COMMENTARY

The bacterial outer membrane is an evolving antibiotic barrier

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The outer membrane (OM) of the diderm "gram-negative" class of bacteria is an essential organelle and a robust permeability barrier that prevents many antibiotics from reaching their intracellular targets (1). The OM is a unique asymmetrical lipid bilayer (Fig. 1): The inner leaflet is composed of phospholipids (PLs), and the outer leaflet consists almost exclusively of a glycolipid referred to either as lipopolysaccharide (LPS, in bacteria that attach long repeats of sugars to the glycolipid) or lipooligosaccharide (LOS, in bacteria that attach only a short oligosaccharide to cap the glycolipid) (1). Assembly of these lipids into a contiguous barrier, and how that barrier is maintained in response to damage, is a fascinating biological problem. Both PLs and LPS/LOS are synthesized inside the cell, so they must first transit the inner membrane (IM) and then traverse the hostile aqueous periplasmic environment before being assembled into an OM. Work over the past decade uncovered a protein bridge that links the IM and OM and allows LPS/LOS to flow directly into the OM outer leaflet (2). How PLs are transported to the OM remains a mystery. Understanding the pathways of OM biogenesis is a pressing goal. New antibiotics against gram-negative bacteria are urgently needed (3). Rates of antibiotic resistance continue to rise unabated, while the last truly novel antibiotic effective against gram-negative bacteria was discovered in the 1960s (3). The hope is that treatments interfering with OM biogenesis will offer new lethal therapeutics or will help permeabilize gram-negative bacteria to existing drugs. Until that promise is realized, clinicians are increasingly forced to rely on last-resort antibiotics that were once sidelined due to their unfavorable toxicity profiles, including the OM-targeting antibiotic colistin (polymyxin E) (4). In PNAS, Powers and Trent (5) provide new insights into how colistin-resistant bacteria evolve improved fitness by altering their OM composition. Remarkably, their work has provided an unexpected insight into PL transport in the cell envelope.

Strict lipid asymmetry in the bilayer is key to the OM barrier function (Fig. 1). LPS/LOS at the cell's surface fortifies the membrane against antibiotics and detergents (e.g., bile salts) in several ways: First, these molecules densely pack the outer leaflet with saturated acyl chains that make it extremely hydrophobic, and, second, the lipid and saccharide portions of individual LPS/LOS molecules each carry negative charges that allow intermolecular bridging interactions to occur through binding of divalent cations (1). These bridging interactions between neighboring LPS/LOS molecules result in tight lateral interactions that seal the membrane from antibiotics and detergents that are otherwise capable of penetrating a typical PL bilayer. Polymyxins, the class of antibiotics that includes colistin, directly damage the OM by interfering with LPS/LOS bridging interactions (6). Polymyxins are cationic molecules that competitively bind negative charges on LPS/LOS, but since they do not allow for bridging interactions, polymyxins weaken lateral LPS/LOS interactions and destabilize the OM (Fig. 1) (6).

Even though it is used sparingly in last-resort treatments, colistin has not escaped the evolution of resistance. Typically, any of several enzymatic modifications of LPS/LOS can reduce its negative charge, and thereby decrease colistin binding (6). *Acinetobacter baumannii* is a common multidrug-resistant human pathogen that is clinically treated with colistin (6). Powers and Trent (5) examine *A. baumannii* strains that have taken the remarkable step of inactivating LOS production entirely to become highly colistin resistant. For most gram-negative bacteria, LPS/LOS production is essential for viability; *A. baumannii* is among a small group that can tolerate loss of LOS (7). This drastic resistance strategy is not without significant fitness costs. Absence of LOS drastically alters the OM: PLs replace LOS in the outer leaflet, and the OM becomes a symmetrical PL bilayer. As a result, LOS deficiency causes severely reduced growth rates in vitro, cells become permeable to large antibiotics, and virulence is markedly attenuated (8).

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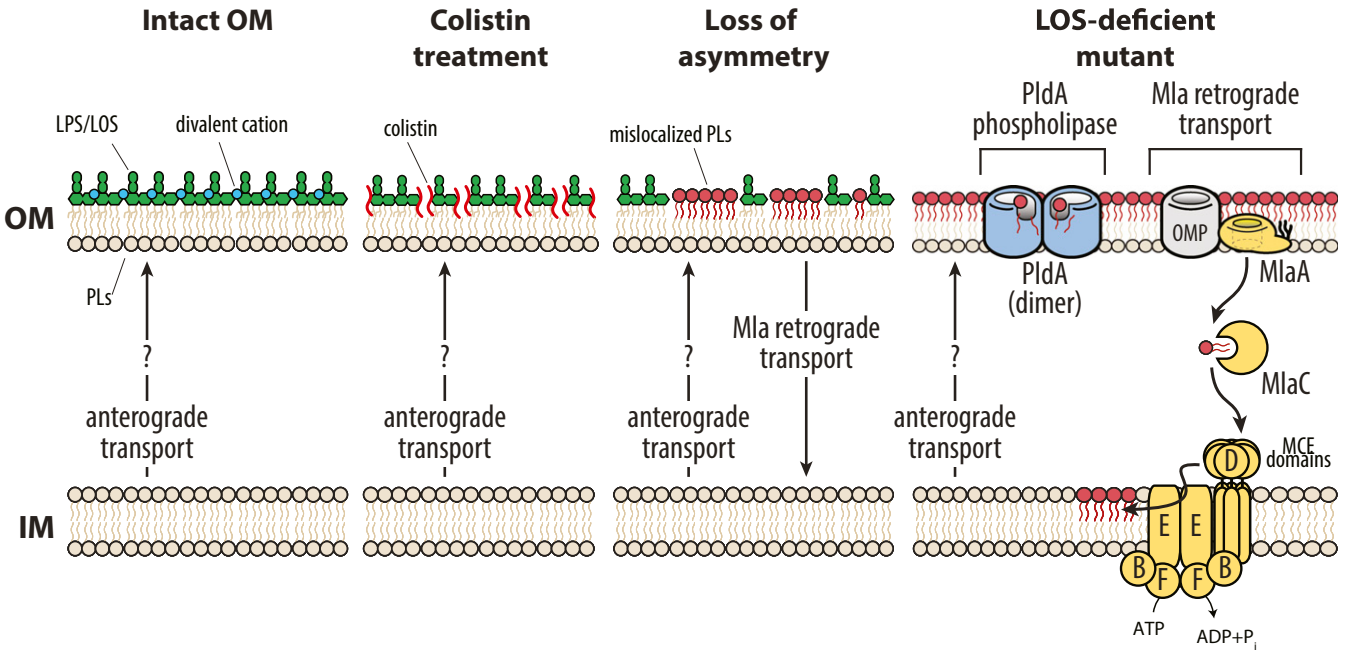

Fig. 1. Architecture of the gram-negative envelope. The OM and IM are separated by an aqueous periplasm. OM lipids are symmetrically distributed, with the surface glycolipids (LPS/LOS) being held together via divalent cation bridging. PLs are in the inner leaflet but can mislocalize when the OM is damaged. The PldA and Mla pathways work together to remove mislocalized PLs and restore asymmetry. In LOS-deficient cells, constitutive activity of PldA and Mla is deleterious as the cell attempts to maintain an OM lipid bilayer.

The consequences of LOS deficiency are stark, but *A. baumannii* strains remain viable. What allows some bacteria to survive without LPS/LOS but not others? Potentially, the answer may come from examining how *A. baumannii* adapts to loss of LOS. Powers and Trent (5) sought to glean insights into such adaptation by serially culturing LOS-deficient *A. baumannii* and examining the spontaneous mutations that arise to improve fitness. Within 120 generations, their data converge on one central conclusion: When the OM is faced with a lipid deficit (because LOS is absent), two systems, the Mla pathway and the OM phospholipase PldA, that are proposed to remove PLs from the OM are deleterious for fitness (Fig. 1). Mutations inactivating both Mla and PldA spontaneously arise to boost growth rates of LOS-deficient cells. Even more surprisingly, these mutations also somehow help repair the antibiotic barrier against large antibiotics.

Chemical damage or OM assembly defects allow PLs to translocate to the outer leaflet (1). These mislocalized PLs disrupt lipid asymmetry and impair barrier integrity (1). Genetic evidence from *Escherichia coli* indicated that Mla and PldA function together to preserve OM lipid asymmetry (9). The multiprotein Mla system has components in each compartment of the cell envelope: an integral MlaA OM lipoprotein, a soluble periplasmic chaperone MlaC, and an IM ATP-binding cassette (ABC) transporter complex of MlaBDEF (Fig. 1) (9–13). Absence of any Mla protein inactivates the system and allows PLs to accumulate in the outer leaflet (9). Mislocalized PLs can be detected (albeit indirectly) because they become substrates for an LPS-modifying reaction that occurs only in the OM outer leaflet (14). These PLs are also substrates for the PldA phospholipase whose active site is strategically positioned in the outer leaflet (15). PldA processively degrades mislocalized PLs to remove them from the OM (Fig. 1) (15). In wild-type cells, inactivating *pldA* mutations do not cause significant defects (9). However, combining mutations in *pldA* and *mla* causes severe detergent sensitivity and a marked increase in mislocalized PLs; these defects in the double mutant are greater than observed with either single mutation (9). Moreover, spontaneous suppressor mutations that increase PldA production can complement the defects of mutations in *mla*.

Because their absence results in more PLs in the OM outer leaflet, the Mla and PldA pathways were ascribed roles in removing PLs from the OM: PldA by degrading mislocalized PLs and Mla by transporting them back into the IM. Indeed, the IM protein MlaD belongs to a class of proteins that function in lipid import in diverse organisms. MlaD possesses a mammalian cell entry (MCE) domain that was identified in the diderm *Mycobacterium tuberculosis* as important for virulence, but the domain has since been shown to bind lipids (10, 12). In *M. tuberculosis*, MCE proteins are required for lipid import that allows these bacteria to metabolize host cholesterol (16). An MCE protein is even present in chloroplasts that have inner and outer envelope membranes (likely due to their cyanobacterial origin). In plant cells, lipids are exchanged between the endoplasmic reticulum (ER) and the outer envelope membrane (17). The MCE-containing TGD2 protein is required for the subsequent import of ER-originating lipids from the outer to the inner envelope membrane so that they can be metabolized into chloroplast-specific lipids.

The proposed retrograde transport of PLs (from the OM to the IM) by the Mla system was supported by *mla* mutants exhibiting outer leaflet PL accumulation, complementation of this *mla* mutant phenotype by the PldA phospholipase, and the functions of MCE proteins (9). Recent structural studies of the OM component, MlaA, revealed a central pore opening to the outer leaflet and structures that preclude entry of inner leaflet PLs to the pore (11, 13).

Surprisingly, LOS-deficient *A. baumannii* was earlier found to exhibit a striking increase in transcription of the *mla* genes (18, 19). Why would the cell increase expression of a system that removes lipids from the OM when, in the absence of LOS production, this

organelle is faced with a deficit of lipids? A more sensible approach should be to increase anterograde PL transport (from the IM to the OM) to supply the additional PLs now needed to build the OM. Importantly, bulk transport of PLs to the OM is yet to be accounted for. Up-regulation of *mla* genes when LOS is absent seemed to hint that, perhaps at least in this organism, Mla could function in the anterograde direction or bidirectionally. A fascinating *mlaA* mutation seemed, in part, to further suggest this possibility. The *mlaA** mutation appears to actively facilitate movement of PLs to the outer leaflet (20). However, this activity is completely independent of the Mla system; deleting any other *mla* gene in the *mlaA** mutant does not suppress this activity (20). Rather, the MlaA* mutant protein functions aberrantly to allow passage of inner leaflet PLs to the outer leaflet (11, 13, 20).

The finding that mutations inactivating both Mla and PldA arise to increase fitness of LOS-deficient *A. baumannii* can only suggest one direction of lipid transport for Mla: It must be working in the retrograde manner. Because the LOS-deficient OM has become a PL bilayer, Mla must be constitutively active. Its activity is not helpful, however; the cell is faced with a lipid deficit in the OM. The fitness advantage of inactivating both Mla and PldA allows cells to continue accumulating PLs in the OM in an effort to build the OM, which, after all, remains an essential organelle. The findings of Powers and Trent (5) have the power of using multiple strains and an unbiased approach: LOS-deficient strains are simply cultured, and the best evolutionary solution wins. So, it is striking that the same solution has independently arisen in all but one of the evolved LOS-deficient strains (the holdout carries a mutation in a signal transduction system that is likely pleiotropic). This work is a reminder that gene expression profiles do not necessarily predict key fitness determinants (21).

The evolved LOS-deficient *A. baumannii* also exhibits improved resistance to large antibiotics, suggesting the quality of the OM barrier is somehow improved. Given the rapid evolution of fitness, are we likely to encounter colistin-resistant, LOS-deficient *A. baumannii* in the clinic? Perhaps not. Whether virulence of the evolved strains has been restored is still unclear. Even evolved strains might still be readily cleared by the immune system. However, it is worth noting that LOS-deficient *Neisseria meningitidis* has been isolated from cerebral spinal fluid in the clinic (22). At least this rich and immune-privileged site can support growth of bacteria with a drastically altered OM. Clinically sourced LOS-deficient strains should not be readily discounted. Findings from Powers and Trent (5) are instructive in assessing both the transport of PLs in the gram-negative cell envelope and the bacterial adaption to antibiotic treatment. As we learn more about both of these processes, we will become better equipped to devise strategies aimed at combating antibiotic resistance.

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