Proteins sorted by 'chaos and disorder'

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Proteins sorted by 'chaos and disorder'

Entropy-driven protein sorting traffics membrane-anchored exposed proteins to the surface of Gram-positive bacteria.

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A host of studies has addressed the functions of exported proteins in Gram-negative bacteria and characterized the pathways that they follow, from the ribosome, across the inner and outer membranes, and into the extracellular milieu. Remarkably, relatively little attention has thus far been paid to the mechanisms underlying protein passage through the Gram-positive bacterial cell wall. Protein export in Gram-positive species has been assumed to occur by passive diffusion after energy-driven membrane translocation, with chaperone-mediated post-translocational folding at the *trans*-side of the membrane[1].

Reporting in this issue of *Nature Microbiology*, Halladin et al. combine theoretical modelling and biochemical validation to reveal that entropy, rather than diffusion, powers the partitioning of the actin assembly-inducing protein (ActA) across the cell wall of the notorious food-borne Gram-positive pathogen *Listeria monocytogenes*[2].

The Gram-positive bacterial cell wall is essentially one molecule of cross-linked peptidoglycan that shelters a protoplast within its boundaries and provides support against internal turgor pressures of up to 25 atm[3]. The wall is dynamic, allowing the cell to grow and divide, and serves as a semi-porous filter for uptake of essential nutrients, exclusion of toxic compounds and excretion of metabolic wastes. Importantly, there is continuous passage of proteins from the bacterial cytoplasm into and across the wall.

Exported proteins have diverse functions, including bacterial motility, facilitating adhesion of cells to substrates, provision of nutrients through enzymatic activities and, in the case of pathogens, subversion of the host and its immune defences. The precise repertoire of proteins that traverse the cell wall is thus a reflection of the different ecological niches occupied by bacteria, be it the soil, aquatic environments or the human body. Since many exported proteins of Gram-positive bacteria are secreted directly into the extracellular milieu, non-pathogenic species, such as *Bacillus subtilis*, are exploited to produce a wide range of valuable enzymes that are readily recovered from the fermentation broth[1]. As exported proteins of pathogens are important targets for antimicrobial agents and vaccines[4], developing an understanding of how proteins are translocated across the cell wall could impact fields ranging from biotechnology to host defence.

L. monocytogenes is an important human pathogen that relies on the ActA protein to mediate cell-to-cell transport of bacteria during infection. Upon membrane translocation and cleavage of the N-terminal signal peptide, the newly exposed N terminus of ActA navigates through narrow pores in the wall (<2–3 nm)[5] to become exposed on the outside of the cell. Once *Listeria* has invaded the cytoplasm of a mammalian host cell, the surface-exposed

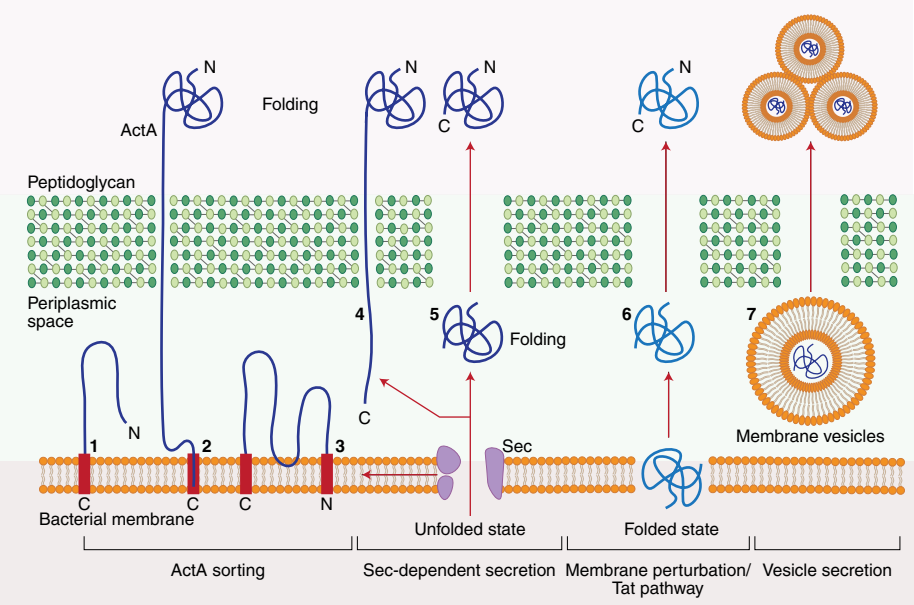

Fig. 1 | Protein sorting across the Gram-positive bacterial cell wall. Most Gram-positive bacterial proteins are exported in an unfolded state from their site of synthesis (the cytoplasm) to extracytoplasmic locations via the Sec pathway (1–5). Other proteins are exported and secreted in a folded state via the Tat pathway (6), through membrane perturbations (6), or through vesicles that are pinched off from the membrane (7). Halladin et al. have now proposed a new mechanism of entropy-driven reversible sorting of exported proteins across the Gram-positive bacterial cell wall, which is based on studies of the ActA protein of *L. monocytogenes*. Upon export from the cytoplasm, ActA adopts a *trans*-wall configuration, but remains C-terminally anchored to the cytoplasmic membrane (2). The *trans*-wall configuration is facilitated by the intrinsically disordered state of ActA, but comes at a high entropic cost. Consequently, shortening of the N-terminal domain by proteolytic or genetic truncation results in retraction of ActA into the periplasm (1), while provision of N- and C-terminal membrane anchors to an ActA–Nsp1 chimera retains the protein at the periplasmic membrane side without cell wall translocation (3). Removal of the C-terminal membrane anchor from the ActA–Nsp1 chimera leads to secretion into the extracellular milieu (4). In contrast to ActA, many secreted proteins of Gram-positive bacteria will fold upon exit from the Sec channel, taking up structures with dimensions that exceed the known size of cell wall pores of <2–3 nm (5). This suggests that larger cell wall pores must be present or created to accommodate the secretion of these and other folded proteins (5–6), or of membrane vesicles with protein cargo (6).

ActA domain promotes actin polymerization and subsequent cell-to-cell propulsion of the bacterium. Importantly, the wall-spanning ActA protein is retained to the bacterial cytoplasmic membrane through a C-terminal transmembrane domain (Fig. 1).

Halladin et al. now provide evidence for entropy-driven threading of the largely disordered ActA protein through pores in the wall, without the involvement of force-generating machinery or chaperones. In essence, their model proposes that cell wall passage of ActA is reversible, and that the free-energy cost of ActA's *trans*-wall state is relatively high due to the entropic penalty for protein confinement within a narrow pore. Consequently, membrane-retained ActA derivatives with shortened N-terminal ends would be excluded from wall pores and retained in the periplasmic space between the membrane and the wall, whereas N-termini that are long enough may allow a *trans*-wall state (Fig. 1).

The authors used proteolytic digestion or genetic truncation of ActA's N-terminal end to validate their model experimentally. This revealed that ActA derivatives shortened by 166 residues or more can no longer extend through the wall, and that wall translocation requires a crucial length of peptide. They then carried out additional validation by decreasing cell wall thickness through mutation of the *walI* gene of *L. monocytogenes*, which enabled traversal across the wall of a truncated form of ActA that lacked 200 residues. Following this, they showed that entropy-driven cell wall sorting also occurs in other Gram-positive bacteria. For example, the authors expressed ActA, or the larger iActA protein from *Listeria ivanovii*, in *B. subtilis*, and found that whilst iActA traversed the thick *B. subtilis* cell wall, ActA from *L. monocytogenes* was too short to cross.

Importantly, previously characterized disordered regions from the nuclear pore complex proteins Nsp1 and Nup1 (ref. [6]) can replace the disordered central region of ActA in promoting wall passage in *Listeria* and the human pathogen *Staphylococcus aureus*[2]. Further, as predicted by the entropy-driven protein sorting model, an Nsp1 chimera with an N-terminal anchor and lacking the C-terminal transmembrane domain also assumed a *trans*-wall orientation, while the presence of both N- and C-terminal membrane anchors prevented cell wall passage upon membrane translocation (Fig. 1). Finally, when no membrane anchor was present the Nsp1 chimera was secreted into the extracellular milieu. Together, these observations imply that sorting of structurally disordered proteins across the Gram-positive bacterial cell wall is driven by entropic forces.

What are the implications of a model that explains entropy-driven protein sorting? First, it provides a theoretical grounding for earlier assumptions that membrane-translocated proteins pass across the wall by Brownian motion[1]. However, currently available data indicate that most proteins passing the membrane-embedded Sec channel in an unfolded state would fold immediately upon entering the periplasm, rendering membrane translocation irreversible. This view is supported by the fact that catalysts for protein folding and quality control, like the peptidyl-prolyl *cis*/*trans* isomerase PrsA, the thiol-disulphide oxidoreductases BdbB,C,D for oxidative protein folding, and the quality control proteases HtrA,B remain attached to the periplasmic side of the membrane[1].

The dilemma, however, is that many fully folded secretory proteins will not fit through wall pores of <2–3 nm. For instance, the efficiently secreted α-amylase AmyE of *B. subtilis* measures roughly $3.5 \times 4.0 \times 7$ nm[7], without taking into account surrounding water molecules. Likewise, the haemoprotein EfeB, which is exported in a fully folded state from the cytoplasm into the extracellular milieu via the twin-arginine protein translocation pathway (Tat), measures roughly $7.5 \times 7 \times 5$ nm[8]. Thus, entropy-driven cell wall passage, as proposed and proven for ActA, seems unlikely to be the case for AmyE, EfeB and many other secreted proteins. Indeed, proteome-wide analyses have revealed alternative protein secretion routes in Gram-positive bacteria, which involve membrane-weakening peptides, cell wall hydrolases, prophages and even vesicles (Fig. 1)[1].

One intriguing question that remains to be resolved is how intrinsically disordered proteins remain stable in the highly proteolytic environment of the membrane and cell wall[1]. Potentially, proline-rich domains could stabilize disordered proteins like ActA, because prolyl-peptide bonds are highly resilient to proteolysis[9]. Furthermore, it is tempting to speculate that such disordered domains and entropic forces could be recruited for the secretory production of recombinant proteins, especially in *Bacillus* species. This may require the use of less proteolytic, genome-engineered strains, possibly with thinner cell walls, in which it will be easier to disentangle simultaneously ongoing events of cell wall biogenesis, turnover and protein passage[10]. Conversely, from a biomedical perspective, it will be interesting to elucidate how factors that impact on cell wall thickness, such as antibiotic therapy or water activity, will modulate exposure of *trans*-wall virulence factors at the bacterial cell surface.

Altogether, the finding that protein sorting across the Gram-positive bacterial cell wall can be driven by 'chaos and disorder' provides renewed impetus for studies on the mechanisms that underlie passage of diverse, industrially and medically important proteins through this complex and dynamic one-molecule matrix. ❐

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Competing interests

The authors declare no competing interests.