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The role of bacterial lipid diversity and membrane properties in modulating antimicrobial peptide activity and drug resistance

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This review provides an analysis of the role that membrane composition and structure plays in the resistance of microorganisms to antimicrobial peptides (AMPs). We describe the current models of membrane disruption caused by AMPs and the changes in the structural properties that microbial membranes undergo in response to AMPs. This is followed by an outline of how the phospholipid composition of microbial membranes contributes to the changes in membrane bilayer structure and how the composition can be analysed in significant detail by modern lipidomic techniques. Finally, we discuss the challenges to fully analyse microbial membrane composition and structure that may occur during the development of resistance to AMPs.

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Introduction

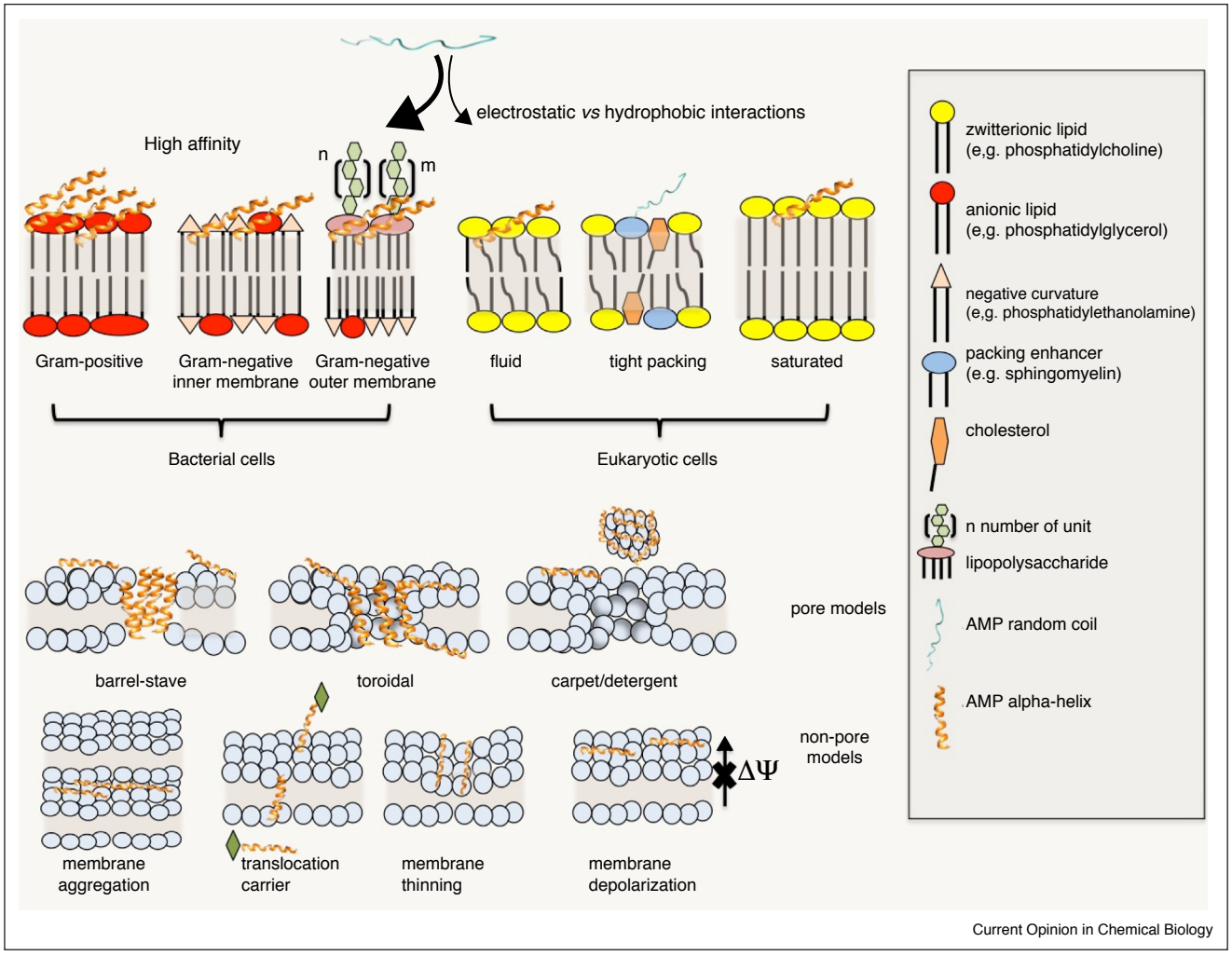
Antibiotic resistance continues to evolve and intensify and, in the absence of new effective drugs, the emerging global healthcare crisis is undeniable (see Tacconelli *et al.* [2]). Antimicrobial peptides (AMPs) are a promising alternative that act via disrupting the lipid membrane. However, as seen for conventional antibiotics, bacteria have evolved a range of resistance mechanisms to AMPs including changes in lipid membranes [3–5]. AMPs and the emerging AMP-resistance mechanisms may have co-evolved to support the delicate host–pathogen balance [6]. Understanding the molecular basis of AMP-resistance mechanisms could, therefore, lead to the development of more sustainable antibiotics. However, very little is known about remodelling of microbial membranes to resist AMP action.

Antimicrobial peptides (AMPs) are the primary line of defence of the innate immune system of many vertebrates, including humans and are typically active against a wide range of pathogens, including Gram-positive and Gram-negative bacteria, mycobacteria, spirochetes, fungi, viruses and protozoa [7–9]. AMPs are secreted by the host from neutrophils and epithelial cells to directly neutralise invading pathogens [10,11]. In response to AMP release, membranes, and thus lipids, are the front-line of the defence against antibiotics and AMPs, and, therefore, undergo significant dynamic remodelling as a major response [12]. This review provides an analysis of the role that membrane composition and structure plays in the resistance of microorganisms to AMPs. Readers are also referred to an excellent special issue of ten articles that review different aspects of bacterial resistance to AMPs [3].

Mechanism of action of AMPs

Over 2600 AMPs have been isolated and some structurally characterised from a wide range of organisms including humans [13]. Unlike antibiotic compounds, such as penicillin which target a specific metabolic pathway, cytolytic AMPs may act by binding to and disrupting the outer membranes of potential pathogens. Most AMPs characterised to date carry a positive net charge, which allows them to bind and insert into the negatively charged bacterial membrane (see Figure 1). Two general mechanisms are commonly used to explain their cytolytic activity [1••,14••,15,16]: either the peptide penetrates the membrane by forming channels or pores in a 'barrel-stave' arrangement or the 'toroidal' form; or the peptide aggregates on the membrane surface and causes lesions in the membrane but does not insert ('carpet' mechanism). In both cases cell death occurs due to the loss of the electrochemical gradient followed by cell lysis (Figure 2 **centre**). While widely accepted, there is increasing evidence that such models are overly simplistic [1••,15] and a number of more complex molecular models have been articulated to describe the action of AMPs [14••,17,18]. These models range from structurally defined mechanisms described above to

Figure 1



General mechanisms by which AMPs insert into and destroy bacterial or host lipid membranes ([1••] Copyright American Chemical Society 21016).

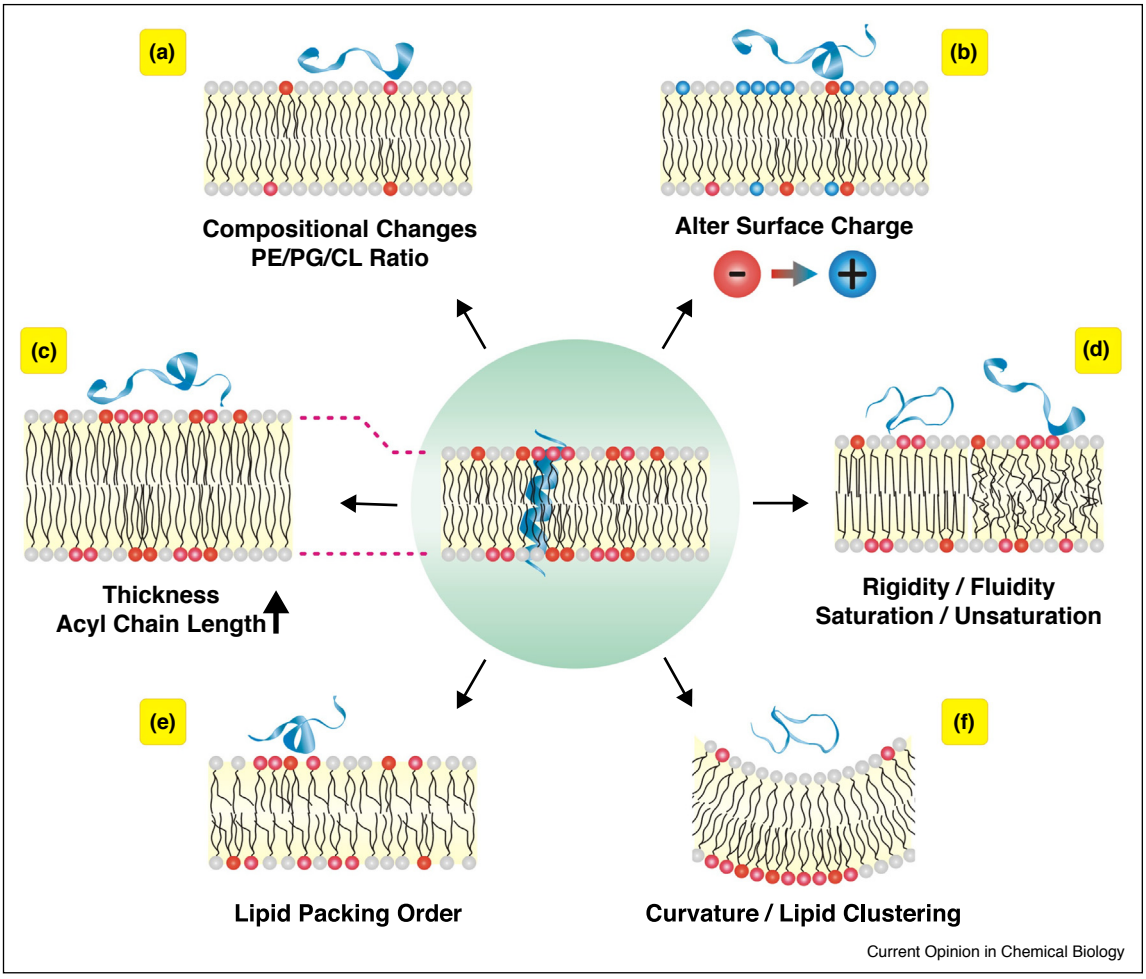
mechanisms, such as lipid segregation into domains, interfacial activity, formation of non-lamellar phases and the non-permeabilising sinking raft model.

Membrane structure changes during AMP binding

There is now indisputable evidence that bacteria modify their membrane to reduce the lytic effects of AMPs (Figure 2) [3–5]. Several studies have recently shown that bacteria have evolved a range of resistance mechanisms to AMPs by changes in their cell membrane, which include thickening of the cell wall, modification of the phospholipid composition, changing the net surface charge, increasing or decreasing the membrane fluidity (see Figure 2), releasing proteases to degrade the peptides and discharging amino acids into the environment to reduce hypo-osmotic stress [3–6,19,20].

More specifically, changes in the lipid composition in terms of both head group and acyl chain structure (discussed below) significantly impact on the membrane structural properties and molecular organisation of membrane lipids [21,22]. These changes can be categorised in terms of their impact on: 1) the net negative surface charge, 2) the membrane thickness, 3) membrane fluidity and ordering [23•], and 4) curvature and domain formation. These properties all are strongly inter-linked and define the ability of a membrane bilayer to respond to the binding of an AMP [24–28,29••,30–32,33•]. As membrane destabilisation is one of the main actions of AMPs, the molecular organisation of membrane lipids, in addition to the membrane surface charge and curvature, play an important role in the selectivity of AMPs. By determining the origin of membrane resistance in detail using lipidomics techniques, a basis for the development of AMPs actively targeted against specific human pathogens can be established.

Figure 2



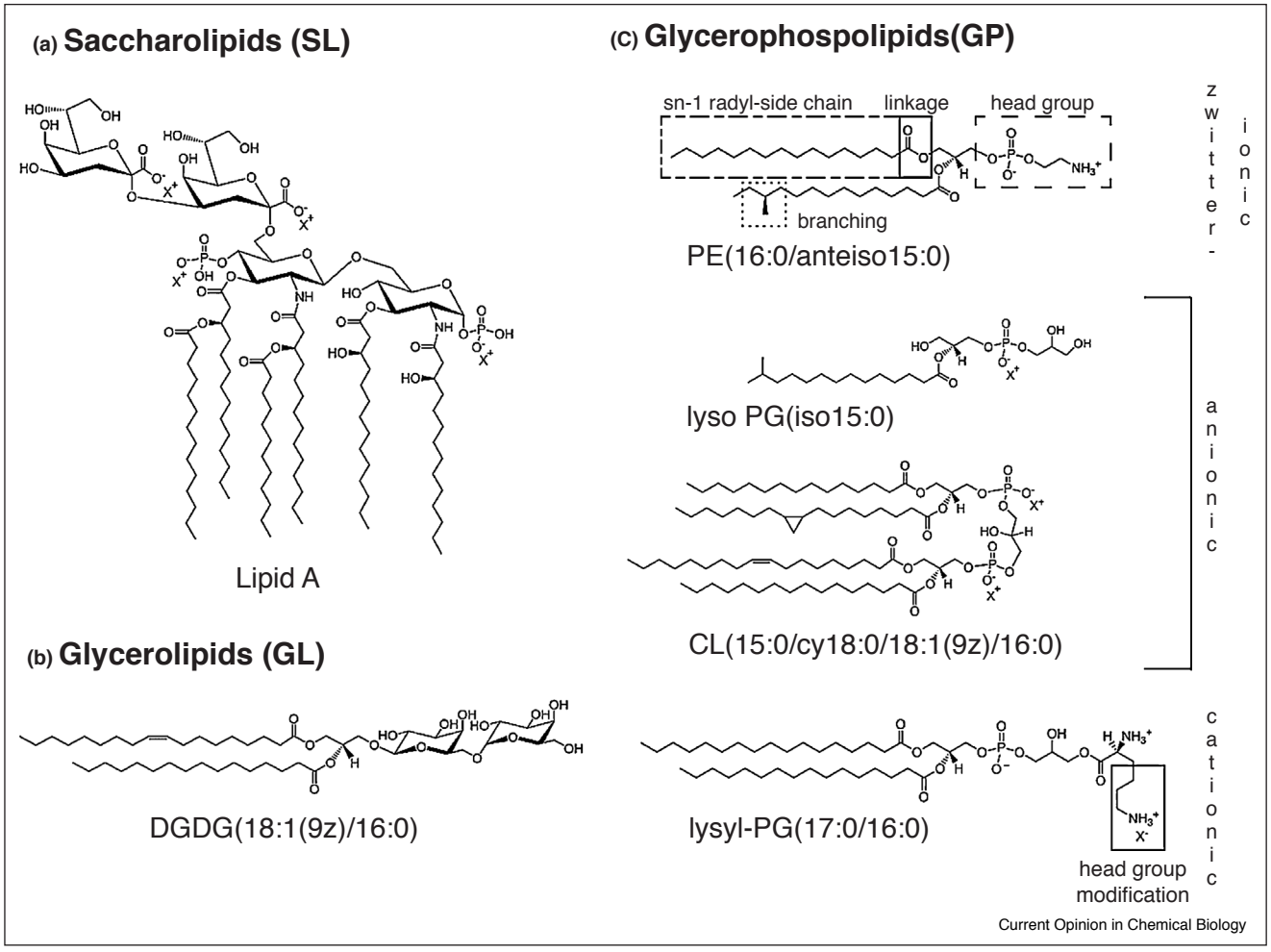
Mechanisms by which bacteria alter membrane composition to repel AMPs.

Characterising bacterial lipid compositions and diversity with lipidomics

Lipids represent a diverse group of hydrophobic to amphiphilic biomolecules whose physicochemical properties enable them to perform a wide variety of cellular functions, for example acting as integral components of cell membranes and membrane proteins, in energy homeostasis, and as active intra-cellular and inter-cellular signaling molecules [34]. Lipids may be divided into eight categories: fatty acids (FA), polyketides (PK), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterols (ST), prenols (PR), and saccharolipids (SL); and further subdivided into lipid class and subclass, depending on the backbone structure, the headgroup identity, and the number and type of acyl side-chain, alkyl (O)-side-chain or alkenyl (P)-side-chain side-chain linkages. Each lipid subclass may potentially also contain a multitude of individual molecular lipid species depending on the length and regiochemistry, the number, position and stereochemistry of C=C double bonds, and the presence of branching, cyclisation, and so on, within their side-chains.

Membrane lipid categories frequently observed in bacteria are SLs, GLs and GPs [35]. The most common SL and GL classes are lipopolysaccharides (e.g. lipid A) and diglycosyldiacylglycerol (DGDG) (Figure 3a and b), respectively, while the most common GP classes are glycerophosphatidylethanolamine (PE), glycerophosphatidylglycerol (PG), and cardiolipin (CL) (Figure 1c) [36,37]. However, the presence and relative abundances can vary significantly depending on the specific bacteria, particularly between Gram-positive versus Gram-negative bacteria [12,29••,35–37,38••,39,40••,41••,42•]. Bacterial membrane lipid extracts, however, are complex mixtures and new lipidomic techniques are required to identify and quantify the components.

Figure 3



Representation of the major categories of bacterial lipids showing diversity of structure and physiochemistry (e.g. charge state): **(a)** Lipid A from the saccharolipid (SL) category, **(b)** diglycosyldiacylglycerol (DGDG) from the glycerolipid (GL) category, and **(c)** glycerophosphatidylethanolamine (PE), glycerophosphatidylglycerol (PG), cardiolipins (CL) and lysyl-glycerophosphatidylglycerol (lysyl-PG) from the glycerophospholipid (GL) category. Structural diversity within the GP lipids include differences in head group identities, the number and length of radyl-side chains, the number, location of C=C double-bonds, and the presence of straight-chained or branch-chained or cyclopropyl rings (cy) [12,29••,35–37,38••,40••,41••,42•].

Lipidomic technologies

Lipidomics aims to systematically identify, characterise and quantitate all lipids in a given sample. This field's progression has been enabled by advancements in NMR [43], mass spectrometry (MS), and sophisticated chromatographic separation techniques [44••,45]. Most commonly, lipid identification and characterisation are performed using liquid chromatography (LC) or 'shotgun' (i.e. direct infusion) sample introduction, coupled on-line with electrospray ionisation (ESI) MS and/or tandem mass spectrometry (MS/MS) methods. Both low-resolution (e.g. triple quadrupole) and high-resolution (e.g. Orbitrap or Q-ToF) MS instrumentation, operating under targeted or untargeted acquisition conditions, respectively, are available. Identification of lipids within complex mixtures, including isomeric lipids, requires chemical derivatisation strategies [45] and new syntheses, for example, Caforio *et al.* have published synthetic methods for formation of *Escherichia coli* [46] and *Mycobacterium tuberculosis* lipids [47]. For lipid quantitation, stable isotope labelled synthetic lipids as internal standards (e.g. deuterated lipids) are required and are increasingly commercially available [44••]. For a comprehensive review outlining the analytical strategies currently available for mass spectrometry-based lipidomics, refer to Rustam and Reid [44••].

Remodelling of the bacterial membrane lipidome profile in drug and AMP resistance

The outer layer of Gram-negative bacteria is composedof lipopolysaccharides (LPS), especially lipid A

(see Figure 3a) and has been shown to be modified due to antimicrobial activity [12,37]. However, LPS are not present in Gram-positive bacteria.

The cationic daptomycin is a last-line lipopeptide antibiotic that inserts into bacterial membranes and daptomycin-resistance has developed among Gram-positive pathogens [29••,38••]. Two recent studies used lipidomics to unravel the impact of membrane lipid remodelling in daptomycin-resistant *Staphylococcus aureus*. Hines *et al.* showed that a loss-of-function mutation of the *pgsA* gene (encoding for PG synthase) leads to decreased levels of anionic CL and PG as well as cationic lysyl-PG [38••]. Jiang *et al.* showed that a gain-of-function mutation in the *cls2* gene (encoding for cardiolipin synthase) leads to increased CL but decreased PG and no significant change in lysyl-PG [29••]. The common pattern between these studies is a reduced level of PG, which is known to be a key factor in the action of daptomycin [48•]. Additionally, glycolipid levels such as DGDG were found to be increased in daptomycin-resistant strains [38••]. This is in accordance with lipidomic studies of other antibiotic resistances [39].

Odd-numbered carbon chain length containing lipid species are reduced more than even-numbered chain length species, together with a shift towards longer chains has also been observed in daptomycin-resistant *S. aureus*, indicating an acyl-chain-dependence on membrane fluidity. A recently published mechanistic study showed a positive correlation between increased acyl-chain length and reduction in daptomycin-induced pore formation [48•]. Collectively, this evidence suggests that daptomycin resistance is driven by reduced PG levels together with changed acyl-chain compositions, rather than primarily a change in membrane charge.

The importance of acyl-chain remodelling due to antimicrobial resistance has also been reported for Gram-negative *E. coli*. For example, Schmidt *et al.* showed significantly decreased odd-chain (cyclopropane-containing) lipid species and an increase in unsaturation in the AMP apidaecin 1b resistant *E. coli* [40••]. Furthermore, decreased levels of cyclopropyl-containing lipids, and also changes in the ratios of iso-branched to anteiso-branched acyl-chains species were observed in *E. coli* after naringenin exposure [40••]. Decreased levels of cyclopropane lipids, and also changes in the ratios of iso-branched to anteiso-branched acyl-chains species, were found in *E. coli* after naringenin exposure [41••]. Hence, lipidomics is providing the tools to study and understand the underlying mechanisms of AMP resistance and can also help to rapidly characterise bacteria such as the ESKAPE pathogens [42•].

In another example, *Legionella pneumophila* is more sensitive to the AMP, warnericin RK, when its membrane contains low levels of branched-chain and short-chain fatty acids [49]. Furthermore, the cell membranes of both humans and bacteria are inhomogeneous. Cell membranes contain patches of distinct compositions and vary over the cell type and lifecycle of the cell and organism [6,11]. The key to understanding how the lipid composition of the membrane of a pathogenic organism reduces AMP action lies in defining how the membrane composition changes the membrane structure to become AMP-repellent. In summary, there is substantial evidence that the membrane is a highly dynamic entity and that this plasticity is exploited by bacterial pathogens to elude the action of AMPs.

Summary and future challenges

Microorganisms have evolved a range of strategies to resist the effects of antibiotics, resulting in a health crisis in terms of an ever-increasing incidence of bacterial resistance to current antibiotics and AMPs may present an alternative counter measure. However, irrespective of the mechanism of action of AMPs, it is now clear that the ability of AMPs to disrupt the membrane bilayer can be diminished by changes in the composition and dynamic structure and packing of bacterial membranes. The challenge is now to achieve high precision lipid analysis and correlate these changes with their impact on bacterial membrane structure and dynamics. The identification of libraries of new lipids in turn will generate the need for synthetic methods to provide access to pure lipids for membrane structural analysis studies. Moreover, if the biosynthesis of these new lipids is involved in the emergence of resistance, then inhibitors of the enzymes that are responsible for biosynthesis of these lipid may represent new targets for inhibitor development and hence underpin the development of new synthetic regimes.

Complete lipidome structural characterisation and coverage

Given that 'structure defines function', a critical requirement of methods for bacterial lipid analysis on a 'lipidome'-wide scale is to provide sufficient information that enables precise structural characterisation, and quantification, of the potentially thousands of individual molecular lipid species that may be present within the bacteria of interest. Conventional MS and MS/MS-based strategies for lipidome analysis fail to achieve this goal, particularly for determining the locations of C=C double bonds or branching, or the sn-positions of their acyl chains, which significantly limits the ability to obtain insights into their underlying biological roles in the development of antimicrobial resistance. Recently, however, an array of alternative MS/MS strategies have been developed, that overcome these limitations and provide increased structural information for 'near-complete' molecular lipid structural characterisation. These include Ozone-Induced Dissociation (OzID) [50,51•],

the Paternò–Büchi reaction [52–54], UltraViolet Photodissociation (UVPD) [55•,56,57], and Electron Impact Excitation of Ions from Organics (EIEIO) [58•]. For example, of particular relevance to understanding the role of individual LPS molecular structures on the virulence of Gram-negative bacteria, Brodbelt and co-workers have reported the utility of 193 nm UPVD-MS/MS for the improved structural characterisation of a series of isomeric Lipid A [59,60]. These techniques, along with novel separation methods that are orthogonal to conventional chromatography, for example ion mobility spectrometry [61•,62], have great promise in further advancing the field toward understanding how bacterial lipids react to membrane-active AMPs.

Membrane bilayer structure analysis

Optical biosensors can now be used to measure the affinity and kinetics of AMP-membrane binding and these techniques have demonstrated that strong binding is not necessarily a determinant of lytic activity [63•]. More recent studies with a dual focus of simultaneous measurement of AMP binding and membrane bilayer structure have revealed a complex interplay between peptide structure, membrane composition and resultant bilayer structure changes upon AMP binding [63•]. The challenge now is to dissect these different structural changes which, together with more detailed lipid analysis, will allow real-time tracking of these membrane changes. A more detailed analysis of the individual membrane packing and fluidity parameters will be required but is currently challenging. While fluorescent techniques provide some insight into domain formation, higher resolution analysis by, for example atomic force microscopy-associated quantum nanomechanical techniques have potential to generate surface mechanical mapping of intact cells. This will allow the kinetics and reversibility of membrane changes to be studied and provide insight into the response of bacteria to AMP exposure in real time and ultimately establish clear relationships between lipid composition changes and membrane-mediated resistance to AMP action.

Conflict of interest statement

Nothing declared.

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