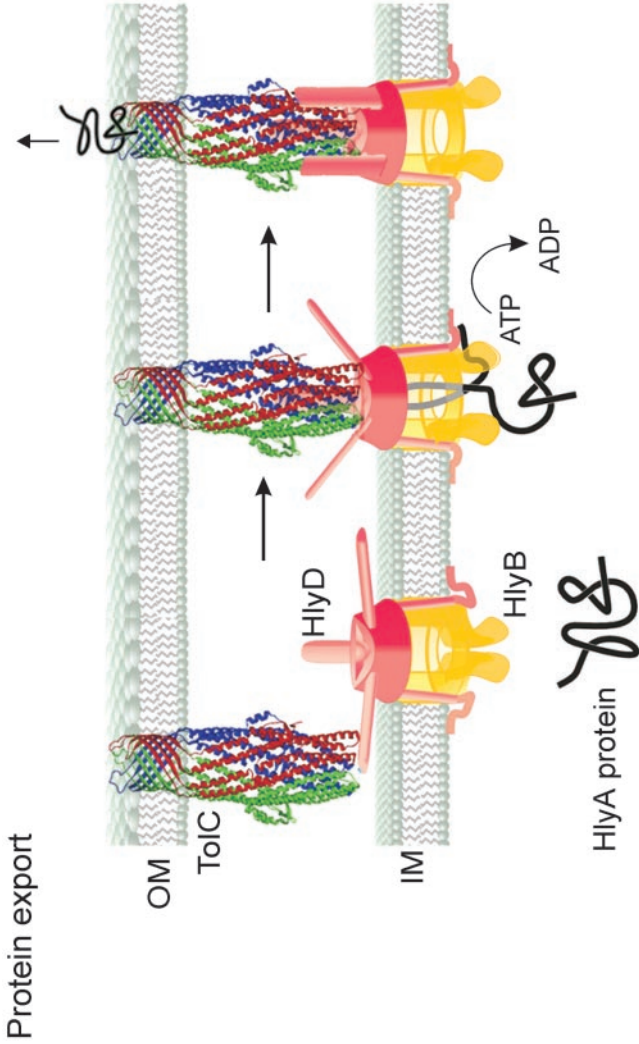
ASSEMBLY OF TolC-DEPENDENT MACHINERIES: RECRUITMENT OF TolC

In vivo cross-linking has defined the sequence of protein-protein interactions underlying export (44). The inner membrane translocase is formed constitutively, i.e., even in the absence of their respective substrates (44, 70, 71). TolC recruitment by the inner membrane translocase is clearly a central step in the mechanism, and it has been shown to occur in response to substrate engagement by the translocase (Figure 5, upper). All three export components undergo conformational changes during the substrate-induced assembly of the machinery

(44). The use of chemical uncouplers has indicated that this pretranslocation assembly of the substrate-bound export complex requires the membrane electrochemical potential but not ATP hydrolysis by the traffic ATPase (72–74). Recruitment is mediated by the adaptor protein, which is a common element in all translocases of TolC-dependent drug efflux and protein export (44, 75). Efflux adaptors are predicted to be anchored to the inner membrane by a transmembrane helix, e.g., MFS EmrA, or by an N-terminal lipid modification, e.g., RND AcrA and MexA (Figure 6) (18), and biophysical studies of AcrA have predicted an extended structure in solution, c.210Å long with an 8:1 axial ratio (76, 77). The adaptor of the *E. coli* type I hemolysin export machinery is HlyD, which comprises a large periplasmic domain (residues 81–478) connected by a single transmembrane helix to a small N-terminal cytosolic domain (residues 1–59) (Figure 6). HlyD seems to assemble to at least a trimer (44, 70), while oligomerization is also indicated by low resolution (20Å) electron microscopy of lipid-reconstituted RND efflux adaptor AcrA (77), although no structure is discernable.

The principle feature of adaptor proteins is a large periplasmic domain, which is predicted to contain long coiled coils that may form an α -helical hairpin (62) (Figure 6). During assembly, the predicted coiled-coil structures of the adaptor could contact the coiled coils of the periplasmic tunnel α barrel, possibly reaching to the equatorial domain to recruit TolC. When the protein substrate is exported, the inner and outer membrane components revert to their resting state (44). It is therefore envisaged that the adaptor has a dynamic function, which effects a transient coupling of the TolC channel tunnel to the energy-providing protein of the cognate translocase.

Notwithstanding the evidence supporting a common role for the adaptors in TolC recruitment, the 3.5Å resolution crystal structure (78) of the *E. coli* RND AcrB [which forms a constitutive inner membrane complex with AcrA (71)] has prompted speculation that this class of antiporter might be able to contact the TolC periplasmic tunnel entrance directly (Figure 5, lower). AcrB is a trimer with a periplasmic domain of length 70Å and an 80Å diameter comprising two large hydrophilic periplasmic loops from each monomer (Figure 7). The contiguous transmembrane domain is composed of 36 α -helices, 12 from each monomer, and is 50Å in length and 100Å in diameter, with an opening at the cytosolic side of the inner membrane (78). As with TolC, there is structural similarity between the N- and C-terminal halves of the 1049 residue AcrB, suggesting that evolution of this protein has also involved a gene duplication event. It is proposed that six hairpins at the top of the AcrB trimer could dock with the six α -helix turn α -helix structures at the base of TolC. Such a docking might not be stable and may be precluded in the protein export pathway and the MFS and ABC efflux machineries because their energy transducing components, traffic ATPases and antiporters, are not predicted to have substantial periplasmic domains (18, 38) (Figure 8). In the various systems, the adaptor may therefore function to stabilize RND antiporter docking, while in all cases effecting the TolC recruitment required to



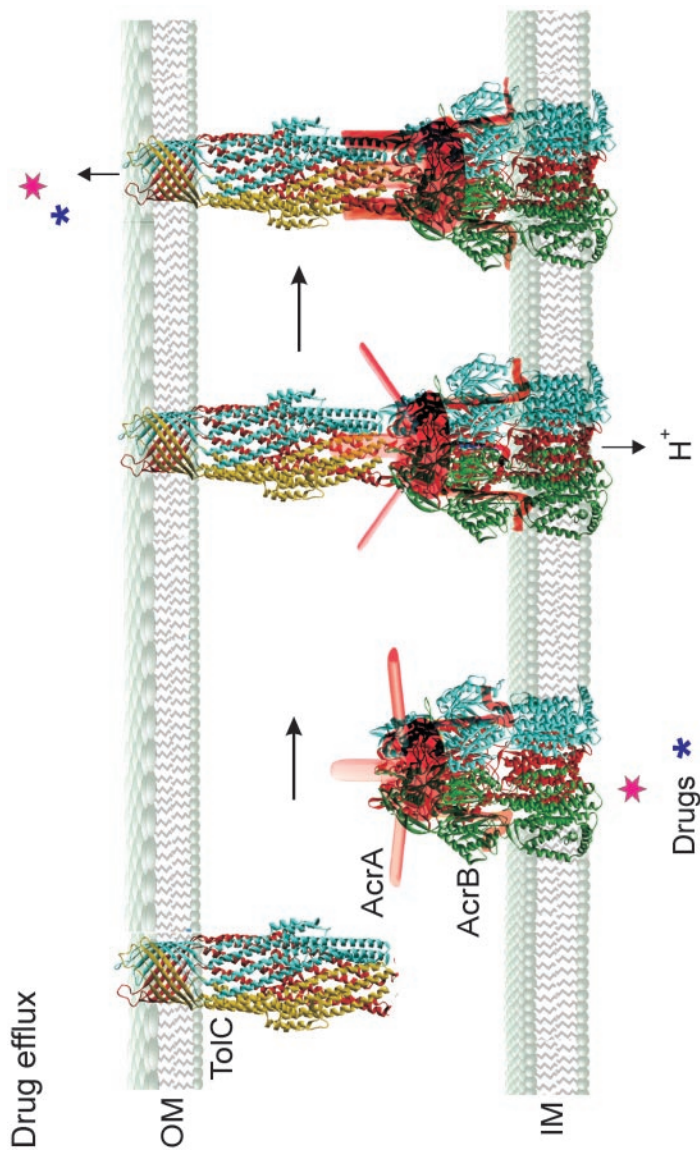


Figure 5 Assembly of TolC-dependent export and efflux machineries. A model indicating reversible interaction of outer membrane TolC or a homologue with substrate-specific inner membrane complexes (translocases) containing an adaptor protein and an energy-providing protein, either a traffic ATPase in protein export (*upper*, indicating the type I hemolysin export) or typically an antiporter in drug efflux (*lower*, indicating the RND pump). In both cases, transport occurs across the inner membrane (IM), outer membrane (OM), and the intervening periplasmic space.

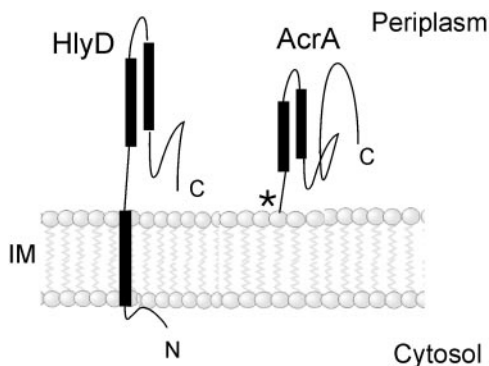


Figure 6 Representation of inner membrane (IM) adaptor proteins. A putative topological representation of the HlyD (type I protein export) and AcrA (RND drug efflux) monomers based on secondary structure predictions. The asterisk indicates lipophilic modification; black boxes are predicted α -helices, which could potentially form coiled coils.

open the periplasmic entrance. The assembled RND drug efflux machinery has not been isolated (37), but recent results show that, though the purified AcrA adaptor has micromolar affinity for the AcrB antiporter and for TolC, no binding was detectable between AcrB and TolC *in vitro* (V. Koronakis, T. Touz , J. Eswaran, E. Bokma, E. Koronakis, and C. Hughes, unpublished), possibly reflecting a need to be stabilized by AcrA *in vivo*. It may be significant that the export adaptor HlyD has additional sequence in the periplasm (62, 75), compared to AcrA; perhaps this compensates for the lack of a periplasmic domain in its translocase partner.

A view of substrate-responsive TolC recruitment by translocases envisages transduction of the substrate-binding signal across the inner membrane from the cytosolic face of the translocase. Protein substrates interact independently with both the traffic ATPase and adaptor *in vivo* (44, 70), although genetic and biophysical studies suggest that the initial interaction involves the substrate export signal and the (c.250 residue) cytosolic ATPase domain of the traffic ATPase (79, 80). This is predicted to be fused to an N-terminal domain encompassing six transmembrane helices, as exemplified by the 707 residue hemolysin B (HlyB) (81, 82) (Figure 8). However, substrate interaction involves not only this initial signal recognition as the substrate is engaged (70) but also a subsequent step in which substrate binding to the adaptor triggers TolC recruitment. Removal of the small N-terminal cytosolic domain of the export adaptor HlyD abolishes protein export and substrate-adaptor interaction, and small deletions within this domain disable TolC recruitment (and therefore protein export), even though substrate is still engaged. Such mutants thus appear to be defective specifically in triggering recruitment in response to substrate engage-

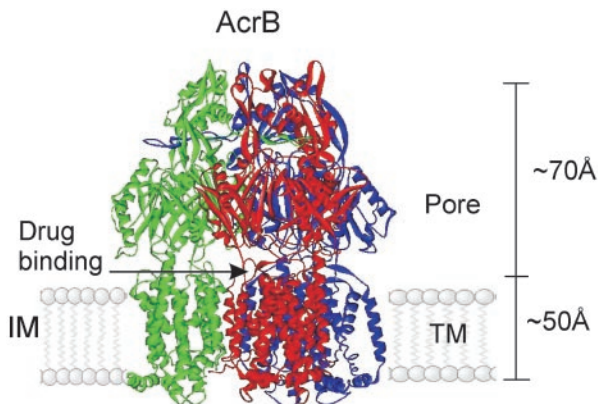


Figure 7 Structure of the inner membrane (IM) drug efflux antiporter AcrB. Ribbon depiction of the crystal structure of trimeric AcrB (78); colors indicate each protomer. AcrB comprises transmembrane (TM) and periplasmic regions, the latter encompassing pore and TolC-docking domains. The arrow indicates a region suggested to bind drug efflux substrates (86).

ment. It is assumed that this engagement signal is transduced by an intramolecular allosteric mechanism to the coiled coils of the adaptor periplasmic domain.

SUBSTRATE TRANSLOCATION THROUGH TolC-DEPENDENT MACHINERIES

Little is known about substrate translocation, although experiments with chemical uncouplers (72) suggest that it does not require the electrochemical potential but is driven solely by traffic ATPase ATP hydrolysis (the HlyB cytosolic domain has an *in vitro* V_{max} of $1 \mu\text{molATP}/\text{min}/\text{mg}$ and a K_m of 0.2 mM ATP) (73, 74). Mutations that abolish hydrolysis of ATP bound at the cytosolic domain disable passage of protein through the TolC-dependent system, causing accumu-

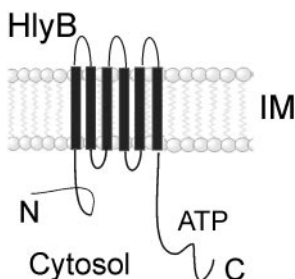


Figure 8 Representation of the inner membrane (IM) traffic ATPase. A putative topology of the HlyB monomer based on secondary structure predictions.

lation of a stalled intermediate complex containing the substrate, translocase and TolC (44). Although this evidence supports a specific role for ATP hydrolysis in substrate translocation through the assembled machinery, little is known yet of how ATP hydrolysis, substrate (un)folding, and substrate movement are coupled (82). It is possible that large protein substrates pass through the translocase in a partially unfolded state, resulting in “ratcheted” translocation driven by ATP hydrolysis, analogous to that suggested for mitochondrial protein import (83). This, however, is speculation, especially as no contacts have been defined between internal regions of the substrate and any of the export proteins. Although no high-resolution structure of a traffic ATPase has been solved, i.e., complete with its transmembrane domain, the structures of several ATP-binding proteins and isolated domains (84, 85) are compatible with biochemical evidence suggesting they function as homodimers (73, 84, 85). But it is not yet possible to discern the nature of interaction between the ATPase monomers or their transmembrane and cytosolic domains (84).

Drug efflux systems, such as AcrAB-TolC, expel a wide variety of small structurally unrelated compounds (13, 16) (Table 2). It seems likely that these substrates could enter the efflux channel through the opening at the cytosolic face of AcrB (78), but it has also been proposed that substrates might enter AcrB laterally from within the membrane, on both the cytosolic and periplasmic sides (78). The crystal structure of AcrB liganded with diverse substrates revealed binding at nonidentical positions in the transmembrane domain (86). Although this could partly explain how diverse drug efflux substrates are accommodated, hybrid transporter studies indicate that the antiporter periplasmic domain plays the major role in determining substrate specificity (68, 69), and the drug binding sites in the transmembrane domain may be only a snapshot of a transient point in the efflux process. Once past the TolC entrance, the substrates, even large proteins, can readily pass through what is effectively the external environment. Nevertheless, substrates have a wide variation in charge and other physicochemical properties, and it has been proposed that the strikingly electronegative surface of the TolC tunnel could affect movement of substrates out of the bacterium (47).

TOLC OPERATION: TRANSITION TO THE ENTRANCE OPEN STATE

In both protein export and drug efflux, substrates are channeled through the translocase to the periplasmic entrance of TolC. It is not known how small molecules, such as drugs, trigger opening of the entrance, but as in protein export, this transition to the open state must occur because the resting state entrance aperture is too small for their passage (47) (Figure 9, left). Opening of the entrance is therefore key to the function of TolC and to the export and efflux

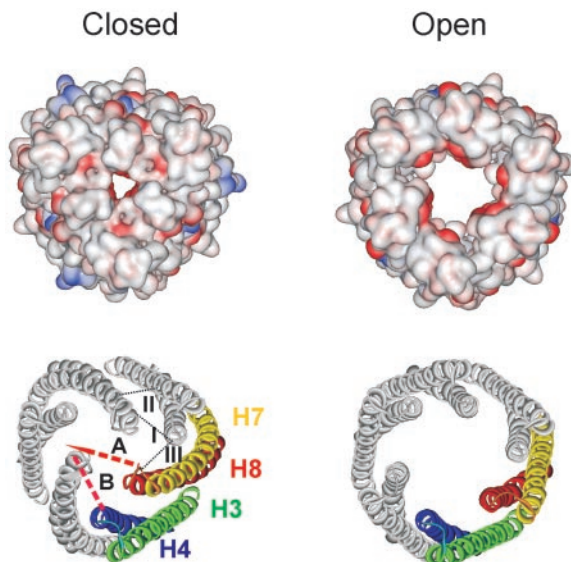


Figure 9 The closed and modeled open states of TolC. Space-filled (*upper*) and ribbon (*lower*) depictions of the closed (*left*) and modeled open (*right*) states (47) of the tunnel entrance, viewed from the periplasm. The coiled coils of one protomer are colored (H3/4 and H7/8; numbering taken from Reference 47) and show the constraining intramonomer (I and II) and intermonomer (III) links. The crystal structure (*closed*) shows the coiled coils closing the end of the tunnel. The open-state model (*open*) illustrates how the channel may be opened.

machineries. The dense packing of the helices at the periplasmic entrance suggests a very stable structure, and our *in vitro* analyses of TolC in planar lipid bilayers show that opening can not be induced by high voltage, low pH, or even urea (60). TolC must therefore undergo a conformational change to allow passage of substrate. An allosteric mechanism has been proposed for TolC opening (47). This is based on the observation that the three inner coiled coils (comprising helices H7 and H8) differ from the outer coiled coils (H3/H4) only by small changes in superhelical twist, and it envisages that transition to the open state is achieved by the inner coil of each monomer realigning relative to the outer coil, thereby enlarging the aperture diameter (Figure 9, right). Comparison of the resting closed state of the entrance observed in the crystal structure with the modeled open state (47) identifies inter- and intramolecular bonds that constrain the three inner coils in the closed conformation (Figure 9). Links I and II connect each inner coiled coil to the outer coil of the same monomer by hydrogen bonds between Asp¹⁵³-Tyr³⁶² and Gln¹³⁶-Glu³⁵⁹, respectively. Link III connects Arg³⁶⁷ of each inner coiled coil to the outer coil of the adjacent monomer by a salt bridge to Asp¹⁵³ and a hydrogen bond to Thr¹⁵². In this model

of entrance opening, these links must be disrupted for the inner coiled coils to move outward and enlarge the entrance diameter.

This model is supported by data from both *in vivo* and *in vitro* experiments. Formation of the salt bridge and hydrogen bonds was prevented by substituting critical residues, and because the periplasmic entrance is the sole constriction of the 140Å long pore (47, 60), change in the diameter of the entrance aperture was monitored as the conductance of purified TolC proteins in black lipid bilayers. Elimination of individual connections I and II caused only small changes in conductivity, whereas significant increases resulted from disruption of the R³⁶⁷-D¹⁵³ salt-bridge of intermonomer connection III (Figure 10). When both components of link III were disrupted simultaneously with the intramonomer link I, there was a synergistic effect, dictating a 6- to 10-fold increase over wild-type conductance (87). This would be compatible with an aperture of 16Å, which corresponds to the modeled open state. These results support a view of transition to the open state by an iris-like realignment of the entrance helices, generating an aperture large enough to allow passage of diverse substrates. Complementary *in vivo* evidence was obtained by introducing disulphide bonds to constrain the entrance coiled coils in the closed state (88). Type I hemolysin export from *E. coli* was abolished by introducing intermonomer disulphide bridges cross-linked at the narrowest point of the entrance constriction, either between Asp³⁷⁴ of adjacent monomers (link A) or between Asn¹⁵⁶ and Ala³⁷⁵ to connect the inner coil of each monomer to the outer coiled coil of its adjacent monomer (link B). When the TolC entrance was locked and there was no export, the hemolysin protein substrate was still bound at the inner membrane translocase and triggered recruitment of the locked TolC (88). These results confirm that untwisting the entrance helices is essential for TolC function and show that this acts specifically to open the entrance and allow passage of substrate engaged at the inner membrane complex.

In the bacterium, transition to the TolC open state is linked to recruitment of TolC by the inner membrane translocase adaptor, although the recruitment of locked TolC shows that the opening step can be uncoupled. The target for the adaptor interaction could be the TolC entrance itself, but interaction with the periplasmic equatorial domain could also mediate opening. Its strands and helices pack against the inner set of coiled coils, and any change in this relationship, induced by interactions with the adaptor protein, could activate an allosteric transition in the coiled coils. The adaptor coiled-coil domain could repack against the untwisted coiled coils at the base of TolC, stabilizing the open state.

PUMPS AS DRUG TARGETS: IS IT POSSIBLE TO BLOCK THE TolC ENTRANCE?

Knowledge of the structure and function of the machineries will not only further the understanding of the mechanism underlying protein export and drug efflux, but it may permit rational design of potential antibacterial agents for the treatment of

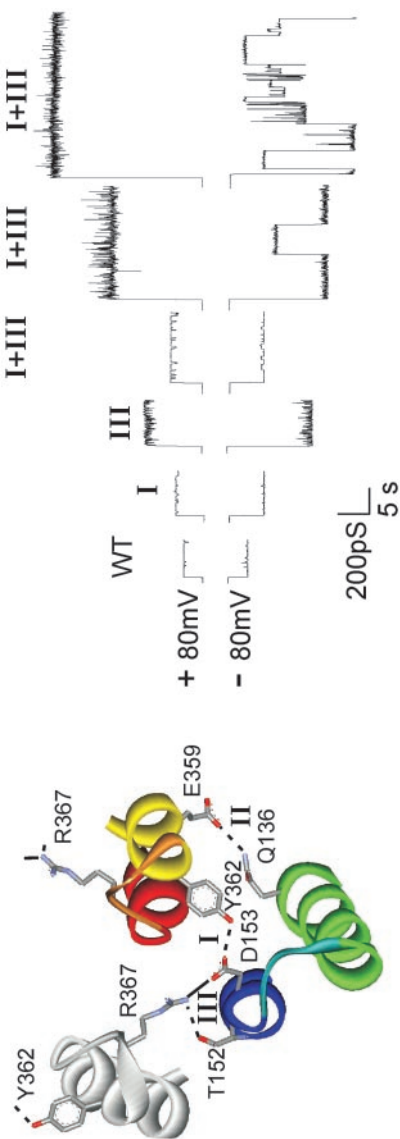


Figure 10 The entrance opening mechanism. Experimental evaluation of the “twist-to-open” mechanism for transition from the closed to open state of ToIC. The four helices of one protomer are colored as in Figure 9. Experiments shown are in vitro disruption of entrance constraining links I and III using different amino acid substitutions. The corresponding changes in conductance of the purified ToIC derivatives in planar lipid bilayers are compared with the ToIC wildtype (87).

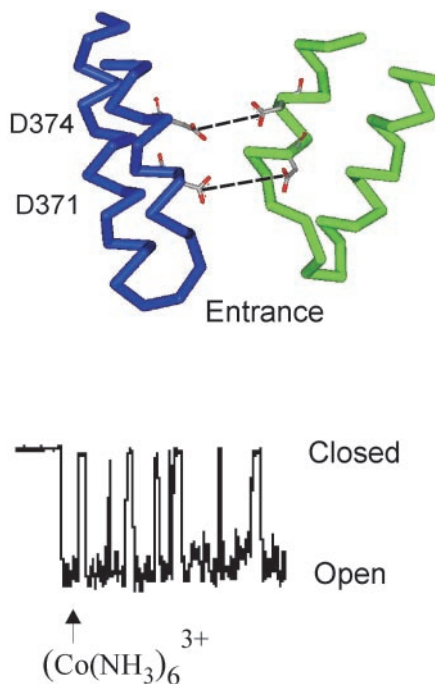


Figure 11 Blocking the electronegative entrance. The electronegative aspartate rings are formed by residues D^{371} and D^{374} of each monomer (two adjacent monomers are shown) (*top*). Conductance in lipid bilayers of wild-type TolC shows single reversible blocking events in the presence of 3 nM hexaamminecobalt trichloride (*bottom*).

multidrug-resistant bacterial infections. The importance of channel tunnels to bacterial survival, especially during infection, suggests they may present a possible target. The periplasmic entrance of TolC is the sole constriction in the exit duct (Figure 1), and the negatively charged residues at the entrance might be liganded to effect irreversible closure of the tunnel, thus reducing virulence and drug resistance. Initial investigation of this possibility shows that TolC pore function in artificial lipid bilayers is severely inhibited by divalent and trivalent cations introduced into the channel from the extracellular side (89). Trivalent cations are most potent, with hexaamminecobalt binding at nanomolar affinity. The TolC entrance constriction is lined by a ring of six aspartate residues, D^{371} and D^{374} from each of the three monomers (Figure 11) (46, 47). When either or both of the entrance aspartates are substituted by alanines, high-affinity binding is abolished, and blocking of the membrane pore is alleviated (89). This is compatible with the inhibitor binding to the entrance aspartate ring, which is also indicated by X-ray crystallography of the liganded TolC (M.H. Higgins and V. Koronakis, unpublished). These results may suggest a strategy to develop

bioactive molecules, especially as the electronegative entrance is widely conserved throughout the TolC family of gram-negative bacteria.

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