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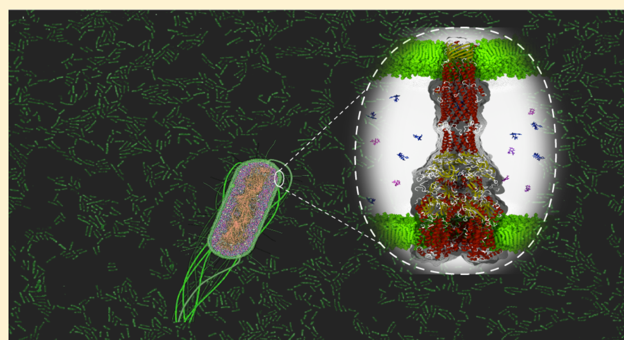
Permeability Barrier of Gram-Negative Cell Envelopes and Approaches To Bypass It

Helen I. Zgurskaya,[*,†] Cesar A. López,[§] and S. Gnanakaran[§]

[†]Department of Chemistry and Biochemistry, University of Oklahoma, 101 Stephenson Parkway, Norman, Oklahoma 73019, United States

[§]Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, New Mexico 87545, United States

ABSTRACT: Gram-negative bacteria are intrinsically resistant to many antibiotics. Species that have acquired multidrug resistance and cause infections that are effectively untreatable present a serious threat to public health. The problem is broadly recognized and tackled at both the fundamental and applied levels. This paper summarizes current advances in understanding the molecular bases of the low permeability barrier of Gram-negative pathogens, which is the major obstacle in discovery and development of antibiotics effective against such pathogens. Gaps in knowledge and specific strategies to break this barrier and to achieve potent activities against difficult Gram-negative bacteria are also discussed.



KEYWORDS: *Gram-negative resistance, permeability barrier, outer membrane, multidrug efflux*

GRAM-NEGATIVE PATHOGENS AND CHALLENGES OF ANTIBIOTIC DISCOVERY

Drug resistance presents an ever-increasing threat to public health and encompasses all major microbial pathogens and antimicrobial drugs.[1,2] Some pathogens have acquired resistance to multiple antibiotics and cause infections that are effectively untreatable. Among pathogenic Gram-negative *Enterobacteriaceae*, *Acinetobacter*, and *Pseudomonas*, species have emerged that are resistant to all good antibiotics.[3] One of the most troubling event is the worldwide spread of carbapenem-resistant *Klebsiella* spp.[4] Infections caused by these resistant variants have a mortality rate of up to 50%. By 2013, 17% of *Escherichia coli* infections became multidrug resistant. In some regions, fluoroquinolones are no longer on the lists of recommended treatment options.[5,6] Such environmental species as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex (Bcc), and *Acinetobacter baumannii* are intrinsically resistant to antibiotics. Among these species, *P. aeruginosa* is a common nosocomial pathogen, the causative agent of many life-threatening infections and the major reason for the shortened life span of people with cystic fibrosis (CF). *P. aeruginosa* infections can be successfully treated by only a few specific representatives of fluoroquinolones, β-lactams, or aminoglycosides. However, even these few antibiotics fail against antibiotic-resistant *P. aeruginosa* isolates. Thus, there is a strong need for new therapeutic options, particularly those directed against multiresistant Gram-negative bacteria.

The discovery of new antibiotics effective against Gram-negative bacteria is a major challenge, primarily because of a low hit rate during screening of compound libraries, which is up to 1000-fold lower in *P. aeruginosa* than against Gram-positive bacteria.[7] The major reasons for such a low hit rate are the low permeability barrier of two-membrane cell envelopes of Gram-negative bacteria and insufficient chemical diversity of compound libraries to probe this barrier. Gram-negative bacteria vary significantly in their permeability to antibiotics, but one could expect that the basic principles established by extensive studies of *E. coli* would apply equally to such "impermeable" species as *Burkholderia* spp. or *Pseudomonas* spp. It remains unclear, however, whether permeation rules,[8] in analogy with Lipinski's rules,[9] if such existed and were applied to structure−activity relationships or to filtering compound libraries, would yield compounds that permeate all Gram-negative barriers. Here, we briefly review the current state of understanding of molecular bases of low-permeability barriers of the problematic Gram-negative pathogens and current efforts to define the physicochemical properties that enable uptake of various compounds into bacterial cells.

THE TWO-MEMBRANE BARRIER OF GRAM-NEGATIVE BACTERIA

The susceptibility of Gram-negative bacteria to antibiotics is defined by two opposing fluxes across the two membranes of these species (Figure 1).[10−12] The influx and uptake of antibiotics are significantly slowed by the elaborate outer membrane (OM). This membrane is an asymmetric bilayer of

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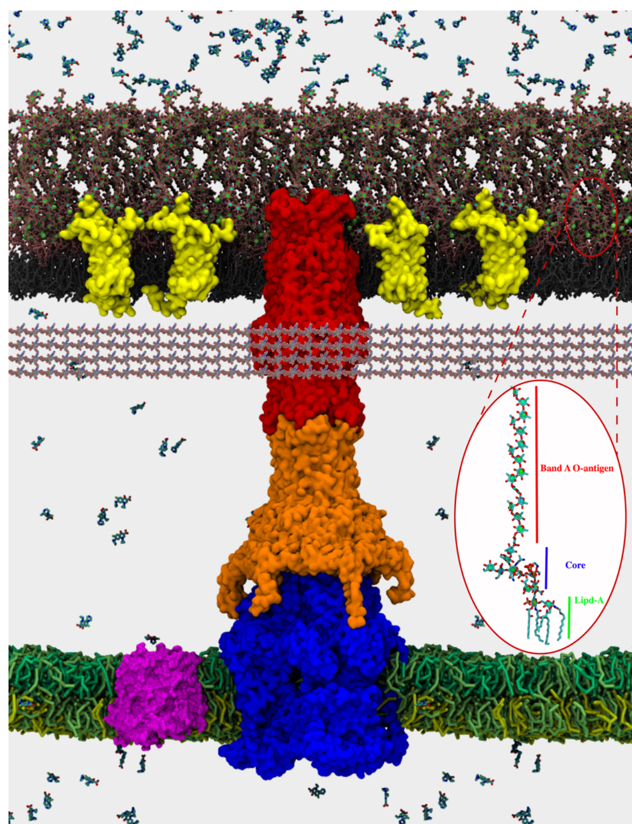

Figure 1. Cross-section view of a modeled *P. aeruginosa* cell envelope. The outer leaflet of the outer membrane is assembled of LPS (pink color) corresponding to the band A antigen,[25] and the inner leaflet contains glycerophospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE). Green spheres are magnesium ions bound to O-chains and core polysaccharides of LPS. The inner membrane contains an equimolar mixture of cardiolipin, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE)b and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG). Embedded in the outer membrane are the main porin OprF (PDB 4RLC) in a yellow surface density. In the inner bilayer is localized MdfA transporter, represented in a magenta surface (PDB 4ZOW). The modeled structure of the assembled MexAB-OprM multidrug efflux pump (MexB, blue; MexA, orange; OprM, red) spans both the inner and outer membranes. The structure of MexAB-OprM is from a long (1 μs) all-atom molecular dynamics simulations with the tripartite complex embedded in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers mimicking inner and outer membranes (see Figure 3). The MexA component lacks the N-terminal lipid modification. The outer and inner membranes are based on separate protein-free all-atom MD simulations with the above composition. Other structures were taken from the Protein Data Bank. Ciprofloxacin molecules were added to illustrate a difference of concentrations created by slow diffusion across the outer membrane through porins and LPS-containing bilayer and the active efflux across the outer (MexAB-OprM) and inner (MdfA) membranes. (Inset) Single representative LPS molecule from the simulations. The peptidoglycan is pictorially represented by fiber-like structures underneath the outer membrane. The image was composed and posteriorly rendered using the VMD Molecular Viewer.

lipopolysaccharides (LPS) and phospholipids, into which nonspecific porins and specific uptake channels are embedded.[13,14] The LPS-containing bilayers are more rigid than normal bilayers, slowing passive diffusion of hydrophobic compounds, whereas narrow pores limit by size the penetration of hydrophilic drugs. The slow influx of drugs across the OM is further opposed by active efflux mediated by multidrug efflux transporters. Multidrug efflux transporters are structurally and functionally diverse, with some transporters pumping antibiotics across the inner membrane and reducing concentrations of antibiotics in the cytoplasm, whereas others expel antibiotics from the periplasm into the external medium. The latter transporters confer resistance to antibiotics by associating with the periplasmic and OM accessory proteins to form trans-envelope complexes (Figure 1).[15,16] These complexes enable conversion of the energy stored in the inner membrane into active efflux of antibiotics across the OM. Efflux of antibiotics across the inner membrane acts synergistically with the trans-envelope efflux and, as a result, inactivation of efflux pumps leads to dramatic sensitization of Gram-negative bacteria to antibiotics. The clinical relevance of efflux of multiple antibiotics has also been established. For example, in clinical isolates of *E. coli* and *Klebsiella pneumoniae*, fluoroquinolone resistance is linked to overproduction of the AcrAB-TolC efflux pump, whereas multiple efflux pumps confer antibiotic resistance in *P. aeruginosa*, *Burkholderia* spp., and *A. baumannii*.[17−20] The interplay between uptake and efflux defines the steady-state accumulation level of antibiotics at targets.

Composition and Properties of LPS-Containing Bilayers. Despite a similar structural organization, outer membranes of Gram-negative bacteria differ dramatically in their permeability properties. These differences are largely attributed to differences in the permeability properties of general porins[12,14] (see also below), but the chemical structure and properties of the LPS-containing bilayer also play an important role (Figure 1). Typical LPS comprises a basic lipid A structure containing an N- and O-acylated diglucosamine bisphosphate backbone. Chemical variations of lipid A among species involve the number of primary acyl groups and the types of fatty acids substituting the primary and secondary acyl groups (Figure 2). *Escherichia* lipid A is most frequently described as a hexa-acylated molecular species, although penta- and tetra-acylated molecules are also present in various amounts.[21] Most of the laboratory-adapted strains of *P. aeruginosa* synthesize a penta-acylated (band A, 75% of the molecules) LPS (Figure 1), with some proportion made as a hexa-acylated LPS (25% of the molecules).[22,23] Growth conditions, notably magnesium levels, can affect the acylation pattern of *P. aeruginosa* lipid A (Figure 2). Among isolates from chronically infected CF patients, which are known to be mutants generally unable to synthesize O-antigen side chains, a hexa-acylated LPS form predominates, although a hepta-acylated lipid A has been isolated, containing an additional palmitoyl (C16:0) group linked to the primary 3-hydroxydecanoic acid group at position 3′ of glucosamine 2.[24,25] The hexa- and hepta-acylated lipid A moieties also contain cationic 4-amino-4-deoxy-L-arabinose sugars. Similarly, the major lipid A species in *Burkholderia* spp. (*B. cepacia*, *B. mallei*, *B. pseudomallei*) consists of a biphosphorylated disaccharide backbone, but it is constitutively modified with 4-amino-4-deoxy-L-arabinose and penta- or tetra-acylated.[26] Lipid A of *A. baumannii* is often modified by phosphorylethanolamine and an unusual sugar galactosamine and hexa- and hepta-acylated[27] (Figure 2).

Early studies showed that the permeabilities of *E. coli* and *P. aeruginosa* OM to hydrophobic steroid probes are similar, suggesting that the differences in the lipid A acylation state and the length of fatty acids do not affect significantly the OM permeability to small planar molecules.[28,29] On the other hand,

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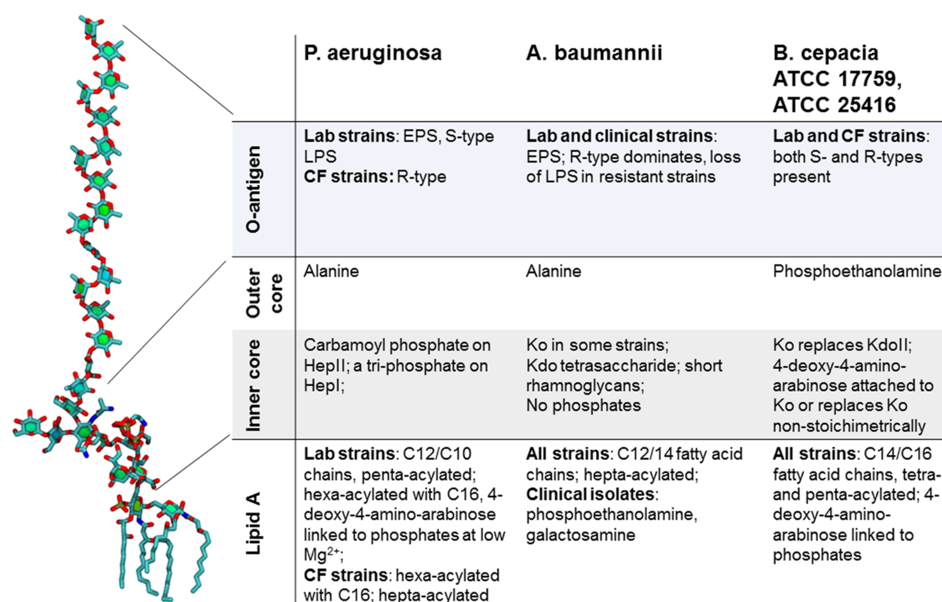

	P. aeruginosa	A. baumannii	B. cepacia ATCC 17759, ATCC 25416
O-antigen	**Lab strains**: EPS, S-type LPS **CF strains:** R-type	**Lab and clinical strains**: EPS; R-type dominates, loss of LPS in resistant strains	**Lab and CF strains**: both S- and R-types present
Outer core	Alanine	Alanine	Phosphoethanolamine
Inner core	Carbamoyl phosphate on HepII; a tri-phosphate on HepI;	Ko in some strains; Kdo tetrasaccharide; short rhamnoglycans; No phosphates	Ko replaces KdoII; 4-deoxy-4-amino-arabinose attached to Ko or replaces Ko non-stoichimetrically
Lipid A	**Lab strains**: C12/C10 chains, penta-acylated; hexa-acylated with C16, 4-deoxy-4-amino-arabinose linked to phosphates at low Mg^{2+}; **CF strains**: hexa-acylated with C16; hepta-acylated	**All strains**: C12/14 fatty acid chains; hepta-acylated; **Clinical isolates**: phosphoethanolamine, galactosamine	**All strains**: C14/C16 fatty acid chains, tetra- and penta-acylated; 4-deoxy-4-amino-arabinose linked to phosphates

Figure 2. Diversity of chemical structures and modifications in LPS. The *P. aeruginosa* band A LPS molecule is shown for comparison. Abbreviations: Kdo, α-3-deoxy-D-manno-oct-2-ulosonic acid; Hep, heptulose; NGal, galactosamine; EPS, extracellular polysaccharide; S-type, "smooth" LPS containing O-chains; R-type, "rough" LPS lacking O-chains; Ko, D-glycero-D-talo-oct-2-ulosonic acid.

amphiphilic and charged molecules are likely to interact with the backbone of lipid A and LPS cores, and their permeation could be sensitive to modifications of both the lipids and polysaccharides of the LPS-containing bilayers.

LPS cores of enterobacteria typically consist of 8−12 often branched sugar units (Figure 2).[30] The sugar at the reducing end is always α-3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) (2→6)-linked to lipid A. At the C-4 position of Kdo, there may be one or two Kdo groupings. Three L-glycero-D-manno-heptose residues are also (1→5)-linked to the first Kdo. A heptose residue may be substituted by a phosphate, pyrophosphate, or phosphorylethanolamine group or by another sugar to make up the inner core. In *Pseudomonas* spp. cores, one often finds an alanyl group substituting a galactosamine residue and a carbamoyl group on heptose I.[31] *P. aeruginosa* PAO1 serotype O5 and its two rough-type mutants share these characteristics but have, in addition, three phosphomonoester groups on their heptose.[31,32] It was suggested that these groups play a role in interactions with antibiotics because their presence correlates with resistance to β-lactams but not to aminoglycosides. Some *Acinetobacter* and *Burkholderia* spp. produce LPS cores devoid of heptose. *B. cepacia* and *A. hemolyticus* synthesize and incorporate the Kdo analogue D-glycero-D-talo-oct-2-ulosonic acid (Ko).[33−35]

The O-chains determine the specificity of each bacterial serotype. A combination of monosaccharide diversity, the numerous possibilities of glycosidic linkage, substitution, and configuration of sugars, and the genetic capacities of the diverse organisms have all contributed to the uniqueness of the great majority of O-chain structures.

It is broadly accepted that LPS cores and lipid A of enterobacteria and *P. aeruginosa* are modified in response to specific growth conditions and stresses (Figure 2).[36−38] These modifications are critical under the low magnesium ion conditions that destabilize the LPS leaflet and increase permeability of the OM. They also play an important role in the development of resistance against cationic antimicrobial peptides and polymyxins.[36,39] However, in *Acinetobacter* spp. and *Burkholderia* spp. some of the modifications that reduce the negative-charge character of LPS are constitutive (Figure 2). How the phosphate, pyrophosphate, or phosphorylethanolamine groups on Kdo and heptoses, terminal sugars, amino acid, and other groups occurring nonstoichiometrically correlate with specific growth conditions of these bacteria remains unclear. Even less is known of how these modifications affect the packing and rigidity of the LPS layer and its interactions with proteins and small molecules in the context of the outer membrane.

Recent advances in molecular dynamics (MD) simulations of asymmetric bilayers containing LPS offer first glimpses into the packing and dynamics of such structures.[40−42] The inner leaflet of the outer membrane is composed of phospholipids with a composition similar to that of the cytoplasmic membrane. Hence, the geometry of the LPS leaflet should match that of the phospholipid leaflet. On the basis of MD simulations, LPS-containing bilayers appear to be more disordered and thinner than bilayers assembled from phospholipids only (Figure 1). Magnesium ions form a layer of ionic bonds with phosphoryl moieties of lipid A and the core and stabilize LPS molecules in the bilayers. Also, LPS changes the hydration profile at membrane−water interfaces.[43] The packing and rigidity of LPS bilayers are expected to affect permeation of amphiphilic and hydrophobic antibiotics. However, this picture remains incomplete because about 50% of the *E. coli* OM mass consists of protein and the OM resembles a LPS−protein aggregate. Changes in hydration and electrostatic profiles due to LPS may affect the environment of embedded proteins and influence protein−protein interactions.

Outer Membrane Proteins and Permeability. In *E. coli*, a few integral membrane proteins, such as OmpA and the general porins OmpF/C, are expressed at high levels (Table 1). Besides these, there are minor proteins whose synthesis in some cases is strongly induced when they are needed, such as specific porins (e.g., PhoE and LamB), TonB-dependent receptors (e.g., FhuA and FepA), components of several protein export systems, proteins involved in the biogenesis of

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Table 1. Major Porins and Efflux Pumps of Representative Gram-Negative Bacteria

species	relative OM permeability (%)	major general porins	major efflux pumps
E. coli	100	OmpF/OmpC	AcrAB-TolC
P. aeruginosa	1−8	OprF	MexAB-OprM, MexXY-OprM
B. cepacia	11	OpcP1/OpcP2	AmrAB-OprA, BpeAB-OprB, BpeEF-OprC
A. baumannii	1−5	OmpA-AB	AdeABC and AdeIJK

flagella and pili, and enzymes (e.g., OmpT protease and phospholipase A).[14,44] The permeability properties of *E. coli* membranes are largely defined by general porins that have an exclusion limit at about 600 Da for OmpF. Most hydrophilic and amphiphilic antibiotics reach the periplasm through porins, whereas larger antibiotics, for example, erythromycin or novobiocin, are believed to cross the outer membrane through lipid bilayer by diffusion, which is expected to be slow.[14,45]

The existing paradigm that only small molecules (MW ∼ 600 Da) can cross the membrane by passive diffusion has recently been challenged by studies of the CymA channel from *Klebsiella oxytoca*.[46] CymA mediates diffusion of cyclodextrins with diameters up to 15 Å by a novel mechanism, which involves a mobile N-terminal peptide acting as a periplasmic gate. Binding of the incoming substrate displaces the N-terminal constriction and allows diffusion of the bulky molecule without compromising the permeability barrier of the OM. One could imagine that antibiotics could be modified in a way to mimic the interactions of substrates with specific porins and facilitate their own diffusion across the OM.

In addition, some leakage of large antibiotics is possible through export systems. *E. coli* cells lacking the TolC channel, which is involved in efflux of antibiotics and export of proteins as a part of the type I secretion pathway, are more resistant to vancomycin (2−4-fold increase in minimal inhibitory concentrations) than the wild-type cells, suggesting some penetration of this ∼1200 Da molecule through TolC.[47,48] Interestingly, this penetration requires the presence of active AcrAB transporter, further suggesting that vancomycin slips through TolC when it is engaged by the AcrAB pump (see also below). It is possible that permeation through other specific porins and TonB-dependent channels and the Bam complex responsible for the assembly of the outer membrane and at protein−LPS interfaces also contribute to the intracellular accumulation of antibiotics.

The overall compositions and architectures of *Pseudomonas* spp. and *Acinetobacter* spp. outer membranes are similar to those of other Gram-negative bacteria.[49] About 160 various proteins are embedded into the asymmetric bilayer and play a role in generalized and specific uptake and export of various compounds and polypeptides. Unlike enterobacteria, outer membranes of Pseudomonads and *Acinetobacter* spp. do not contain general trimeric porins such as OmpF and OmpC of *E. coli*. It is estimated that the permeability of *P. aeruginosa* and *A. baumannii* outer membranes is only 1−8% of that *E. coli* and that nonspecific "slow" porins OprF and OmpA-AB, respectively, restrict access of molecules larger than ∼200 Da, which is the size of a typical monosaccharide.[49−51] However, the growth rate of *P. aeruginosa* as well as of *A. baumannii* in a rich medium is comparable to that of *E. coli*, and hence to sustain the high rate of metabolism, the net influx of nutrients is expected to be comparable for the three species. *P. aeruginosa* likely solves this conundrum of the low permeability of the outer membrane despite a high nutrient uptake in several ways: (i) by secreting degrading enzymes that convert larger molecules into monounits (pseudomonads are masters of biodegradation) and (ii) by producing a larger fraction of substrate-specific channels (such as OprB and OprD).

OprF is one of the most abundant proteins in *P. aeruginosa* with a copy number of 200 000 per cell.[49] This protein plays a structural role by associating with LPS and peptidoglycan. *P. aeruginosa* mutants deficient in OprF synthesis have an almost spherical appearance, are shorter than wild-type cells, and do not grow in low-osmolarity medium. OprF also plays an important nonspecific uptake function by existing in closed and open conformations (400 open conformers of 200 000 total OprF).[52,53] Much less is known about the major porin OmpA of *A. baumannii*. This protein has a permeability similar to that OprF and also plays both structural and uptake roles in the outer membrane, but whether or not it exists as different conformers is unclear.[54]

In addition to "slow" porins, *P. aeruginosa* and *A. baumannii* use specific porins for uptake of small molecules. The large number of specific porins provides an advantage in nutrient-deficient environments, but could be limiting in rich growth media.[55−57] *P. aeruginosa* OprB, specific for glucose uptake, and OprD, specific for the diffusion of basic amino acids and peptides, are best characterized. OprD is the primary channel for the entry of carbapenems across the OM, and the reduced expression or loss of OprD has been frequently observed in carbapenem-resistant clinical isolates. *A. baumannii* produces an OprD homologue CarO, but whether or not this porin provides a path for carpenems remains under debate.[58,59]

Interestingly, although the outer membrane permeability of *A. baumannii* to cephalothin and cephaloridine, measured in intact cells, was found to be about 100-fold lower than that of *E. coli* K-12,[54] these cells are more susceptible to large antibiotics such as novobiocin and erythromycin.[28,60] Many *Acinetobacter* spp. are capable of using long-chain hydrocarbons as growth substrates, suggesting that their LPS−phospholipid bilayers of the outer membrane are more permeable for the hydrophobic molecules.[60]

Outer membranes of *Burkholderia* spp. contain a trimeric general porin Omp38 (OpcP), a homologue of *E. coli* OmpF.[61−63] The two studies of Omp38 permeability produced contradictory results: this porin is either similar to *E. coli* OmpF or by 1 or 2 orders of magnitude less permeable than OmpF. Further studies are needed to analyze the properties of porins and outer membranes for challenging Gram-negative bacteria such as *A. baumannii* and *Burkholderia* spp.

Efflux Pumps. *Efflux across the Outer Membrane.* All Gram-negative bacteria examined so far contain at least one multidrug efflux transporter responsible for protection against a variety of antimicrobial agents.[10,11] AcrAB-TolC of *E. coli* is the best characterized efflux pump, and its homologues are broadly represented in enterobacteria and other Gram-negative species.[16,64−66] In this three-component complex, AcrB is a proton-motive force driven transporter from the resistance−nodulation−division (RND) superfamily of proteins, TolC is an outer membrane channel, and AcrA is a periplasmic membrane fusion protein (MFP). The three proteins form a trans-envelope complex that expels multiple antibiotics from *E. coli* cells (Figure 3).[67,68] Extensive structural and functional analyses, including our own studies, showed that AcrB captures

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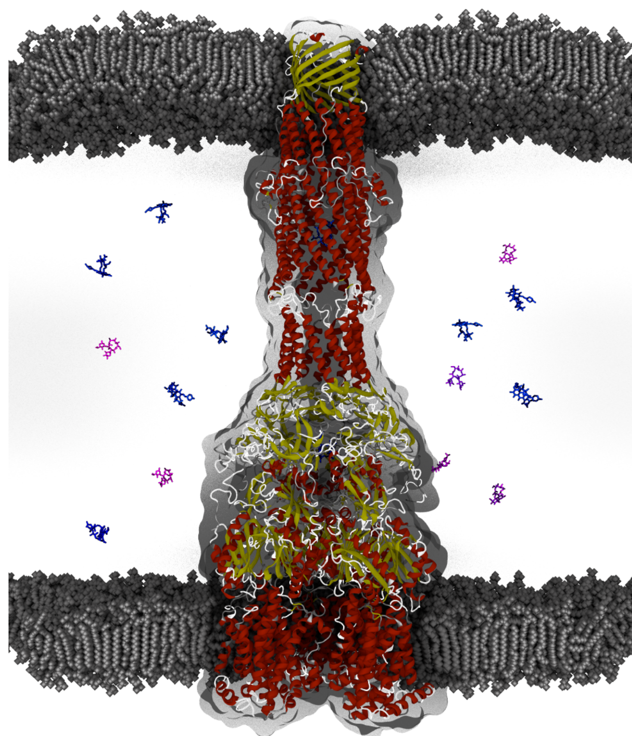

Figure 3. Model of the assembled MexB-MexA-OprM multidrug efflux pump from *P. aeruginosa*. MexA and other MFPs are proposed to translate the conformational changes in MexB transporter driven by a proton-motive force into opening of the outer membrane channel OprM that enables efflux across the outer membrane. The tripartite complex shown is a snapshot of a 1 μs MD simulation of the complex embedded in a bilayer composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). Proteins are colored according to secondary structure. For visual clarity water molecules are not shown. Accompanying drugs are shown for illustration purposes only (blue, rifampicin; pink, erythromycin). The gray shape corresponds to the AcrAB-TolC density obtained by the cryo-EM imaging. The equilibrated MexAB-OprM complex in the bacterial envelope maintains the expected distance of the *P. aeruginosa* periplasm. Image was initially exported from VMD Molecular Viewer in STL format and posteriorly rendered in Blender (https://www.blender.org). The gray shape corresponds to the AcrAB-TolC density obtained by the cryo-EM imaging.[124]

its substrates from the membrane and from the periplasm and expels them through the TolC channel into the external medium with the help of AcrA protein.[69−71] At least two substrate binding sites in AcrB, the proximal and the distal, are both located in the periplasmic domains of the protein. Thus, unlike other typical secondary transporters, AcrB with the help of AcrA and TolC expels substrates across the outer membrane while driven by the proton transfer across the cytoplasmic membrane. All RND-type multidrug efflux pumps are likely to share this mechanism, but it remains unclear whether some of these protein complexes can actually pump substrates not only across the outer but also across the inner membrane, thus performing the trans-envelope efflux of antibiotics from the cytoplasm directly into the external medium.

Among AcrAB-TolC substrates are organic solvents, antibiotics, detergents, dyes, and even hormones. Examination of chemical structures, as well as cocrystallization and molecular dynamics studies, significantly advanced the understanding of the molecular mechanism of multidrug recognition by AcrB.[72,73] However, physicochemical properties of compounds that would distinguish AcrB substrates from nonsubstrates remain elusive. The current consensus is that AcrAB-TolC is the most effective against amphiphilic compounds that diffuse slowly across the OM. Recent attempts to determine the affinity of AcrAB-TolC toward its substrates demonstrated convincingly that minimal inhibitory concentrations of antibiotics is a very poor, if at all, measure of whether an antibiotic is a good or a bad substrate of an efflux pump.[74] Several uptake assays were developed to analyze drug efflux, with most of them informative in both *E. coli* and *P. aeruginosa*. However, further characterization of the kinetic behavior of efflux pumps in the context of two-membrane envelopes of Gram-negative bacteria is needed.

P. aeruginosa MexAB-OprM is a close homologue of AcrAB-TolC and the major "house-keeping" efflux pump (Figure 3), but it is not the only pump that is expressed and contributes to antibiotic resistance in this species. The constitutive expression of MexAB-OprM confers intrinsic antibiotic resistance,[17−19] whereas elevated expression of MexXY-OprM is the major cause of aminoglycoside resistance in the absence of modifying enzymes.[75] In addition, efflux pumps MexCD-OprJ and MexEF-OprN are often overproduced in multiresistant clinical isolates. MexEF-OprN and MexGHI-OprD play important roles in *P. aeruginosa* physiology and are involved in the secretion of quorum-sensing signals and biofilm formation.[76−78] Furthermore, *P. aeruginosa* lacking either MexAB or MexHI is attenuated in animal models.[78,79]

As in the case of *P. aeruginosa*, genomes of *Burkholderia* spp. and *Acinetobacter* spp. contain multiple operons encoding RND-type efflux pumps. Some of these pumps are constitutively expressed and confer intrinsic antibiotic resistance, whereas others are inducible and expressed under specific physiological conditions. AmrAB-OprA *B. pseudomallei* and its homologues in other *Burkholderia* spp. are largely responsible for intrinsic antibiotic resistance.[80] AmrAB-OprA is closely related to MexXY-OprM of *P. aeruginosa* and, in addition to various antibiotics, confers resistance to aminoglycosides. In the most comprehensive genetic analysis, deletion of the 16 putative RND operons from *B. cenocepacia* strain J2315 showed that these pumps play differential roles in the drug resistance of sessile (biofilm) and planktonic cells. These studies revealed that (1) RND-3 (a homologue of AmrAB-OprA) and RND-4 play important roles in resistance to various antibiotics, including fluoroquinolones and aminoglycosides, in planktonic populations; (2) RND-3, RND-8, and RND-9 protect against the antimicrobial effects of tobramycin in biofilm cells; and (3) RND-8 and RND-9 do not play a role in ciprofloxacin resistance.[81]

The *A. baumannii* genome encodes seven RND-type efflux pumps. AdeIJK confers intrinsic resistance to various antibiotics including β-lactams, fluoroquinolones, tetracyclines, lincosamides, and chloramphenicol. In addition, AdeABC and AdeFGH are overproduced in clinical multidrug resistant isolates.[82] Surprisingly, overproduction of AdeABC and AdeIJK could also alter bacterial membrane composition, resulting in decreased biofilm formation but not motility.[83] When expressed in *E. coli* at levels comparable to those of AcrAB-TolC, AdeIJK but not AdeABC was more effective in the removal of lipophilic β-lactams, novobiocin, and ethidium bromide.[83]

Efflux across the Cytoplasmic Membrane. Highly abundant in genomes of various Gram-negative bacteria, single-component transporters extruding drugs from the cytoplasm

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into periplasm remain understudied in clinical settings, in part because their drug efflux activities are important only in the context of intact OM and in the presence of active efflux across the OM. Inactivation of single-component transporters that pump antibiotics across the inner membrane usually does not lead to significant changes in susceptibilities to antibiotics.[84] However, inactivation of multiple such pumps in *E. coli* is able to negate even the activity of AcrAB-TolC.[85]

Drug-specific transporters, such as TetA, are also spread by plasmids, and their contribution could be either additive or multiplicative depending on the combination of transporters expressed in host cells.[86] Experimental data and kinetic modeling showed that the coexpression of one- and three-component transporters leads to a synergistic loss of susceptibility to shared substrates.[86,87]

One-component transporters can belong to one of the three protein families: small multidrug resistance (SMR), major facilitator superfamily (MFS), or multidrug and toxic extrusion (MATE). *E. coli* SMR transporter EmrE, MFS MdfA, and MATE transporter NorE are the best characterized representatives.[88−90] MdfA (Figure 1) and NorE are commonly expressed in fluoroquinolone-resistant isolates and contribute to resistance against these antibiotics.[18] The properties and mechanisms of these proteins are well-characterized, but very little is known about their homologues in "difficult" Gram-negative pathogens.

■ APPROACHES TO BYPASS OR BREAK THE PERMEABILITY BARRIER

In general, several approaches are envisioned as to how antibiotic penetration across the permeability barrier of Gram-negative bacteria could be improved, but all of these approaches suffer from intrinsic limitations that need to be further addressed for new therapeutics to emerge.

Inhibition of New Accessible Targets. The most traditional approach would be to identify new accessible targets on cell surfaces or in the periplasm that could be interrogated in drug discovery efforts. The major bottleneck here is that such targets are optimized over millions of years of evolution to be resistant to antibiotics. On the other hand, recent breakthroughs in understanding the molecular mechanisms of the cell envelope assembly promise that such a goal is attainable, albeit through a more target-oriented approach. LPS and its biosynthesis pathway have been considered highly attractive targets in Gram-negative bacteria for decades.[7,91] Polymyxins, cationic cyclic lipopeptides, bind to LPS and permeabilize OM. These peptides recently re-emerged in clinics to treat multidrug-resistant Gram-negative infections, and their less toxic and "improved" variants attract significant attention.[92,93] *Burkholderia* spp. are intrinsically resistant to cationic peptides, but they have high efficacy against susceptible enterobacteria, *P. aeruginosa* and *A. baumannii*. There are two drug candidates in clinical trials that target the LPS pathway in *P. aeruginosa*.[94] ACHN-975 (Achaogen) is a synthetic molecule that inhibits LpxC deacylase, and POL7080 (Polyphor) is a synthetic peptide targeting the outer membrane protein LptD, involved in exporting LPS molecules across the periplasm. In addition to targeting essential enzymes, these inhibitors are expected to be synergistic with other antibiotics by enabling their permeation across the cell envelope.

Identification of Uptake Pathways and the "Trojan Horse" Approach. Another approach to bypass the permeability problem is to achieve fast or facilitated uptake of an antibiotic. Such efficient uptake would negate the impact of efflux and increase the concentration of an antibiotic at the target. As discussed above, *P. aeruginosa* and other "impermeable" Gram-negative species rely on specific porins and an active uptake system to fulfill their metabolic demands and to maximize their growth rates.[55,57] These pathways could be potentially exploited for delivery of antibiotics to their targets through the characterization of specificity determinants and modification of antibiotics to enable their uptake through such specific systems. In the recent study of carbapenem penetration into *P. aeruginosa*, molecular metadynamics simulations were used to delineate the lowest energy path of native substrates and carbapenem antibiotics through OccD1(OprD) and OccD3 channels and to identify molecular features in antibiotics that enable diffusion through specific channels.[95] Subsequent synthesis of carbapenem analogues to chemically probe some of the required features of permeation while maintaining the inherent activity led to an analogue of meropenem, for which a simple substitution on the side chain resulted in diminished dependence on OccD1 for translocation. This study is the first success story and demonstrates that a rational design of compounds with enhanced OM permeability is feasible. The major limitation of such an approach is a high frequency of resistance because the pathways are redundant and nonessential. However, identification and exploitation of uptake systems that are essential during establishment and proliferation of infections could facilitate the development of new therapeutics.

The "Trojan Horse" approach, which is largely derived from siderophore-conjugated antibiotics, is an example of such a strategy. Siderophore-conjugated antibiotics can be actively transported into the periplasm and cytoplasm by various iron-uptake systems.[96] A common pathway of bacterial siderophore transport systems in Gram-negative bacteria has been identified.[97] An outer membrane transporter binds the Fe^{3+}−siderophore complex with an affinity in the range of 1 nM and translocates this complex across the outer membrane with the help of TonB protein anchored in the cytoplasmic membrane. This process is driven by the cytoplasmic membrane potential. The Fe^{3+}−siderophore in the periplasm is bound by a binding protein that delivers its cargo via the cognate ABC transporter into the cytoplasm. Alternatively, the reduced iron is unloaded in the periplasm, as in the case of yersiniabactin and *P. aeruginosa* pyoverdines.[98] Hence, siderophore-conjugated antibiotics could be delivered by such uptake pathways either into the periplasm or all the way into the cytoplasm.[99] BAL30072 (Basilea) is the only candidate currently in clinical trials.[100] This compound is a monocyclic β-lactam conjugated to a dihydropyridone siderophore moiety, which has a potent activity against multidrug resistant Gram-negative pathogens including "impermeable" species.

Studies of natural peptide antibiotics suggested additional pathways that could be exploited for bypassing the "impermeable" cell envelopes. Pacidamycins are uridyl peptide antibiotics, which specifically kill strains against *P. aeruginosa*.[101−103] These antibiotics inhibit MraY translocase by catalyzing the first step in peptidoglycan synthesis. With MW > 800 Da, pacidamycins cannot penetrate the OM but exploit a specific uptake system to reach the target. The ABC transporter NppABCD belonging to the PepT family of transporters is implicated in the uptake of pacidamycin and other peptidyl nucleoside antibiotics, such as blastidin S, albomycin, and microcin C, across the inner membrane.[104] These antibiotics are likely to cross the OM

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through the TonB-dependent receptors involved in the uptake of siderophores.

Rules of Permeation. It is likely that permeation of different classes of compounds is affected by the outer membrane barrier and by the active efflux to different degrees. At present no rules exist to predict whether increasing uptake or reducing efflux would be the most efficient way to increase the potency of a specific class of compounds. It is believed that these rules will emerge when we address a critical gap in knowledge about what physicochemical properties and specific functional groups define the permeation of compounds across cell walls of Gram-negative pathogens.[8] Having such rules to guide medicinal chemistry efforts could potentially facilitate the discovery of novel anti-Gram negative drugs.[105] Although the task is complex, recent success in the development of a predictive model for drug accumulation in *Caenorhabditis elegans*[106] is inspiring. Several investigators[107,108] led the efforts to develop experimental approaches and to generate representative data sets of compound penetration into difficult Gram-negative pathogens. These studies complement the activity-based approaches to identify physicochemical properties that facilitate efflux of compounds.[109]

In addition, the first bacterial membrane models reflecting the biological complexity are emerging and could be used to address a wide range of questions about protein−lipid packing and dynamics that are not accessible by experiments[110] (see also Figure 1). With these models and methods it is now possible to study interactions of antimicrobial peptides and antibiotics with membranes and to identify their preferable penetration routes.[111] The "ideal" rules of permeation will also have to account for genetic and cellular processes that change the expression of efflux pumps and influx porins or modify the permeability of the OM in response to the uptake of antibiotics.

Efflux Pump Inhibitors (EPIs). The unquestionably significant impact of multidrug efflux pumps on bacterial physiology and the resistance to antibiotics in clinical settings makes them attractive targets for inhibition. Several classes of EPIs have been reported in the literature and are being pursued in drug development programs.[112−116] Phenylalanyl-arginyl-β-naphthylamide and analogous arylamines are broad-spectrum EPIs with activities against Gram-negative bacteria;[113,117,118] arylpiperidines[119] and more recently pyrinopyridine[120] are inhibitors of AcrAB-TolC from *E. coli*. Likewise, pyridopyrimidines are specific inhibitors of *P. aeruginosa* MexB and *E. coli* AcrB.[121,122] Importantly, broad-spectrum EPIs that target multiple efflux pumps have been reported not only to restore activities of antibiotics but also to reduce frequencies of antibiotic resistance.[123] As with many combination therapies, the task of EPI development is not easy, and multiple hurdles must be overcome, starting with the choice of an antibiotic for potentiation and all the way to matching the pharmacological properties of an EPI/antibiotic pair.[115] At the same time, some efflux pumps are critical for virulence and biofilm formation, for example, MexGHI-OpmH and MexEF-OprN from *P. aeruginosa*. EPIs effective against such transporters, if available, could be useful alternative therapeutics on their own.

CONCLUSIONS

The emerging multidrug-resistant Gram-negative pathogens present a significant challenge in clinics that should be addressed in a timely manner. Several decades of antibiotic discovery experience suggested that the permeability barrier is the major hurdle in the development of new therapeutics against these pathogens. Significant efforts are currently directed both at understanding at the molecular level the permeability properties of the OM from different bacterial species and at finding correlations between physicochemical properties of compounds and their permeation across the two membrane cell envelopes in the presence of efflux. Ultimately, the development of effective therapeutic modalities against multidrug-resistant Gram-negative pathogens will be strongly facilitated by a system-level approach that integrates the rules of permeation at the molecular level with supramolecular genetic and cellular processes of the organism. The task is complex and multifaceted but could be achieved by combined efforts at the government, industry, and academic levels.

AUTHOR INFORMATION

Corresponding Author

*(H.I.Z.) Phone: (405) 325-1678. Fax: (405) 325-6111. E-mail: elenaz@ou.edu.

Notes

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