Opinion

Targeting bacterial outer-membrane remodelling to impact antimicrobial drug resistance

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The cell envelope is essential for survival and adaptation of bacteria. Bacterial membrane proteins include the major porins that mediate the influx of nutrients and several classes of antimicrobial drugs. Consequently, membrane remodelling is closely linked to antimicrobial resistance (AMR). Knowledge of bacterial membrane protein biogenesis and turnover underpins our understanding of bacterial membrane remodelling and the consequences that this process have in the evolution of AMR phenotypes. At the population level, the evolution of phenotypes is a reversible process, and we can use these insights to deploy evolutionary principles to resensitize bacteria to existing antimicrobial drugs. In our opinion, fundamental knowledge is opening a new way of thinking towards sustainable solutions to the mounting crisis in AMR. Here we discuss what is known about outer-membrane remodelling in bacteria and how the process could be targeted as a means to restore sensitivity to antimicrobial drugs. Bacteriophages are highlighted as a powerful means to exert this control over membrane remodelling but they require careful selection so as to reverse, and not exacerbate, AMR phenotypes.

Antimicrobial resistance is a phenotype

The global impact in loss of human life from increases in AMR has distracted many interested parties from the obvious, simple message that a key definition of AMR is that it is a phenotype. As with other phenotypes, it is a transient description of a given population of bacteria (or fungi, or parasites, but here we focus on bacteria) which is subject to evolution, based on selection under a given set of selective pressures. When we push the evolution of a bacterial population by increasing the exposure to antimicrobials we are selecting for an AMR phenotype. Any strategy that would slow or select against the AMR phenotype would be a better idea.

For bacteria, four broad mechanisms are recognized by which AMR phenotypes are driven to evolve (Box 1). Firstly, mutations that alter the target of the antimicrobial drug to inhibit drug-binding to said target will generate an AMR phenotype. Secondly, the modification of existing genes or acquisition of new genes encoding efflux pumps (see Glossary) provides an AMR phenotype. Thirdly, the acquisition of new genes encoding enzymes that hydrolyze or modify the drug provides an AMR phenotype, with perhaps the most salient example seen in the multigenerational β-lactam developments towards carbapenems (Box 1). Fourthly, membrane remodelling to prevent drug influx at the cell surface, thereby protecting the internal compartments where most drug targets reside.

In this opinion piece we explore the relationships between bacterial membrane remodelling and AMR. As our awareness grows that many of the worst-case scenarios of bacterial superbug lineages spreading globally are composite phenotypes, addressing the issue of membrane

Highlights

How porins are assembled into the bacterial outer membrane is now understood in molecular detail. We also have knowledge of the signals that dictate which porin-encoding genes are activated under specific environmental stimuli, including the presence of antimicrobial drugs. These signals change the protein-specific composition of the outer membrane, a process referred to as outer-membrane remodelling.

The general mechanisms by which mutations and/or adaptations confer AMR phenotypes on bacteria are known. One of these mechanisms is outer-membrane remodelling. Its impact on AMR, particularly carbapenem resistance, is a central feature of several of the bacterial pathogens currently rated as being in urgent need of research and new treatments.

New therapies are canvassed here and warnings around phage therapy, based on considerations of outer-membrane remodelling, are made clear.

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rem(225,691),(250,743)(250,691),(275,743)(275,691),(300,743) could be an effective means to resensitize bacteria to existing antibiotics. It is worthy of discussion: the 20th century has taught us that the silver bullet approach of a new antibiotic drug can never be a sustainable solution to AMR, with each new drug failing due to AMR after just a few years in clinical use. This remains true in this first part of the 21st century. Yet we almost know enough about bacterial cell biology to be inventive in how we might push back on evolution to restore susceptibility to some existing drugs within our antibacterial arsenal. It is our opinion that understanding just a few more features of the fundamental biology of bacterial outer membranes will both assist in the development of novel therapies and restore our ability to use existing drugs to treat what would otherwise be AMR infections.

Bacteria remodel their membranes in response to external cues

All of the antibiotic drugs in widespread clinical use in the 20th century targeted bacterial cell processes via binding to targets in internal compartments of the bacterial cell (Figure 1). In order to do so in Gram-negative bacteria, these drugs have to permeate the bacterial outer membrane [1,2]. The means by which this otherwise highly impermeable lipopolysaccharide (LPS)-phospholipid barrier can be breached by drugs is via the major porins. Almost all of the Gram-negative bacteria studied to date express a major porin: ‘major’ in the sense that it is the most abundant protein integrated in the outer membrane, and ‘porin’ in the sense that it has a central luminal space that serves as an aqueous pore through which nutrients and water-soluble drugs can pass. In the case of Escherichia coli, the major porin represents more than 50% of the total protein integrated in the outer membrane [2–4], allowing passage of drugs such as β-lactams and fluoroquinolones [5,6]. Major porins have also been studied in Klebsiella pneumoniae [7,8], in Neisseria gonorrhoeae [9,10], and so on. There are exceptions of course, such as Caulobacter crescentus that facilitates small-molecule uptake with a series of TonB-dependent β-barrel proteins instead of any major porin [11]. However, for all of the Gram-negative species that feature as urgent pathogens, as judged by the WHO and the US-CDC, major porins are used for nutrient and drug entry into the bacterium.

Studies on E. coli showed that while there are two genes (i.e., ompC and ompF) that encode the major porins, only one of these is expressed under any given set of environmental conditions [12].
The outer-membrane proteome is subject to remodelling wherein either OmpC or OmpF is the major porin. Grown in hyperosmotic conditions or nitrogen-limited media, OmpC is the major porin in the E. coli outer membrane, while under hypo-osmotic conditions, or in glucose-limited media, OmpF is the major porin expressed in the membrane [13]. Structurally, OmpC and OmpF are almost identical, with the very limited sequence variation confined to a few of the inter-strand loops that sit at the cell surface [14], and we therefore tend to refer to these porins with the compound term OmpC/F.

Studies in E. coli also showed that OmpF expression is turned on (and OmpC turned off) in response to carbapenems and tetracycline [15,16]. Conversely, OmpC expression is turned on (and OmpF turned off) in response to nalidixic acid or 3% ethanol [16,17]. The OmpC/F major porin system in E. coli is a useful model for this outer-membrane remodelling phenomenon, and it is not unique. For example, K. pneumoniae has four genes (i.e., ompK35, ompK36,
ompK37, and ompK38) encoding major porins that are structurally near-identical [8]. It is not yet clear which environmental conditions induce expression of each of these genes, although ompK35 is sensitive to osmotic conditions [18]. Through enforced remodelling of the outer membrane, these porins (i.e., OmpK35, OmpK36, OmpK37, and OmpK38) were shown to differ in which drugs they permit to enter into K. pneumoniae, with consequences and causality for AMR phenotypes [1,8]. Of greatest concern, it is becoming clear that many clinical strains of Klebsiella have remodelled their outer membrane to express no major porin, and this makes these strains highly resistant to carbapenems [1,8,19,20].

How and why do bacteria remodel their membranes?
Remodelling the proteome of the outer membrane depends on two biomolecular processes: the removal and degradation of existing membrane proteins, and the integration of molecules of new proteins into the outer membrane (Figure 2, Key figure). Relatively little is known about the mechanism for membrane protein removal from the outer membrane, although a set of proteases – BepA, DegP, YcaL – do degrade damaged outer-membrane proteins [21–23]. The latter process, the integration of porin molecules into the outer membrane, is well understood. The process starts in the cytoplasm, with the transport of newly synthesized porin precursors across the inner membrane, and into the periplasmic space, before arrival at the outer membrane. Here, the porin polypeptide is integrated into the membrane by the beta-barrel assembly machinery (BAM) complex [24–28]. The BAM complex is a surface-exposed target for antimicrobial compounds [29–31] that may be developed into a new class of antimicrobial drugs. As far as is known, both the integration process and the degradation process are constitutive without selectivity, and so the selective changes needed to have new protein components in the outer membrane are likely to occur at a transcriptional level: the induction of transcripts encoding the proteins to be introduced into the proteome, or mechanisms that silence transcription for proteins that would, thereby, no longer be integrated into the outer membrane.

At a transcriptional and post-transcriptional level, the genes encoding porins are regulated by a complex system of regulators, including two-component signalling systems (e.g., OmpR, CpxR), small noncoding RNAs (e.g., micF, micC, micA), and other factors. These transcriptional and post-transcriptional signals regulate porin production in response to several environmental stimuli [13,32,33], creating a genuine systems-level of control. By way of example, the two-component EnvZ–OmpR regulatory switch responds to environmental sensing of osmolarity, or ethanol, or various antimicrobial drugs, to remodel the porin composition of the outer membrane [21,34,35]. CpxR and other components of the Cpx envelope stress response also signal into this system, sensing osmolarity or antimicrobial drugs [36,37], and the expression of micC increases in the presence of drug treatment, acting synergistically with the Cpx envelope stress response to remodel the outer membrane in response to β-lactam antibiotics [38]. These small RNAs can be manipulated to remodel the outer membrane in several species of bacteria, and biotech applications are already exploiting this membrane remodelling system [39,40]. There is also the prospect that the remodelling system signals out to the protein integration machinery: intriguing proteomics analysis of E. coli in response to either β-lactam drugs or tetracycline treatment suggested that two components of the BAM complex, BamC (aka NlpB) and BamD (aka YifO), are upregulated coordinately when the ompC gene is induced to remodel the outer membrane [41].

New treatments and good ideas
Outer-membrane remodelling matters because it is a tractable target to override AMR phenotypes and/or effectively reverse the evolutionary trend towards AMR. The question then becomes ‘How’ can the remodelling of bacterial outer membranes be controlled to resensitize bacteria in an infection site or a biofilm to be susceptible to existing drugs. Encouragement that this is a
A worthy question and a tractable issue comes from the use of this strategy in biotech applications coupled with a growing awareness that AMR phenotypes are costly to maintain, suggesting that so-called ‘superbugs’ are not as super as they might appear.

Experimental studies have demonstrated that changes in the outer-membrane proteome of *K. pneumoniae* and *Acinetobacter baumannii* confer antibiotic resistance, but with an associated fitness cost and physiological burden for these clinically important bacteria. Under selection, in an environmental condition where drugs are present, strains that remodel the outer membrane to become drug-resistant would have a competitive advantage against drug-sensitive bacteria. What is less clear is whether, in an environment where drugs are absent, the remodelled
outer membrane would impose sufficient cost on the strain to make it less able to compete against other, drug-sensitive bacteria. In theoretical modelling, mutations conferring AMR are very costly in the absence of the drug [46]. The predicted reversal of an AMR phenotype could occur by either of two means: (i) a genetic change, by which the outer membrane is restored through mutations that reverse (or modify) the outer-membrane proteome to allow evolution of a drug-sensitive phenotype, or (ii) a population- level change, where the carbapenem-resistant strain fails to compete against a new, carbapenem-sensitive strain that can thrive in a drug-free environment.

The gene-regulatory mechanisms that control membrane remodelling are worthy of further investigation since, for example, factors that can switch on porin expression would be a valuable means of restoring carbapenem sensitivity in an infection site. The unmet need to drive these further investigations is here: many carbapenem-resistant strains of Klebsiella, Enterobacter, Acinetobacter, and Pseudomonas are evolving in hospitals to have an inactivated gene (e.g., ompK36) but with a functional albeit silent gene (e.g., ompK35) that – if switched on – would provide for drug-resensitization [1,8,39,47–49].

**New treatments and potentially bad ideas**

The current move towards phage therapy promises a powerful means to treat infections with AMR phenotypes since the drug-resistance mechanisms (Box 1) do not impact bacterial susceptibility to phage infection and ensuing bacterial death [50]. However, we note one issue that must be taken into account when selecting which phages to use in these treatments. In our opinion, phages that use major porins as their receptor must be avoided. If used in therapy, these phages would place selective pressure on the bacterial strain to become porin-defective in order to become phage-resistant, since the prime cause of phage-resistance is the downregulation of the surface receptor [51,52]. The unintended corollary therefore is that phages that use a major porin as their receptor would be mediators to select for AMR phenotypes in situ, a terrible prospect that can be avoided by consideration of outer-membrane remodelling (Figure 3). Advances are being made in this direction, resensitization to antibiotics shown in the case of phage treatment of multidrug-resistant A. baumannii strains [51,53].

**Concluding remarks and future perspectives**

The increase in prevalence of AMR is a major concern worldwide. According to the WHO it is ‘one of the 10 top global public health threats’ and carbapenem-resistant Gram-negative bacteria of various species are listed as urgent threats by the CDC [4]. The economic burden related to AMR is estimated to be US$4.6 billion in the USA per year, and €1.4 billion in the EU/EEA per year [4]. The costs for other continents are not available but will be at least as high. Globally, the economic impact in healthcare costs is predicted to increase in a range equivalent to US$300 billion to US$1 trillion each year by 2050 and with a corresponding 10 million deaths annually by the same year [54].

Discovery and development of effective drug treatments is declining dramatically. In addition, the 20th century has taught us that a new antibiotic drug will never be a sustainable solution to AMR. We must expand novel therapeutic approaches, such as phage therapy, to combat the ever-evolving mechanisms that multidrug-resistant bacteria use to evade current treatments. In our opinion, deep knowledge of bacterial membrane remodelling and the use of evolution and selection for resensitization to existing drugs are important considerations that need to be in place alongside the current moves for development of new antimicrobial drugs and other new therapies (see **Outstanding questions**). We believe that only with multidisciplinary action will the world combat the urgent threat of AMR.
Figure 3. Collateral impact of phages directed at major porin receptors. In order to initiate infection of a bacterial host, phages first bind to a cell-surface receptor [51,52]. Some phages use a specific surface protein, such as a major porin, while other phages use lipopolysaccharide (LPS) or another surface glycan as their receptor. In the first case illustrated, the phage selected for therapy has, as its receptor, the major porin. The selection pressure from prolonged therapy leads to a phage-resistant phenotype by a remodelling of the outer membrane so that the major porin is no longer present, with a collateral effect of generating a phenotype with increased drug resistance (‘AMR phenotype’). In the alternate case illustrated in the lower panel of the figure, the phage selected for therapy has, as its receptor, a feature of the outer membrane that is not the major porin. It may be a minor protein component, LPS, or other surface glycan. The selection pressure from prolonged therapy leads to a phage-resistant phenotype by a remodelling of the outer membrane so that the receptor is no longer present, but the major porin has not been selected against, so that the collateral effect is generation of a drug-sensitive phenotype, that is, the same or even increased drug sensitivity. Abbreviations: DHF, dihydrofolate; IM, inner membrane; PABA, para-amino-benzoic acid; PG, peptidoglycan; THF, tetrahydrofolate; the red ‘X’ represents the site of action of the drug.

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Declaration of interests

No interests were declared.

Resources

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