Chapter 9
Antibiotic Resistance Due to Reduced Uptake

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1 Introduction

The introduction of antibiotic therapy for the treatment of bacterial infections has led to a greatly increased human lifespan compared to that in the pre-antibiotic era. However, a disturbing trend has also been noted in that, within a very short period of time following the introduction of a new antibiotic, resistance to that antibiotic begins to emerge, a factor that is becoming increasingly meaningful as the discovery of new antibiotics wanes (1–3). There are a number of mechanisms by which a bacterium may become resistant to a particular antibiotic. Generally these include, but are not limited to, modification of the drug to render it inactive, modification of the drug target, such that it is incapable of interacting with the drug and decreased uptake of the antibiotic into the cell, due to reduced transport and/or increased efflux. Recent functional genomic studies have also implied that antibiotics may have more complex mechanisms of action than first thought and we are beginning to appreciate that in addition to the mutation of primary targets, subtle mutations in secondary targets are likely to be influential (4, 5). This chapter will focus on the contribution of a decreased antibiotic uptake to an increase in antibacterial resistance.

2 Envelope Structure

2.1 Cytoplasmic Membrane

The cytoplasmic membrane is common to all bacterial species. For Gram-positive bacteria it is the primary barrier to antibiotic penetration, while an outer membrane further protects Gram-negative bacteria (6). In both cases, the cytoplasmic membrane is the site of essential functions such as nutrient transport, energy generation, the enzymatic assembly of lipid-linked monomers of cell envelope macromolecules (e.g. the peptidoglycan or lipopolysaccharide), and protein secretion. The cytoplasmic membrane is a phospholipid bilayer that acts as a hydrophobic barrier controlling the movement of solutes into the cell and enclosing the cytoplasmic contents of bacteria. This bilayer is studded with integral membrane proteins that carry out essential membrane functions. The density of cytoplasmic membrane proteins is high enough such that proteins are separated from each other by only three or four phospholipid molecules (7).

Phospholipids generally contain a glycerol 3-phosphate backbone attached to a hydrophilic head group and hydrophobic fatty acids. The lipids often have a positive charge to balance the negative charge on the phosphate and are termed zwitterionic, or have no charge on the headgroup giving the phospholipid a net negative charge. Although the type and proportion of phospholipids produced will vary under different environmental conditions, a typical membrane composition for *E. coli* is 75% zwitterionic phosphatidylethanolamine (PE), 20% anionic phosphatidylglycerol (PG), and 5% anionic cardiolipin (CL, or diphosphatidyl glycerol) (8). Membrane lipids are amphipathic and given an appropriate balance of headgroups, will spontaneously form bilayers to create a hydrophobic core that contains the fatty acyl chains separating the polar head groups on both sides of the bilayer. The fatty acyl chains are usually either saturated or contain a single double bond and are termed unsaturated, while the acyl chain may comprise 14–22 carbons. For example, the predominant fatty acids in the cytoplasmic membrane lipids of *E. coli* are saturated palmitic acid (16:0), the unsaturated species palmitoleic acid (cis-$\omega^{9,10}$–16:1) and cis-vaccenic acid (cis-$\omega^{11,12}$–18:1) (7).

The fluid mosaic model describes the properties of a membrane whereby both phospholipids and proteins diffuse laterally along the plane of a membrane, although proteins diffuse at a slower rate than lipids (7). Generally speaking, phospholipids do not readily flip from one leaflet in the

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bilayer to the other, since it is thermodynamically unfavourable for the polar head group to pass through the hydrophobic core. When bacterial cells are grown at increasing temperatures, there is generally an increased production of rigid, saturated fatty acids and a decreased production of flexible, unsaturated fatty acids in order to maintain membrane fluidity at a physiologically appropriate level.

2.2 Periplasm/Peptidoglycan

Located between the cytoplasmic membrane and outer membrane of Gram-negative bacteria, is the periplasm (Fig. 1a). Based on thin section transmission electron microscopy, the periplasm is estimated to be between 13 and 25 nm in width (9–11), depending on the sample preparation method used, and this can be compared to the width of membranes that are about 7–10 nm for the inner membrane and 10–30 for the outer membrane (NB, the membrane bilayer of the outer membrane is only slightly larger than that of the cytoplasmic but the long sugar chains of lipolysaccharide, LPS, can thicken the outer membrane adding a capsule-like aspect to the surface of the outer membrane (12). The peptidoglycan layer is located within the periplasmic region.

Given its position, the periplasm plays an important role in buffering the cell from changes in both the intracellular and extracellular environments. To facilitate this function, the periplasm contains anionic sugar polymers termed membrane-derived oligosaccharides as well as many proteins including (a) specific solute or ion binding proteins for the uptake of sugars, amino acids, peptides, vitamins and ions; (b) catabolic enzymes for the degradation of complex molecules into simpler ones that can be transported across the inner membrane; (c) detoxifying enzymes, like β-lactamases and aminoglycoside-modifying enzymes, for the degradation or modification of potential cell inhibitors; (d) hydrolytic enzymes, like nucleases and alkaline phosphatases and (e) proteins which aid in the assembly or translocation of major envelope proteins, peptidoglycan, LPS or capsules (13).

Despite some disparity in measurements of the size of the periplasmic space, the physiological state of the periplasm is thought to be gel-like. Hobot et al. (9) proposed that the periplasm is organized in a gradient of increasing peptidoglycan polymerization from the cytoplasmic membrane to the outer membrane. This peptidoglycan framework is filled with an aqueous solution containing periplasmic proteins, oligosaccharides and other small molecules. More recently, this model has been refined to propose that periplasmic proteins rather than peptidoglycan polymers account for the gel-like state of the periplasm (14). Measurements of periplasmic protein mobility are consistent with this modification of the model (15). Whatever the physiological state, the periplasm is a dynamic rather than a static environment, and is often underestimated for its significant role in cellular homeostasis.

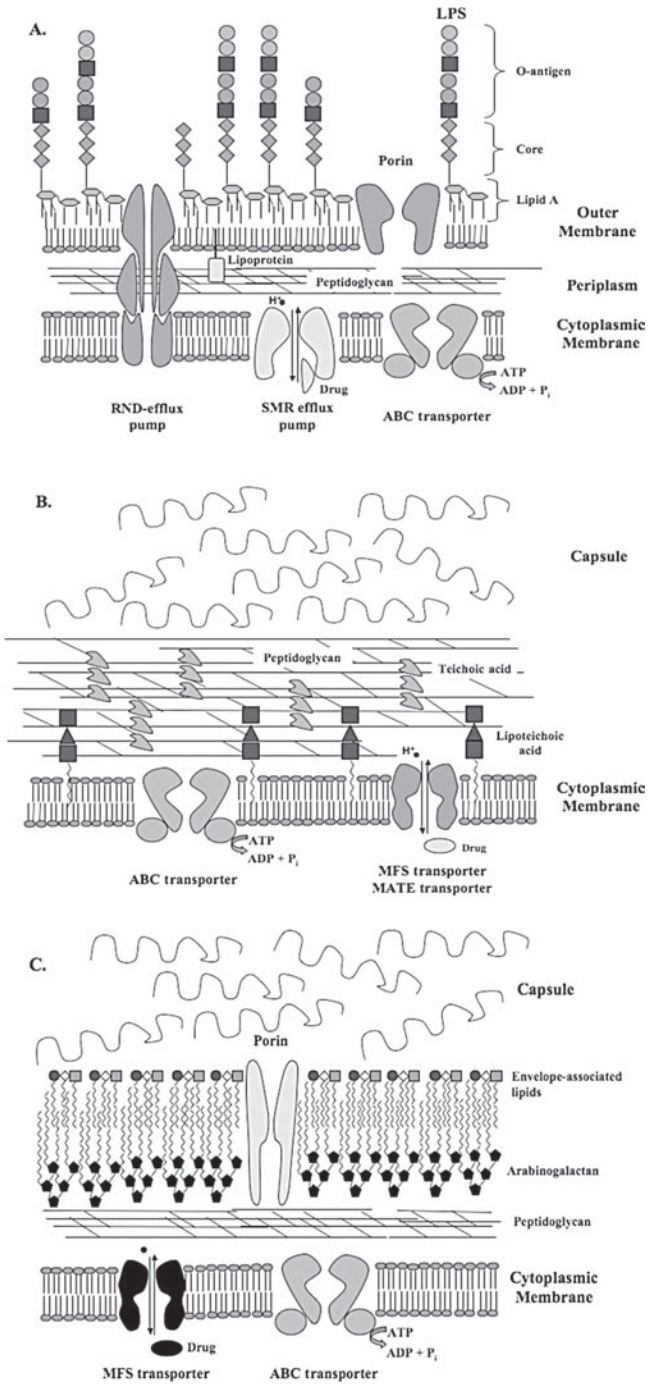

Fig. 1 The structure and arrangement of the cell envelope components of (**a**) Gram-negative bacteria, (**b**) Gram-positive bacteria and (**c**) mycobacteria. Note that although representations of example clinically relevant efflux system are shown, each type of bacterium may contain members of other classes of efflux systems, in addition to those displayed

The term peptidoglycan was first introduced by Weidel and Pelzer (16) to describe a "rigid bag of the volume and shape of the cell." Peptidoglycan is the polymer that encompasses the bacterial cell providing both strength and structure to the cell and is sometimes called the cell wall or murein sacculus. Due to the high metabolic activity and correspondingly high solute concentration within the cell, bacteria must contain an osmotic pressure that is between five and twenty atmospheres and thus greater than that of the surrounding medium. The peptidoglycan layer is the structure that facilitates maintenance of this pressure difference and is therefore absolutely essential to cell survival. Nevertheless, the peptidoglycan layer has sufficient plasticity to allow for both cell growth and division and specific enzymes that can remodel the peptidoglycan locally to permit these essential functions, with which peptidoglycan is intimately involved.

Although it is conserved in all eubacteria, differences exist in the peptidoglycan layer between Gram-positive and Gram-negative bacteria. In Gram-positive organisms, the peptidoglycan layer is multilayered and relatively thick (5–25 nm) (17, 18). Various acidic and/or neutral polymers like teichoic acid or teichuronic acid are covalently attached to the peptidoglycan layer (Fig. 1b). In Gram-negative organisms, the peptidoglycan layer is located between the cytoplasmic and outer membranes and tends to be only a few layers (19) and 1.5–6 nm thick (20), although recent studies suggest that the peptidoglycan chains may be at least partially oriented perpendicularly to the surface of the cytoplasmic membrane (21). Lipoproteins embedded in the outer membrane and peptidoglycan-associated proteins (covalent and non-covalent) anchor the peptidoglycan layer to the outer membrane.

Peptidoglycan is composed of a polysaccharide backbone made up of β, 1–4-linked alternating residues of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), cross-linked through peptide side chains. A short peptide of four amino acids is attached to the carboxyl group of NAM of mature peptidoglycan. Variability in the peptidoglycan structure is largely due to differences in the short peptide, although differences in the glycan backbone and nature of the crosslink are also observed (22). In *Escherichia coli*, for example, the mature stem peptide is composed of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid and D-alanine, whereas in *Staphylococcus aureus meso*-diaminopimelic acid is replaced by L-lysine.

The average glycan strand is about 30 muropeptide units in length (23, 24). Individual strands are cross-linked to each other either directly or indirectly through peptide side chains, and these covalent peptide crosslinks provide the strength required to resist the internal osmotic pressure. In Gram-negative bacteria, for example *E. coli*, direct cross-linking occurs between the carboxyl group of the D-alanine in position 4 of one stem peptide and the free amino group of *meso*-diaminopimelic acid in the adjacent strand. Cross-linking in Gram-positive bacteria is indirect, and occurs through an inter-peptide bridge of five glycines in *S. aureus* for example. The degree of cross-linking and cross-linking position also differs between species of bacteria (25, 26), with Gram-positive organisms having a higher degree of cross-linking than Gram-negative organisms, which have the added protection of the outer membrane.

2.3 Outer Membrane

The outer membrane is an unusual bilayer membrane found only in Gram-negative bacteria (Fig. 1a) (6). What makes this structure unique is the asymmetric nature of the bilayer. The composition of the inner leaflet is similar to that of the cytoplasmic (inner) membrane, with phosphatidylethanolamine being the predominant phospholipid and minor amounts of other phospholipids, e.g. phosphatidylglycerol and cardiolipin (diphosphatidylglycerol). As with the cytoplasmic membrane, the lipid composition of the outer membrane is not static; it varies with the environmental conditions in which the bacteria are found.

There is some dispute as to whether phospholipids are also found in the outer leaflet of the outer membrane, however the most predominant lipidic species of the outer leaflet is a long polymeric glycolipid termed LPS. LPS has a tripartite structure consisting of a Lipid A moiety, a core oligosaccharide and a longer *O*-polysaccharide.

The Lipid A (or endotoxin) backbone usually consists of a diglucosamine residue that is phosphorylated at its C1 and C4′ positions. The disaccharide is covalently *N*- or *O*-linked to anywhere from 4 to 7 fatty acids that anchor it into the membrane. These fatty acids tend to be saturated and hydroxylated at the C3 position. This 3–OH group may have another fatty acid as a substituent, producing an acyl-oxyacyl structure that is a characteristic feature of Lipid A.

The diglucosamine backbone of Lipid A is conserved amongst most Gram-negative bacteria. The fatty acid composition however, is quite variable from species to species. Different environmental conditions can also induce changes in the fatty acid profile. How these differences in fatty acid composition influence their packing behaviour and thus, membrane fluidity and transport, are discussed below. In some bacteria, under particular conditions (e.g. low concentrations of divalent cations in the growth medium), the phosphate groups of the diglucosamine moiety can be substituted with the positively charged sugar arabinosamine, whereas phosphatidylethanolamine substitutions can also occur. These changes increase the resistance of the bacteria to certain cationic antibiotics and are discussed in detail in Sect. 4.2.

The core oligosaccharide of LPS is covalently attached to the Lipid A via the unique sugar molecule 2-keto-3-deoxyoctulosonic acid (KDO). In addition to KDO, this region also includes a variety of other sugar molecules such as L-glycero-D-manno-heptose and its optical isomers, glucose, galactose, rhamnose etc. Some of these sugars may be modified by the addition of phosphate, pyrophosphate, phospholipids (e.g. phophatidylethanolamine, phosphatidylcholine), or amino acids (e.g. alanine). The overall structure of the core oligosaccharide is relatively conserved within a given bacterial genus but may vary somewhat with respect to sugar composition, substitution, and/or connectivity (27).

Between approximately 10 and 25% of the core oligosaccharides are covalently linked to the *O*-polysaccharide (or *O*-antigen), a string of sugar repeat units, that vary substantially even within a species. This diversity is proposed to be driven by selective pressure (e.g. from the immune response or from phage susceptibility) that arises from being exposed to the external environment (28). The basic structure of the *O*-polysaccharide consists of a mono- to octa-saccharide repeat. Over 60 different sugars from different Gram-negative bacteria have been identified as being components of an *O*-polysaccharide. Some examples of these sugars include glucose, mannose, ribose, rhamnose, glucosamine, fucosamine and amino hexuronic acids such as quinovosamine.

The number of *O*-repeats varies from 0 to 50 units and this produces a characteristic ladder pattern when LPS is resolved on an acrylamide gel. Some mucosal pathogens, such as *Bordetella pertussis*, completely lack an *O*-antigen and are thus said to possess LOS (lipooligosaccharide) rather than LPS (29). Other organisms, such as *Pseudomonas aeruginosa*, can have *O*-antigens that extend more than 40 nm from the surface of the cell (12).

In addition to LPS, the outer membrane contains a moderate number of proteins present in high copy number. These proteins are involved in a variety of cellular processes that include selective permeation, cell shape and membrane stabilization, motility, adherence, transport and interaction with the immune system, bacteriophages and other bacteria (6, 30).

An abundant class of outer membrane proteins is the lipoproteins. These are relatively small proteins that are present in high copy number (~7×10^5/cell). They are modified at an *N*-terminal cysteine with an *N*-acyl diacyl glyceride residue that non-covalently inserts into the outer membrane to anchor the proteins. Lipoproteins are thought to stabilize the cell wall by associating either covalently or non-covalently with the peptidoglycan depending on the organism. In *Pseudomonas* species, for example, the lipoproteins examined to date are all non-covalently associated with the peptidoglycan. In *E. coli*, however, a third of the major lipoprotein molecules are covalently linked to the diaminopimelate groups of the peptidoglycan via their C-terminal lysine or arginine residues.

Outer membrane transporters are involved in both the uptake (porins) and efflux (efflux channels) of compounds into and out of the cell. Both of these protein classes adopt a β-barrel structure in the outer membrane although their architecture is very different with the porins containing one water-filled channel per monomer (or often three per trimer) and the efflux channels containing one channel made from three monomers. Efflux channels have an additional α-helical periplasmic domain, which is discussed in a later chapter and elsewhere (31). Amino acids with non-polar side chains form the outer surface of the barrel and interact with membrane lipids, thus stabilizing the structure. Hydrophilic amino acids line the interior of the channels, providing a polar environment for hydrophilic compounds to travel through.

2.4 Mycobacterial Cell Envelope

Although phylogenetically classified as Gram-positive bacteria, the mycobacteria have a uniquely organized cell envelope (Fig. 1c). As with other bacteria, the cytoplasmic membrane forms an inner barrier between the cytoplasm and the environment, and its lipid composition is similar to that of other bacteria. This is surrounded by a layer of peptidoglycan, with a structure similar to that of Gram-negative bacteria (i.e. relatively thin). External to this is the arabinogalactan layer, consisting of a complex branched network of polysaccharide. Each arabinogalactan residue consists of a polymer of galactofuranose, many of which possess five or six covalently attached arabinose moieties (Fig. 1c). Each of the arabinose groups in these terminal groups are ester-linked via the 1′-hydroxyl moiety to lipidic mycolic acids which extend to the bacterial surface. The mycolates attached to the arabinogalactan are very long (60–90 carbons) and may contain unusual cyclopropane moieties within their acyl chains (32). Due to the length of these fatty chains, they are found in the gel state with phase transition temperatures as high as 60–70°C (33). The composition of the membrane varies due to regulation by temperature and/or environment, analogous to lipid compositional changes in other types of bacteria. There is some evidence for the presence of another glycolipid monolayer consisting of trehalose dimycolates, sulfolipids, phythiocerol dimycocerosate and phenolic glycolipids external to the mycolate residues of the arabinogalactan. The approximate thickness of the mycolate bilayer is ~37–90 nm, substantially larger than that of a Gram-negative outer membrane (34, 35). Like the Gram-negative bacterial outer membrane there are porin-like molecules that traverse the mycolic acid layer but they have a rather unique structure (35, 36). In some senses, the envelope of mycobacteria resembles the outer membrane of Gram-negative bacteria and due to the presence of this thickened highly hydrophobic envelope, mycobacteria are

characterized by their extremely low permeability to most hydrophilic antibiotics.

2.5 Capsule

Many bacteria in their natural habitats produce extracellular polysaccharide capsules. Capsular polysaccharides are either homo- or hetero-polymers of repeating sugar units, connected by glycosidic bonds to form the capsule structure. Because of the broad range of monosaccharide units and glycosidic bond configurations possible, bacterial capsules are extremely diverse. Initially capsules were divided into groups (referenced to *E. coli*) based on the presence of common monosaccharides (37), but more recently capsule classification has been based solely on genetic and biosynthetic criteria to divide *E. coli* capsules into four distinct groups (38). This updated classification scheme (again referenced to *E. coli*) accounts for the observation that not all capsules are composed of polysaccharide K antigens; previous classifications were based on the biochemical division of K antigens, which all form capsules.

Capsule layers are highly hydrated, containing over 95% water (39), and as such may function to protect the organism from desiccation. Consistent with this suggestion, mucoid isolates are more resistant to drying than their non-mucoid isogenic counterparts (40), and changes in extracellular osmolarity are known to induce expression of capsule molecules (41, 42). Polysaccharide capsules also function as adherence factors. Capsules facilitate both biofilm formation and niche colonization (43, 44) by promoting the adherence of bacteria to each other and to surfaces. This ability of bacteria to attach to surfaces and establish a biofilm plays an important role in initiating and maintaining infection (45, 46). For example, *P. aeruginosa* infections of the cystic fibrosis lung are often characterized by overexpression of alginate and biofilm formation (47), which probably helps to protect the bacteria from opsonization and killing by neutrophils and macrophages in the lung.

Infections are further maintained through the ability of the capsule to resist both the non-specific and specific immune responses of the host. Polysaccharide capsules are poor activators of the alternative complement pathway (48–50) and furthermore mask underlying cell surface structures, which do typically activate this pathway (51, 52). This reduced ability to activate opsonic fragments of complement (e.g. C3b), and the net negative charge of the capsule surface works to inhibit phagocytosis (53, 54). Capsular polysaccharides also confer resistance to the host's specific immune response, by mimicking the structure of polysaccharides found in the host, and consequently are usually poor immunogens (55–57).

3 Intrinsic Resistance

3.1 Restricted Permeability

3.1.1 Gram-Negative Bacteria

The outer membrane of Gram-negative bacteria is a semi-permeable barrier to the uptake of most hydrophilic molecules larger than a certain size exclusion limit. An analogy is often drawn to this membrane constituting a molecular sieve although this is only really true for negatively charged or neutral polar molecules, as both positively charged and hydrophobic molecules can pass across the outer membrane by other routes. For the former molecules, uptake is limited by the size of the water-filled channels of β-barrel proteins termed porins (58). The total surface area of the outer membrane that is occupied by such channels has been estimated as approximately 0.6% in *E. coli*, and this together with limited diffusion imposed by frictional interactions between molecules passing through the channel and the amino acids lining the channel wall, severely restricts uptake of hydrophilic molecules especially those like β-lactams, trisaccharides, and tetrapeptides that have sizes that are not much smaller than the restricting diameters of these channels in e.g. *E. coli*. Other bacteria, e.g. *Pseudomonas aeruginosa*, have a much smaller number of channels leading to an overall outer membrane permeability that is only 1–8% that of the *E. coli* outer membrane, even though *P. aeruginosa* has larger-sized channels and a larger exclusion limit. Restricted permeability through the outer membrane clearly contributes therefore to the observation that Gram-negative bacteria tend to have higher intrinsic resistance to most antibiotics than their Gram-positive counterparts, a factor that is a major contributor to the drastic dearth of discovery of new Gram-negative selective antibiotics.

It is worth considering the nature of the "fabric" of the outer membrane molecular sieve. As mentioned above, the outer membrane surface largely contains, as its major lipidic molecule, the highly anionic glycolipid LPS, which is partly neutralized, cross-bridged and thus stabilized by divalent cations, predominantly Mg^{2+} and Ca^{2+}. This surface thus tends to repel neutral and anionic polar molecules, but as described below can actually serve to permit self-promoted uptake of cationic molecules. Further evidence that the outer membrane is a barrier to uptake of hydrophilic antibiotics is seen in the fact that increasing outer membrane permeability by cloning in large, abundant porins leads to increased antibiotic susceptibility in *Pseudomonas aeruginosa* (59), while disrupting the fabric of the outer membrane by removal of divalent cations with chelators like EDTA has a similar effect (60, 61).

3.2 Mycobacteria

Based upon the low susceptibility of mycobacteria to most antimicrobials, it is clear that the cell wall of this organism forms a significant antimicrobial barrier. Indeed, early studies examining the permeability of *Mycobacterium chelonae* showed that it was approximately tenfold less permeable to hydrophilic β-lactam antibiotics than was *P. aeruginosa* (62) (i.e. 100- to 1,000-fold less permeable than the *E. coli* outer membrane).

In contrast to the trimeric general porins of Gram-negative bacteria that have a single pore per monomer, MspA is an octamer of small subunits that assemble to form a single central channel (35), and channel numbers tend to be relatively low. In addition, the MspA pore is much longer than for the general porins, presumably due to the thickness of the mycobacterial cell wall. Therefore, substrate interactions with the channel interior may be more pronounced in mycobacteria and might hinder solute diffusion. Indeed, this appears to be the case as the deletion of MspA from *Mycobacterium smegmatis* results in both increased resistance to hydrophilic antibiotics as well as decreased growth due to lowered permeability to nutrients (62, 63).

3.3 Efflux

Intrinsic antibiotic resistance in Gram-negative bacteria is due to the synergy between low outer membrane permeability that restricts the rate of exposure of the interior of the cell to antibiotics, and the presence of additional resistance mechanisms such as drug modification (e.g. β-lactamases) and multidrug efflux systems. Cytoplasmic membrane-localized efflux pumps are widespread among bacteria and are divided into five major classes on the basis of bioenergetic and structural criteria (64) and it is worth noting that in addition to contributing to antibiotic efflux, many of these pumps also have roles in normal cell physiology (65).

The ATP-binding cassette (ABC) superfamily is an ATP-driven efflux system found in Gram-negative and Gram-positive bacteria, as well as in mycobacteria. The major facilitator superfamily (MFS) is another ancient efflux system that uses chemiosmotic energy and functions as a drug-ion antiporter. The resistance/nodulation/cell division (RND) family and the small multidrug resistance (SMR) family are both proton-driven pumps although the former comprises multi-subunit complexes. The fifth system is the multidrug and toxic compound extrusion family (MATE) and also utilizes the chemiosmotic gradient across the cytoplasmic membrane to energize transport. Gram-positive bacteria often employ MFS efflux pumps such as NorA in *S. aureus* (66) which provide resistance to fluoroquinolones.

In Gram-negative bacteria, the RND (resistance-nodulation-division) family of pump proteins are the predominant class (67) involved in intrinsic resistance. RND transporters are tripartite systems consisting of an outer membrane channel-tunnel, an inner membrane pump and a peripheral cytoplasmic membrane/periplasmic linker protein. A broad range of structurally unrelated substrates are known to be pumped out of bacterial cells including most types of antibiotics, biocides, heavy metals, organic solvents, dyes, and detergents (68). Given the ubiquitous distribution of efflux systems in bacteria, there is much interest in determining the natural and intended substrates of these efflux systems (65). In *E. coli* for example, efflux pumps are capable of shuttling toxic fatty acids and bile salts out of the cell and thus it has been suggested that normal metabolic intermediates and noxious compounds that *E. coli* encounters in the gut during infection may be natural substrates (68).

In many bacteria, the expression of efflux system genes is tightly controlled. Although antibiotic efflux is typically described as an intrinsic resistance mechanism, there are a number of mutational events that can lead to increased expression of efflux systems, and therefore increased resistance. For example, *tetR*, the negative regulator of the MFS tetracycline efflux pump is ordinarily bound to the operator sequence upstream of the efflux genes, preventing expression under normal conditions (69). In the presence of its substrate (i.e. tetracycline) the TetR protein is released from the operator and transcription of the gene(s) involved (*tetK*, *tetL*, and/or *tetB*) proceeds. Thus the bacteria do not become resistant to tetracycline unless tetracycline is actually present.

A similar general principle exists for many RND efflux systems in wild-type bacteria in that expression of efflux pumps is tightly regulated, although some pumps are always expressed at basal levels. However, unlike the situation with the TetR protein described above, the actual efflux genes are often not induced by the known substrates of the particular efflux pump. Rather, what often occurs is that a mutation appears in the regulator of the efflux system following antimicrobial therapy, such that the genes encoding for the pump components are expressed constitutively at higher levels leading to increased resistance to all substrates that the pump can efflux. The mutations are often stable point mutations that reduce the DNA binding affinity of particular repressors for their target regulatory regions within promoters and lead to constitutive expression of efflux components (70). Many clinical isolates of the cystic fibrosis pathogen *P. aeruginosa* have multidrug resistance phenotypes due to regulatory mutations that are probably selected for in the lungs of CF patients who are often on chronic antimicrobial therapy (68).

4 Antibiotic Penetration and Resistance Mechanisms

4.1 Porin Pathway

Porins permit the diffusion of a variety of compounds into the periplasm. There are three classes of porins; general, specific, and gated (Fig. 2). Uptake through general porins is considered passive, as it involves passive diffusion through the aqueous channels of the porin and is dependent only on the physicochemical properties of the solute (that is, size, charge, polarity, and the magnitude of the concentration gradient across the membrane) relative to the side chains of the amino acids lining the pore and especially those side chains found at the most constricted part of the channel. The crystal structures of several general porins have been solved and reveal that they are trimers of 16 stranded anti-parallel β-barrels that enclose a pore lined predominantly with hydrophilic amino acids (71, 74, 75). These β-strands tend to be connected by short (3–4 amino acid) turn regions on the periplasmic side of the porin and much longer loops of amino acids on the external side of the outer membrane. The cross-section of the channel interior somewhat resembles an hourglass and can be conceptually divided into three zones; the external mouth, the constriction zone or eyelet, and the exit. The mouth of the general porin pore acts as a crude filter. This region is rich in charged amino acids and may be somewhat restricted by one or more extracellular loops that fold into it. The purpose of these two features is to constrict the opening, both physically and electrostatically, such that large, hydrophobic, and/or highly charged compounds cannot enter the cell. The eyelet is the narrowest part of the channel, usually formed by a single loop 3 that folds from the external surface back into the porin channel. The size of this eyelet determines the maximum size i.e. the exclusion limit of molecules that can pass through the channel. For the prototypic bacterium, *E. coli*, the exclusion limit determined by the major porins OmpF and OmpC is around 600 Da (equivalent to a trisaccharide or tetrapeptide), although there are subtle differences in channel size for these two proteins. Therefore, for this and other enterobacteriaceae, it is presumed that small, hydrophilic antibiotics such as chloramphenicol, tetracycline, fluoroquinolones and β-lactams (including cephalosporins and carbapenems) might utilize these channels as entry points. This fact has been confirmed by the isolation of mutants, both in the clinic and in vitro, that are resistant to the above-mentioned antibiotics due to either a complete loss of or diminished porin expression (76–78).

Specific porins are similar to general porins with one major exception; they have stereospecific binding sites for their substrates, which are located in part in the eyelet. This specificity narrows the structural range of molecules that can pass through these channels. The crystal structure of the LamB channel of *E. coli* has been solved and shows that this

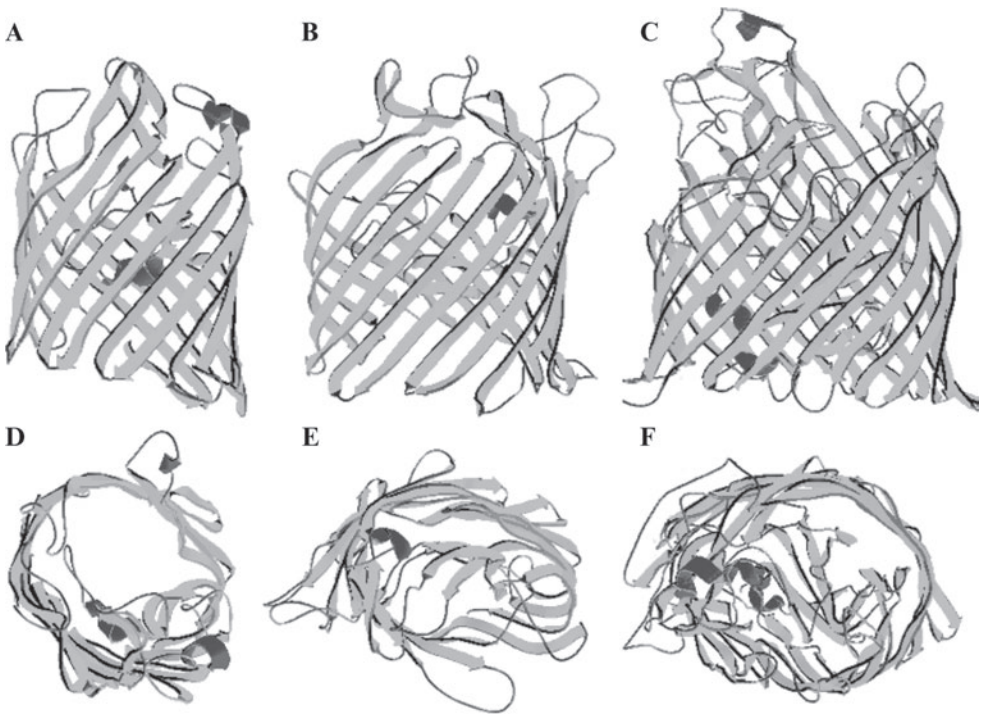

Fig. 2 Representative structures of the porin molecules of *Escherichia coli*. Side (**a**) and top (**d**) view of the OmpF general porin (71). Side (**b**) and top (**e**) view of the maltodextrin-specific channel, LamB (72). Side (**c**) and top (**f**) view of the gated porin FhuA (73). Note the varying degrees of channel constriction imparted in each porin type by the inward folding of various extracellular loops or domains (see text for complete description)

porin is highly specialized for the uptake of maltodextrins (72). The eyelet of this porin is more constrained than in general porins due to the folding of two additional loops into the mouth of the channel. Six contiguous aromatic amino acids (the greasy slide) form a path through the channel, down which the sugar molecules travel. In addition, the channel interior is lined with polar amino acids (the polar track) that stabilize the hydroxyl groups of the sugars (79). An analogous design is found for the phosphate-specific porin OprP of *P. aeruginosa* (80). The channel interior of this trimeric porin is quite constricted and reveals a phosphate-binding "arginine ladder" comprising eight arginine residues that span from the extracellular surface down to a constriction zone where phosphate is coordinated. Lysine residues also coat the inner periplasmic surface of this channel creating an "electropositive-sink" that pulls the phosphates through the eyelet and into the cell.

Due to their specialized nature, the only antibiotics that should be able to penetrate specific porins are those that mimic the channel's natural substrates. Indeed, this is true for the Tsx channel of *E. coli*. Specific for nucleosides, this porin also takes up the structurally related antibiotic albicidin (81, 82). Similarly, the OprD porin of *P. aeruginosa* is specific for the uptake of the basic amino acids arginine and lysine and basic dipeptides, as well as the structurally analogous carbapenem antibiotics imipenem and meropenem (83, 84). Recently we also demonstrated that the tricarboxylate-inducible porin OpdH, a homolog of OprD, appeared to be involved in the uptake of the bulky cephalosporin ceftazidime (85). It should also be noted, that low levels of structurally unrelated compounds can also diffuse through specific porins. This is especially the case for non-fermentative organisms, like *Pseudomonas*, which lack classic general porins. For example, the OprD porin in addition to taking up basic amino acids is the major facilitator involved in the diffusion across the outer membrane of compounds up to 200 Da in mass (59).

Gated porins, also known as TonB-dependent receptors are monomeric proteins consisting of 22-stranded β-barrels, and permit the specific entry of larger compounds such as iron-siderophore complexes into the cell. The mouth of these channels is blocked by a globular domain termed the plug (86, 87). Uptake is initiated once a substrate docks onto a gating porin. This binding, in conjunction with energy input from the TonB energy transducing protein, results in a series of conformational changes in the plug domain that culminate in both the release of the substrate and the revelation of a translocation pathway (73).

Due to their large channel sizes, gated porins may seem like the ideal conduits for antibiotic uptake; however, this use is generally limited by the specificity of substrate docking. It is known that there are certain gated receptors that have somewhat lower selectivity, e.g. Cir and FhuA. However although providing antibiotics with iron binding groups (e.g. catechol or heme groups) can improve uptake across the outer membrane, and consequently lower MICs, none of these substituted drugs have been clinically successful and this may reflect mechanisms of toxicity and/or interference with iron metabolism in the host. Specific antibiotics that can be taken up by ferric-siderophore receptors include albomycin, a structural analogue of ferrichrome, which is taken up by the FhuA gated-porin receptor. Interestingly, rifamycin CGP 4832 (a rifampin derivative), a structurally unrelated antibiotic, is also taken up by FhuA (88). The crystal structures of FhuA in complexes with both of these antibiotics indicate that despite differences in structure, both antibiotics bind to the same residues of the porin (89), indicating that gated porins tolerate some structural flexibility.

As described above, mycobacterial envelopes contain a class of porins that although structurally unrelated to Gram-negative porins, serve as the major pathway for hydrophilic antibiotics. There are two types of mycobacterial porins represented by OmpATb, which is not well-studied, and MspA, which has been crystallized (36). MspA from *M. smegmatis*, the best-characterized mycobacterial porin, is the major route of entry for hydrophilic compounds into this organism (36). However, the medically important mycobacteria, *M. tuberculosis*, and *M. bovis* BCG seem to lack MspA-type porins, and depend exclusively on OmpATb-type porins, an observation that may explain the intrinsically lower susceptibility of these organisms to hydrophilic antibiotics compared to *M. smegmatis*. The diameter of the MspA channel from *M smegmatis* is apparently larger than that of the OmpATb porin from *M. tuberculosis*, which is not well characterized, and cloning of the *M. smegmatis* MspA protein into *M. tuberculosis* increases the sensitivity of *M. tuberculosis* to β-lactams by up to 16-fold (90). Additionally, the growth rate of *M tuberculosis* expressing *M smegmatis* MspA is increased; suggesting that nutrient uptake in this species is also limited by the small pore size of OmpATb. Regardless of which porin proteins a particular strain expresses, the porin pathway seems to be involved in the uptake of pyrazinamide (91) and β-lactams (92).

4.2 Self-Promoted Uptake and Regulatory Mutants

The self-promoted uptake pathway is limited to Gram-negative bacteria and generally pertains to the passage of cationic amphipathic molecules across the outer membrane. Self-promoted uptake involves the interaction of polycations with sites on the surface of the outer membrane at which divalent cations cross-bridge adjacent LPS molecules. Displacement of these divalent cations leads to local distortion of outer membrane structure and this provides sites for

uptake of other polycationic antibiotic molecules; thus these polycations promote their own uptake rather than diffusing across the outer membrane through water-filled channels.

Recently, it has become clear that self-promoted uptake is quite effective in many species of bacteria including *E. coli*, *P. aeruginosa*, *Salmonella enterica* and *Yersinia sp*., which all seem to have the potential to be killed by antibiotics that access the self-promoted uptake pathway (61). Other species such as *Burkholderia cenocepacia* and *Helicobacter pylori* show a significantly lower rate of killing by antibiotics that would normally enter via this pathway (93, 94). For species that are normally sensitive to killing via the self-promoted uptake pathway, the organism in question generally maintains a level of control over the effectiveness and/or accessibility of this pathway (95–98), as discussed in more detail below.

The characteristics of the LPS of a particular bacterial strain primarily determine whether or not a particular bacterium possesses an effective self-promoted uptake pathway. As described in Sect. 3.2, the structure of bacterial LPS is complex and species-specific. The LPS of many bacteria is characterized by a large number (3–12) of negatively charged phosphate groups and anionic sugars (e.g. KDO) in the core oligosaccharide and usually two additional phosphates attached to the Lipid A moieties of the LPS (28). These negatively charged groups are ordinarily bridged by divalent cations, which serve to stabilize the outer membrane by preventing the LPS molecules from repelling one another. Studies carried out with chelators of divalent cations, such as EDTA, have shown that when the cell is rapidly depleted of the divalent cations bound to the LPS, there is a massive disruption in outer membrane integrity, with a concomitant loss of ~50% of the LPS (61). Thus, these divalent cations are an integral component required for maintenance of outer membrane structure.

Cationic antibiotics and the cationic antimicrobial peptides can also disrupt the bacterial outer membrane. The cationic peptides are ubiquitous in nature and form an important component of the human innate immune system (99). Basically, these are small peptides that have a net positive charge due to the presence of a number of lysine or arginine residues in their sequence. Soil-dwelling bacteria, lactic acid bacteria, plants, insects, fish, birds, amphibians, and other animals also produce cationic peptides. Studies with the cationic lipopeptide antibiotic polymyxin B showed that when bacteria are exposed to this antibiotic the integrity of the bacterial outer membrane is rapidly destroyed, indicating that the outer membrane might be a primary determinant by which these compounds gained access to Gram-negative cells (100, 101). Cationic antimicrobial peptides have a number of physical properties that are important for their activity. As suggested by their name, the cationic nature of the molecule is very important and substituting uncharged for the charged amino acids severely impairs their antimicrobial ability. Additionally these peptides usually contain up to 50% hydrophobic amino acids and consequently can insert into membranes while folding into an amphipathic structure that contains both a highly polar face and a hydrophobic face.

Regulation of self-promoted uptake has been studied in a number of organisms including *E. coli. enterica* and *P. aeruginosa*. The genetics of resistance are perhaps best understood in *E. coli* and *S. enterica* and these systems will serve as the model for the remainder of this discussion, with important exceptions being highlighted where applicable. Early work in *S. enterica* showed that there were two loci responsible for increased resistance to polymyxin B and other cationic antimicrobial peptides and that these mapped to two systems named *pmrAB* (*p*oly*m*yxin *r*esistance gene *A* and *B*) and *phoPQ* as reviewed elsewhere (102). Both of these systems are two-component regulatory systems that normally turn on genes in response to a given environmental condition, limiting concentrations of divalent cations for the *phoPQ* system (103), and high concentrations of ferric iron in the case of the *pmrAB* system of *S. enterica* (104). *S. enterica* are intracellular pathogens that encounter limiting divalent cation concentrations and high concentrations of antimicrobial peptides when engulfed by the host cell. Thus the bacterium senses the limiting divalent cation concentration and responds in a way that makes it more resistant to cationic peptides. Alternatively it was recently demonstrated that cationic peptides can bind directly to PhoQ and regulate their own resistance (105). Although the precise mechanism underlying signalling by cationic peptides is not completely defined, it appears to involve interaction with a cytoplasmic-membrane-facing polyanionic domain of PhoQ. Clearly, direct regulation by a host molecule would appear to provide a distinct advantage to the bacterium in a host at a site where Mg^{2+} is not limiting and where the concentration of antimicrobial peptides is very high, such as for example the granules of cells or the lumen of the lung. When these systems are turned on by any of the mentioned conditions, the expression of a number of genes is modified, including those that affect susceptibility to cationic peptides that are taken up by self-promoted uptake.

To decrease susceptibility to agents taken up by self-promoted uptake, bacteria regulate gene sets, through PhoPQ or PmrAB or both, that alter their LPS in a number of important ways. The most important is reduction of the requirement for divalent cation cross-bridging of the LPS. Bacteria accomplish this by masking the negatively charged groups via the synthesis and addition of N_4-aminoarabinose and phosphoethanolamine to the Lipid A phosphates (106). In addition to this modification, activation of the *phoPQ* system leads to increased expression of the *pagP* gene. The PagP protein catalyzes the addition of an extra acyl chain to the

hydrophobic portion of lipid A (107). The addition of this extra fatty acid increases the amphipathicity of the Lipid A, thereby making the outer leaflet more stable in the presence of bulky cationic peptide molecules. Both of these additions lead to substantially increased resistance to molecules that utilize the self-promoted uptake pathway. The PhoPQ system in *Salmonella* also regulates the production of an outer membrane protease, PgtE (108). When this protein is expressed, it is capable of degrading certain cationic peptides that access the cell via the self-promoted uptake pathway, thus providing another way of reducing influx of the antibiotic.

Although the system described above is essentially conserved for Enterobacteriaceae, there are major differences in other organisms. In *P. aeruginosa* for example, LPS modification genes responsible for the addition of N_4-aminoarabinose are also regulated by sub-inhibitory concentrations of cationic antimicrobial peptides, but this regulation is independent of either the PmrAB or the PhoPQ systems (109). Additionally, in *Pseudomonas* the PmrAB system is regulated by the presence of limiting divalent cation concentrations, similar to PhoPQ (109), in contrast to *E. coli*, *Salmonella* and *Erwinia* where it is regulated by high concentrations of Fe^{3+}. Although the precise mechanism by which this signalling takes place is ill-defined, it would appear to provide a distinct advantage to the bacterium in the CF lung, where Mg^{2+} is not limiting and where the concentration of antimicrobial peptides is very high. Overall these systems seem to be arranged in such a way as to limit bacterial susceptibility to self-promoted uptake in environments where the bacterium is likely to encounter cationic antimicrobial peptides or limiting divalent cation concentrations.

As Gram-positive bacteria do not possess outer membranes they utilize other mechanisms for decreasing uptake into the cell and consequently have different resistance mechanisms for cationic peptides. These include the modification of peptidoglycan or lysinylation of phosphatidylglycerol in *S. aureus* (110). The general principle appears to be the same however, in that by decreasing the affinity of envelope components for catonic peptides, resistance is promoted.

4.3 Hydrophobic Pathway

As suggested by the name, the hydrophobic pathway involves the passage of antimicrobial compounds through the hydrophobic interior of the lipid bilayer. The hydrophobic pathway of antimicrobial uptake tends to be more important in Gram-positive bacteria than it is in Gram-negative bacteria, since slowed hydrophobic passage through the Gram-negative outer membrane can be counteracted by active efflux through RND efflux systems. In contrast, the peptidoglycan layer of Gram-positive bacteria has a diffusion limit of approximately 50 kDa and decreased uptake very seldom contributes to resistance. The hydrophobic pathway is especially important for molecules that are active on intracellular targets, but that do not access a specific transporter. In Gram-positive bacteria, this includes many commonly used antibiotics including fluoroquinolones (which can be present at low concentration in an uncharged form), and macrolides.

As mentioned above, bacterial outer membranes have somewhat diminished hydrophobic uptake through the outer membrane bilayer primarily due to the reduced fluidity of the LPS monolayer compared to the cytoplasmic membrane. However, certain mutants that affect LPS core biosynthesis, e.g. *lpxA* and *lpxD*, exhibit up to 1,000-fold increased sensitivity to hydrophobic antimicrobials (111), largely by increasing uptake to an extent where it overwhelms efflux systems. In addition, a study with a series of isogenic LPS mutant strains of *E. coli* and *Salmonella enterica* demonstrated that the susceptibility of each mutant to hydrophobic antibiotics increased as the length of the LPS decreased (112). This study further supports the role of the LPS of Gram-negative bacteria as major determinant of reduced permeation of hydrophobic antibiotics.

Although the porin-mediated pathway described above is somewhat important in mycobacteria, it is believed that many clinically relevant antibiotics used for anti-mycobacterial therapy access the cytoplasm via the hydrophobic pathway. The general rate of diffusion across the mycobacterial envelope is slower due to the high rigidity of the mycolate bilayer, but does not seem to be reinforced by a broad spectrum efflux system that pumps out hydrophobic compounds as in Gram-negatives. Consequently, rifampin, isoniazid, and hydrophobic fluoroquinolones are thought to access the cell via the hydrophobic pathway (113).

4.4 Inner Membrane Transporters

A small number of antibiotics use specific membrane transporters to get across the cytoplasmic membrane, leading generally to a requirement that cells be energized for uptake. Usually this involves structural features that are conserved between the antibiotic and the normal substrate for the transporter. Thus, the antibiotic D-cycloserine is transported across the bacterial cytoplasmic membrane via the D-alanine transport system in a manner that is dependent upon the proton motive force (114). Fosfomycin, an antibiotic that inhibits the biosynthesis of peptidoglycan, crosses the cytoplasmic membrane using the glycerol-3-phosphate or hexose phosphate transporters (114). The antibiotic streptozotocin is also taken across the inner membrane via an active transport process involving the phosphoenol-pyruvate phosphotransferase system.

Aminoglycoside antibiotic uptake is still fairly poorly characterized. The drugs are taken up in a three-step process whereby the first step involves electrostatic LPS interactions on the surface followed by two energy-dependent phases of uptake (EDP I and EDP II) (115). EDP I is believed to represent the initial stages of aminoglycoside passage across the cytoplasmic membrane and binding to the ribosome. It is thought that some aspect of electron transport drives the vectorial transport of aminoglycosides across the cytoplasmic membrane during EDP I, possibly the shuttling of ubiquinones across the membrane (116). At this point the aminoglycoside triggers an event that initiates cell death and at the same time promotes an acceleration of energy dependent aminoglycoside uptake in the EDP II. Many aminoglycoside resistant mutants are altered in the energization of uptake, while a very common mechanism known as impermeability type resistance has been associated with disregulation of RND efflux pumps in *P. aeruginosa* (117).

5 Synergy

Synergy between antimicrobials is a common theme that is clinically utilized in the treatment of complicated infections. Often this is stated to be because one antibiotic assists the uptake of another. In many instances there is little direct evidence for this. However it should be noted that it has been well established that those molecules that access self-promoted uptake and act by increasing outer membrane permeability, also have the capability to increase permeability to other antibiotics. Deacylated polymyxin B is the prototype for such molecules (118) and it has also been shown that cationic peptides have this property as do other polycations and divalent cation chelators (61).

6 Conclusions

It is now well established that decreased outer membrane permeability is a common mechanism leading to clinical resistance. Because in Gram-negative bacteria this often involves uptake pathways of broad significance, these mutants tend to be cross-resistant to several antibiotic classes. While we still have exploitable mechanisms (e.g. self-promoted uptake) that can be manipulated to increase uptake in poorly susceptible bacteria, a recent meta-analysis has described an increase in the rates of resistance to polymyxin B in MDR isolates of *P. aeruginosa*, *Acinetobacter baumanii* and *Klebsiella pneumoniae*, suggesting that even these drugs of last resort may become decreasingly effective as their use becomes more widespread (119). Only through continued research will we be able to overcome these setbacks and effectively exploit the uptake systems described in this review.

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