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Restoring Permeability Barrier Function to Outer Membrane

A recent issue of *Cell* published two papers resulting from the collaboration between the Kahne and Silhavy laboratories [1, 2]. These studies, possibly initiated as an effort to identify the target of action of vancomycin with lipophilic substitutions, resulted in the discovery of a protein complex involved in the assembly of outer membrane proteins.

Daniel Kahne's group showed earlier that chlorobiphenyl (CBP)-vancomycin, which is effective on vancomycin-resistant gram-positive bacteria, unexpectedly acts by inhibiting the transglycosylation reaction of peptidoglycan biosynthesis, rather than by the "classical" mechanism of binding to the D-Ala-D-Ala portion of the peptidoglycan-biosynthetic intermediate [3]. Researchers in Tom Silhavy's laboratory had used the genetic approach, specifically the isolation of mutants in *Escherichia coli*, in the study of assembly of the cell envelope. It was natural for these two laboratories to try to isolate CBP-vancomycin-resistant mutants of *E. coli*, which could produce confirmation of the identity of the target of this antibiotic. The results were not only unexpected, but even more, they were intriguing and important. Gram-negative bacteria such as *E. coli* are surrounded by an outer membrane (OM) permeability barrier that normally prevents the entry of vancomycin and CBP-vancomycin (Figure 1A), and thus an *imp* mutant, defective in the assembly of lipopolysaccharide (LPS) into the OM (Figure 1B), was used in this experiment. In retrospect, therefore, it is not surprising that the resistant mutants had OM with better barrier properties rather than alterations of the drug target. However, the mutations occurred in a gene of hitherto unknown function, *yfgL* [1], whose product was found to assemble into a multiprotein complex, involved in OM protein assembly, containing three other proteins (YaeT, YfiO, and NlpB) [2]. Of these, YaeT is a homolog of Omp85, whose function in OM protein assembly was previously reported [4], but the functions of the other three proteins were totally unknown before this work. Not only is the discovery of this multiprotein complex exciting, the data show functional interaction between the LPS assembly machinery and the protein assembly machinery of OM.

In order to understand the results of Ruiz et al. [1] and Wu et al. [2], we must realize that the OM, a unique structure for Gram-negative bacteria (such as *E. coli*), is an effective permeability barrier and makes these bacteria inherently resistant to many antibiotics. Building membranes of very low permeability, however, is not an easy task. First, the phospholipid bilayers that form the matrix of most biological membranes allow rapid permeation of lipophilic molecules, and most antibiotics (except those of the β-lactam class and a few others) must be lipophilic as they have to traverse the cytoplasmic membrane quickly and spontaneously to reach their targets in the cytoplasm, such as ribosomes, RNA polymerase, or DNA topoisomerases. In order to overcome this problem, the OM bilayer is asymmetric, and its outer leaflet is almost completely composed of an unusual lipid, LPS [5]. The lipid portion of LPS ("lipid A") contains up to seven residues of saturated fatty acids, which produce a lipid interior of very low fluidity [6]. Thus the OM's LPS/phospholipid bilayer is much less permeable to lipophilic solutes than the phospholipid bilayer [7]. However, the steps involved in the assembly and maintenance of this extremely asymmetric bilayer are not known. Second, bacteria must allow the passage of hydrophilic nutrient molecules across the OM, and for that purpose they produce narrow nonspecific channels (porins) as well as specific channels and transporters [6]. Presumably because these proteins must be inserted into the OM after their full export across the cytoplasmic membrane, practically all OM proteins have the final conformation of membrane-spanning β-barrels, in contrast to the inner membrane proteins that cross the bilayer usually as transmembrane helices. But again, the mechanism of assembly of these proteins into the OM is not clear.

Defects in the OM can lead to increased permeability to inhibitors. The classical case is that of the "deep rough" mutants, which produce LPS of severely truncated sugar chains and become hypersusceptible to lipophilic inhibitors such as novobiocin, rifampicin, and erythromycin. (Apparent octanol/water partition coefficients are 48.8, 16.0, and 0.89 for these compounds, respectively [8].) The most obvious explanation of these results is that the outer leaflet composed of the defective LPS serves as a less effective barrier. However, extensive studies in 1970s showed that the OM permeability is increased via an indirect mechanism [9]. Thus the presence of these defective LPS molecules inhibits the insertion of many OM proteins, and the empty spaces in the OM become filled with phospholipids, generating phospholipid bilayers that have a much higher permeability than the normal LPS/phospholipid asymmetric bilayer (Figure 1B). (The alternative solution of shrinking the surface area of the OM is not possible, because the rigid peptidoglycan is located underneath the OM.) In these mutants the OM shows little signs of leakiness for periplasmic proteins, and the integrity of its structure is maintained so that the cells remain resistant to vancomycin and bacitracin (except in some strains with extreme LPS defects) [9].

There are, however, *E. coli* mutants that become hypersusceptible to peptide (or glycopeptide) antibiotics such as vancomycin and bacitracin, which are quite hydrophilic overall with measured octanol/water partition coefficients less than 0.1 [8]. Since such compounds cannot easily dissolve in the hydrocarbon interior of the OM, one must assume that the mutant OM contains transient imperfections or "cracks" that allow the diffusion of these hydrophilic drugs into the periplasm (Figure 1B). Indeed, this interpretation is consistent with

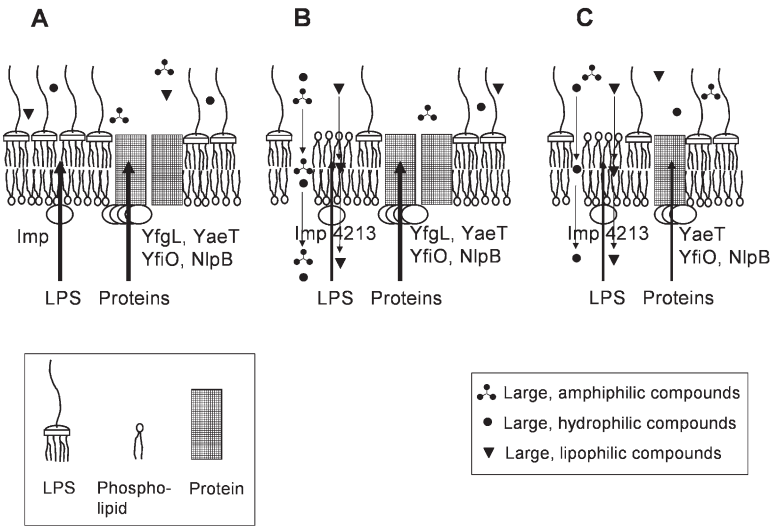

Figure 1. Permeation of Large Inhibitors across OM

(A) Wild-type *E. coli*, where the asymmetric LPS/phospholipid bilayer and the narrow porin channel effectively block the entry of most large inhibitors.

(B) The *imp4213* mutant. Here the decreased incorporation of LPS into OM by the mutant Imp4213 protein generates both the bilayer containing a phospholipid bilayer domain, and transient openings in OM. The former allows the diffusion of lipophilic drugs (erythromycin, novobiocin, and rifampicin), and the latter allows the diffusion of both hydrophilic antibiotics (vancomycin and bacitracin) and amphiphilic compounds (CBP-vancomycin, moenomycin, and bile salts).

(C) The *imp4213 yfgL* double mutant. The inactivation of YfgL alters the export of OM proteins, and the effective width of the gaps in the OM is narrowed. The phospholipid bilayer still allows the passage of lipophilic compounds, and the gap allows the entry of both vancomycin and bacitracin. However, the entry of large, amphiphilic compounds, shown here hypothetically as micelles, now becomes difficult.

the finding that strains with this phenotype have defects that affect the physical assembly and enlargement of the cell envelope, for example leaky mutations in the early genes of the synthesis of the lipid A (such as *lpxA* and *lpxC* [earlier called *envA*]) [8, 9], mutations in peptidoglycan metabolism [10], or strains in which exogenous channels or misfolded proteins are inserted into the OM [11, 12]. Most of these strains are also hypersusceptible to lipophilic agents. Another example is the mutants that are defective in the incorporation of the unsaturated fatty acid chain into lipid A [13] and that become hypersusceptible to vancomycin at low temperature, presumably because the LPS leaflet becomes frozen and brittle, a situation reminiscent of the cold $CaCl_2$ treatment procedure for *E. coli*, which allows the entry of not only DNA but also proteins [14] through the transient cracks of OM.

In the study of Ruiz et al. [1], the defect in Imp protein, which assembles LPS into the OM, leads not only to hypersusceptibility to hydrophobic agents but also to vancomycin and bacitracin. This is what is expected from the phenotype of strains exporting insufficient amounts of LPS to the OM, as described above. What is most interesting, however, is that the mutants with increased resistance to CBP-vancomycin and moenomycin (another large, amphiphilic antibiotic) contain loss-of-function mutations in the gene *yfgL*, whose product was shown by Wu et al. [2] to be a part of multiprotein complex involved in the assembly of OM proteins. In fact, *yfgL* mutants were shown to export OM proteins more slowly [1]. The *imp yfgL* double mutant is still susceptible to vancomycin and bacitracin [1]: the integrity of OM therefore should still be compromised (Figure 1C). The resistance of the double mutant to CBP-vancomycin, moenomycin, and bile salts could mean that the cracks in OM have now become "narrower" (Figure 1C), excluding these amphiphilic compounds that may form micelles. The mechanism whereby decreased assembly of OM proteins produces this effect is still unclear, especially because mutations in other genes in the OM protein export complex (*yaeT*, *yfiO*, *nlpB*) do not produce this phenotype. One would actually expect wider, not narrower, cracks if the export of both LPS and proteins to OM is decreased, although the requirement for slow growth for the double mutant at least is as expected from this line of reasoning. Perhaps the way we imagine the transient cracks as in Figure 1B is overly naive (and energetically too unfavorable); the real opening may arise in between the proteins, and thus the nature, in addition to the size, of the opening might be at issue. In any case these studies not only led to the discovery of new multiprotein machinery for OM protein assembly, but also suggest that there is much more to be explored on such seemingly simple processes as the spontaneous diffusion of drugs across OM. The approach of using small toxic chemicals to understand the dynamics of OM assembly and function indeed looks quite promising. In fact, an inner membrane protein needed for vancomycin resistance of *E. coli* has previously been reported [15]. Could this protein perhaps be a part of the OM protein assembly complex or at least interact with it?

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Hiroshi Nikaido
Department of Molecular and Cell Biology
University of California
426 Barker Hall
Berkeley, California 94720-3202

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