Review

CellPress

Special Issue: Microbial Translocation

Assembly and operation of bacterial tripartite multidrug efflux pumps

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Microorganisms encode several classes of transmembrane pumps that can expel an enormous range of toxic substances, thereby improving their fitness in harsh environments and contributing to resistance against antimicrobial agents. In Gram-negative bacteria these pumps can take the form of tripartite assemblies that actively efflux drugs and other harmful compounds across the cell envelope. We describe recent structural and functional data that have provided insights into the transport mechanisms of these intricate molecular machines.

Throughout the course of the past billion or so years, microorganisms have likely explored vast expanses of chemical space in synthesizing toxic compounds that are useful for defending established ecological niches or for invading and dominating new environments. In response, competing microorganisms have generated mechanisms to evade the cytotoxic effects of these compounds as well as other environmental hazards. This complex evolutionary interplay has generated numerous resistance mechanisms, including the chemical modification and degradation of the toxic compounds, mutation or overexpression of the vulnerable cellular targets, reduced uptake across the cell envelope, and active efflux of a wide range of toxic compounds from the cell by efflux pumps [1].

Five families of transmembrane (TM) transporters have been identified thus far that are involved in active efflux of antimicrobial agents, and these proteins are widely distributed across life (Figure 1) [2–6]. One such family comprises the ATP-binding cassette (ABC) proteins, which are 'primary-active' transporters in that they use the free energy of ATP binding and hydrolysis to catalyze drug extrusion [7]. Members of this family are widely distributed in all domains of life, where they provide analogous functions; for instance, in human cancer cells ABC transporters contribute to resistance against chemotherapeutic agents. The 'secondary-active' multidrug transporters are antiporters, driven by proton and/or sodium motive force, that couple drug efflux to the influx of protons or sodium ions down their electrochemical gradients across the cytoplasmic membrane. These secondary-active transporters comprise four distinct protein families, namely the major facilitator (MFS), small multidrug resistance (SMR), resistance/nodulation/cell division (RND), and multidrug and toxic compounds extrusion (MATE) families [8,9]. These resistance pumps are widely distributed among bacterial lineages including major pathogenic species [10]. To date, hundreds of bacterial drug transporters have been characterized, some of which can extrude a wide range of chemically diverse compounds, including antibiotics, antiseptics, dyes, detergents, and solvents [11,12].

Tripartite multidrug efflux pumps in Gram-negative bacteria span the cell envelope

The envelope of Gram-negative bacteria, which is a formidable protective barrier against myriad environmental hazards, has three principal layers: the outer membrane with an associated lipopolysaccharide (LPS) coat on the cell exterior, the inner membrane that adjoins the cytoplasm, and the peptidoglycan cell wall that is in the interstitial periplasm between the two membranes, which contributes to mechanical rigidity [13,14] (Figure 1). Moving molecules through this barrier, whether it is the uptake of nutrients, the expelling of small toxic compounds, or the transport of large effector proteins such as virulence factors, requires special machinery to negotiate a course through the different layers [15–19]. Cytotoxic compounds, including clinical antibiotics, can be driven from the cell by intricate nanomachines that are tripartite assemblies spanning the width of the envelope. Such machines generally include an outer-membrane protein, an inner-membrane protein, and a periplasmic 'membrane fusion' protein that provides a connection between the other two components. The inner-membrane proteins, which couple the transport process to metabolic energy, typically belong to the MFS, RND, or ABC families. Well-studied examples of each of the three families transporters-based tripartite efflux pumps are the MFS-type efflux pump EmrA–EmrB–TolC, the RND-type acridine efflux pump AcrA–AcrB–TolC, and the ABC-type macrolide pump MacA–MacB–TolC [20–22].

Structural information on tripartite pump components has advanced our understanding of how the individual pump components might work in the assembly. A recent cryo-electron microscopy (cyro-EM) structure of a RND-type tripartite multidrug efflux pump has shown how the components fit together into an operating machine

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Keywords: membrane proteins; drug resistance; transport mechanism.

0966-842X/
 http://dx.doi.org/10.1016/j.tim.2015.01.010

[22]. We discuss each of the components in turn before describing the full assembly.

Outer-membrane proteins

A representative outer-membrane component of the tripartite multidrug efflux pump is TolC from *Escherichia coli* (Figure 1, top right). TolC assembles as an elongated homotrimer and can be succinctly described as having three distinct structural domains: β-barrel, α-helical barrel, and a mixed α/β-fold known as the equatorial domain [23]. The protomer has a structural repeat, originating from gene duplication, such that the tube-like architecture has approximate sixfold symmetry – this will become important later when we consider the assembly of the transporter. Another notable aspect of the tube is that it bears a large interior cavity that is mostly solvent-filled with an average accessible interior diameter of roughly 20 Å. The tube is well shaped to form an exit duct – except for the fact that it is tightly constricted at the end distal to the outer-membrane. The effective diameter here is only ~3.9 Å, which is much too small for the transport substrates to negotiate. This constriction arises from the inwardly curving trajectory of pairs of coiled-coil helices, which meet at an apex and associate through salt bridges. Carboxylates decorate the interior surface near this constriction and provide a 'gating ring' with selectivity for cations [24,25]. Clearly this constriction must somehow open at some stage of the transport process. Insight into the channel opening process has been provided from crystal structures of partially opened states of TolC [26,27] and the homologous *Neisseria gonorrhoeae* MtrE (a component of the MtrC–MtrD–MtrE tripartite pump) [28]. These structures show that the opening is associated with a change in the superhelical trajectory of the coiled coils and with prying apart of the gating salt bridges that stabilize the closed state. Presumably the energy costs associated with these conformational switches is provided by interactions with the other components of the efflux pump, a point to which we will return below.

Membrane fusion proteins

Crystal structures of membrane fusion proteins (MFP) from diverse species reveal that many have four linearly

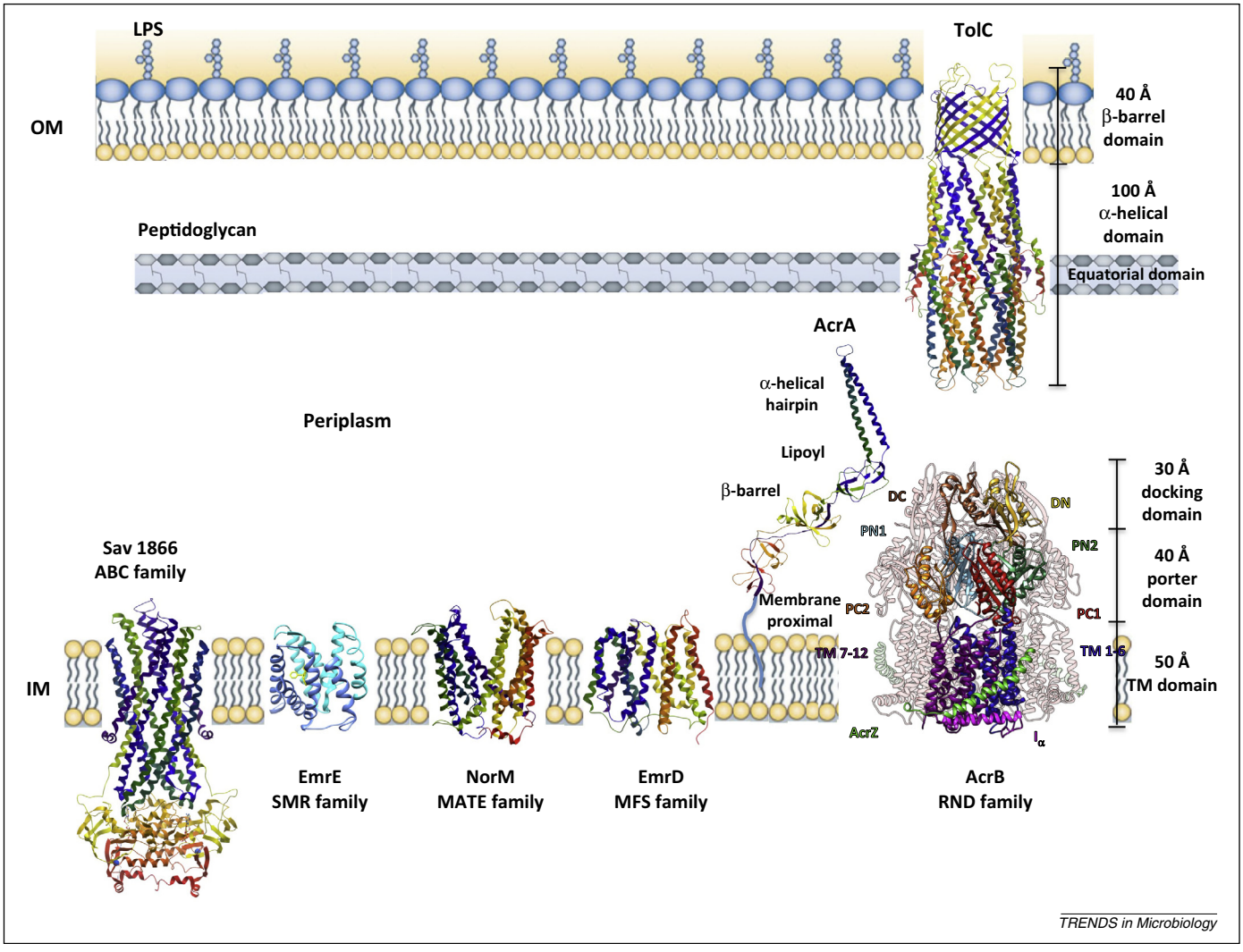

Figure 1. A structural gallery of the drug-transporter repertoire of bacteria. The cell envelope of Gram-negative bacteria: the outer membrane (OM) is an asymmetric bilayer, with the lipids on the inner and outer leaflets composed of phospholipids and glycolipids (principally lipopolysaccharides – LPS), respectively. The outer membrane is anchored through abundant lipoproteins to the underlying peptidoglycan, which is composed of repeating units of the disaccharide *N*-acetyl glucosamine-*N*-acetyl muramic acid, conferring mechanical robustness to the dual membrane system. The inner-membrane layer (IM) is a phospholipid bilayer, consisting largely of phosphatidylethanolamine (PE), phosphatidylglycerol, and cardiolipin. The two membrane layers delimit an aqueous cellular compartment densely packed with proteins, the periplasm [13,14], with a width estimated to be roughly 320 Å, judging from electron cryo tomography images [65].