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[1]Department of Life Sciences, Imperial College London, London, England, UK

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Email Correspondence

zhiwen.xiao20@imperial.ac.uk

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Zhiwen Xiao[1]

Antimicrobial Resistance Mechanisms of Gram-Positive and Gram-Negative Bacteria

Abstract

The discovery of antibiotics has long helped humans in the battle against bacteria. However, the misuse of antibiotics in industries and medical systems has unintentionally provided an ideal environment for bacteria to develop resistance mechanisms through mutations and gene transfer, resulting in the emergence of antimicrobial-resistant bacteria. This has proven to be an urgent and pressing issue in the global healthcare system, resulting in increased mortality. Therefore, increasing resources are invested to study their different resistance mechanisms and develop corresponding novel drugs and treatment methods. This review briefly introduces several key resistance mechanisms with examples from both Gram-negative and Gram-positive bacteria, the current and novel methods for treating multiple drug-resistant bacteria as well as the potential actions that could be done to improve the situation.

Introduction

The discovery of penicillin in 1928 spearheaded the continuous discovery and development of antibiotics, resulting in various families of antibiotics[1]. Being a crucial and effective method to treat bacterial infections, antibiotics have been widely used in healthcare and the husbandry of livestock, saving countless lives and preventing dramatic economic loss. However, increasing cases of antibiotic misuse contribute to the occurrence of antimicrobial resistance (AMR) in bacterial pathogens through a process of selective evolution[2,3]. Mutations in the genetic material can occur spontaneously during processes like DNA replication in asexual reproduction, at times resulting in AMR in bacteria which is then selected in their respective environments. Once resistant strains occur, the spreading of the resistance can be vertically and horizontally transmitted to other bacteria[2,3]. The infections of these AMR bacteria are difficult to treat and have become worldwide medical issues, with an annual approximate of 700,000 deaths and a predicted increasing trend, reaching 10 million annual deaths by 2050[4]. One infamous multiple drug resistant (MDR) bacteria strain, methicillin-resistant *Staphylococcus aureus* (MRSA) has resulted in more than 100,000 deaths in 2019[5]. Together with other MDR bacteria, they are also known as "superbugs". To date, the six most threatening AMR bacterial pathogens identified by the World Health Organization (WHO) are *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species, which together are referred as "ESKAPE".

Additionally, an article published by the WHO[6] included 12 of the most troublesome bacteria in hospitals classified into 3 priority levels (critical, high and medium) according to the therapeutic difficulty and the urgency of the need to develop effective antibiotics against these bacteria. In Table 1, other pathogens are included in addition to the "ESKAPE." By classifying them based on Gram staining, it is apparent that there are more Gram-negative bacteria than Gram-positive bacteria. This classification represents structural differences; there is an additional outer membrane enveloping the cell wall in the Gram-negative bacteria.

When treating infections caused by these MDR strains, clinicians often have no options but to administer a combination of less effective antibiotics or repurpose previous drugs with suboptimal efficacy or toxicity[7]. The reasons behind this may not only be because of the lack of financial support but may also be the difficulties and challenges that are accompanied by new antibiotic development[5]. Therefore, the development of new antibiotic drugs has become less attractive to pharmaceutical companies, leading to a decline in antibiotic options when treating MDR bacterial infections.

More importantly, the study of the underlying AMR mechanisms is vital when trying to overcome resistance. Understanding the factors that contribute to the AMR phenotype in the bacteria can aid in guiding the development of antimicrobial drugs. This review aims to briefly cover the main mechanisms and strategies that some of the Gram-positive and Gram-negative bacteria use to survive upon antibiotic treatment.

Common Antimicrobial Resistance Mechanisms

To ensure their survival, bacteria evolved several resistance mechanisms to antibiotics categorized into 3 main types: intrinsic, acquired and adaptive resistance. Intrinsic resistance refers to the cellular and genetic regulation of expression of some antibiotic-inactivating enzymes, cell permeability and efflux of drugs[2,3]. Acquired and adaptive resistance require external assistance or stimuli; the former includes horizontal gene transfer from other species as well as genetic mutations whereas the latter refers to the gradual change of bacterial protein expression and phenotypes over continuous exposure to antibiotics or other environmental stress factors[8]. Despite the structural differences between Gram-positive and Gram-negative bacteria, some of their resistance mechanisms are common (Figure 1): both can degrade or modify antibiotics, or reduce the affinity or susceptibility of the drug target via enzymatic activities. The genes encoding the resistant enzymes can also exist in a plasmid which are transferred from other bacteria via conjugation, transformation or transduction[8]. Additionally, the porins on the outer membrane proteins of Gram-negative allow the passive pas-

Table 1. A prioritised summary of antibiotic-resistant pathogens proposed by the World Health Organisation[2,6].

Priority	Gram-Positive	Gram-Negative
Critical	None	Carbapenem-resistant *A. baumannii*
		Carbapenem-resistant *P. aeruginosa*
		Carbapenem-resistant *Enterobacteriaceae*
High	Vancomycin-resistant *E. faecium*	Clarithromycin-resistant *Helicobacter pylori*
	MRSA/VISA*/VRSA†	Fluoroquinolone-resistant *Campylobacter spp.*
		Fluoroquinolone-resistant *Salmonellae*
		Cephalosporin/Fluoroquinolone-resistant *Neisseria gonorrhoeae*
Medium	Penicillin-non-susceptible *Streptococcus pneumoniae*	Ampicillin-resistant *Haemophilus influenzae*
		Fluoroquinolone-resistant *Shigella spp.*

* VISA: vancomycin-intermediate *S. aureus*.
† VRSA: vancomycin-resistant *S. aureus*.

sage of small molecules which may be the potential entrance for hydrophilic antibiotics. In other words, mutations or downregulation of porins lead to AMR[9]. Although there is no such restrictive permeability barrier in Gram-positive bacteria, other strategies exist such as the thickening of their peptidoglycan cell wall to block antibiotics or producing enzymes that can degrade antibiotics[8]. Interestingly, a group of complex bacterial machinery, efflux pumps, are present in both types of bacteria to actively extrude antibiotics or other drugs specifically or broadly. Bacterial signalling molecules like autoinducers can also be transported via efflux pumps to regulate responses to antibiotics and facilitate defence actions[2]. According to their protein sequences, energy source and substrate range, they are categorized into five superfamilies, including the major facilitator superfamily (MFS), the resistance nodulation division superfamily (RND), the multidrug and toxic compound extrusion family, the small multidrug resistance family and the ATP-binding cassette (ABC) transporters. All efflux pumps except ABC use proton or sodium gradients as energy sources, while ABC pumps rely on ATP hydrolysis. For MDR Gram-positive bacteria, such as S. pneumoniae and S. pyogenes, MFS transporters are crucial for the extrusion of macrolide-family antibiotics. Additionally, RND transporters are primarily found in Gram-negative bacteria and contribute to their MDR, while members of this superfamily also exist in other organisms. For instance, RND transporters extrude tetracyclines in *Enterobacteriaceae* and *P. aeruginosa*. Moreover, genes encoding RND pumps are identified in genomes of various clinical isolates of pathogenic Gram-negative bacteria, indicating their importance in the bacterial intrinsic AMR. Different from efflux pumps in Gram-positive bacteria, a model RND transporter in Gram-negative bacteria spans across the periplasms and consists of a transporter on the inner membrane, periplasmic linker proteins and a protein channel on the outer membrane[10].

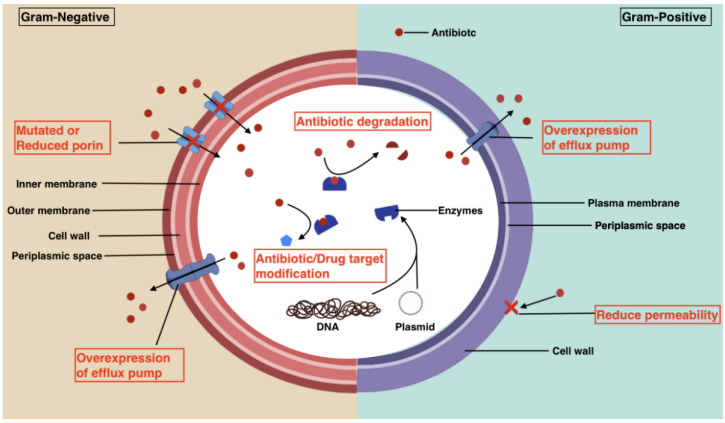

Figure 1. The schematic diagram of Gram-positive (right) and Gram-negative (left) bacteria and their antibiotic resistance mechanisms. Key AMR mechanisms are highlighted in red. Created with BioRender.com.

AMR of ESKAPE Pathogens

Gram-Positive Bacteria

Among the priority list of bacterial pathogens, Gram-positive bacteria are only included in the medium and high ranking, which might be due to the lack of an outer membrane that covers the peptidoglycan cell wall and acts as an additional selective barrier in Gram-negative bacteria[2]. Nonetheless, many MDR bacteria are still Gram-positive[9], and two examples are described below.

S. aureus has multiple resistant strains, including AMR to methicillin, vancomycin and fluoroquinolone. MRSA is resistant to all beta-lactam antibiotics like penams and cephams, and this is mainly due to the penicillin-binding protein (PBP) 2a, which is a transpeptidase that can function in the presence of beta-lactam antibiotics. Therefore, the synthesis of the nascent cell wall is unaffected, allowing bacterial growth[11]. Moreover, although vancomycin is considered the last resort for treating MRSA, VISAS and VRSA have also emerged independently with different mechanisms. The former thickens its peptidoglycan cell wall that contains decoys of the actual drug target of vancomycin, which are two D-alanine residues. The latter adopted an external gene that allows modification of the target site so that vancomycin cannot function[9], as in Figure 2. The decoys produced by VISA can sequester vancomycin and therefore reduce the chance of vancomycin binding to the key cell wall synthesis site, but there is still binding of free D-alanine-D-alanine, which can further reduce the drug efficacy[12].

E. faecium, another problematic pathogen, has strains that are resistant to betalactam and vancomycin as well[13]. For the beta-lactam resistance, both mutations and overexpression of low-affinity PBP5 were detected in the resistant *E. faecium* strains. Similarly, the vancomycin resistance in *E. faecium* is contributed by the same modification of the target site, replacing the terminal D-alanine with D-lactate. Additionally, *E. faecium* also are resistant to ribosome-targeting antibiotics, like aminoglycoside (kanamycin, gentamycin) and streptogramins (quinupristin-dalfopristin), by enzymatic modification of antibiotics and drug efflux pumps, respectively[13].

Gram-Negative Bacteria

Different from Gram-positive bacteria, Gram-negative bacteria have an outer membrane and thinner layer of the peptidoglycan cell wall. The outer membrane is attached with endotoxic lipopolysaccharide (LPS) chains and contains porins for the transportation of small molecules[2]. Most of the bacteria on the list, including all members in the critical priority, are Gram-negative bacteria, as some of them are responsible for severe nosocomial infections and the presence of resistance only worsens the situation[2].

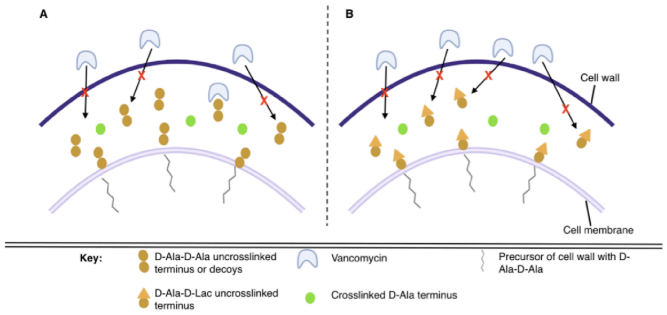

Figure 2. AMR resistance mechanisms with VISA and VRSA. (A) The schematic shows how VISA continuously thicken its cell wall by producing a large amount of many D-Ala-D-Ala terminus. (B) VRSA has a mutated terminus, which becomes D-Ala-D-Lac in its uncrosslinked form, so vancomycin is unable to bind. Created with BioRender.com.

A. baumannii, as a member of ESKAPE, is an aerobic pathogen that is commonly found in hospitals and causes opportunistic infections of the skin, bloodstream and other soft tissues, which contributes to up to 20% of nosocomial infections in critically ill patients in the intensive care units globally[2,14]. Moreover, epidemiological studies showed that injured soldiers or veterans who returned from the Middle East war zones were infected with *A. baumannii*, and this gave rise to MDR *A. baumannii* in the civilian hospitals of their home countries[15]. The AMR of *A. baumannii* is achieved by the expression of: (1) Four classes of beta-lactamases, which are enzymes for breaking the beta-lactam ring of betalactam antibiotics[10,14]. Although encoded in both Gram-negative and -positive bacteria, beta-lactamase is more commonly associated with AMR of the former[10]. There are both narrow- and broad-spectrum beta-lactamases. One of which (PER-1) had also been reported to be a virulent factor with adhesive function[14]; (2) Four classes of multidrug efflux pumps except ABC transporters. Studies also demonstrated that overexpression of an efflux pump (AdeFGH) in *A. baumannii* promotes biofilm formation when exposed to subinhibitory antibiotic administration[14]; (3) Aminoglycoside-modifying enzymes[14]. In addition, *A. baumannii* reduces outer membrane permeability by downregulating porin expression. For instance, the reduced expression of multiple porins such as CarO and several other members in the outer membrane protein family had been shown to relate to AMR towards nalidixic acid, chloramphenicol, aztreonam and imipenem; Antibiotic site alteration is another AMR strategy used by *A. baumannii*, which include chemical modifications in bacterial gyrases, PBPs, 16S rRNA and LPS[14].

Another example is *P. aeruginosa*, which is an opportunistic pus-causing pathogen and commonly found on surfaces of medical equipment. It has been proven to be the fourth most common nosocomial pathogen that is found in monitors, dialysis tubing and ventilator buttons, leading to 10% of nosocomial infections[16]. Four classes of betalactamases are identified in *P. aeruginosa*, catalyzing the breakdown of penicillin, cephalosporin and carbapenems. Another enzyme-mediated resistance is against aminoglycosides, as *P. aeruginosa* has enzymes that are responsible for adenylation, phosphorylation and acetylation of the antibiotics[16]. The AMR of *P. aeruginosa* involves a complex network of pathways that regulate the expression of well-studied resistance genes in its chromosome, which code for cephalosporinases, porins and multidrug efflux pumps[17].

Novel Therapeutic Approaches

Fortunately, with the growing incidence of MDR bacterial infections, more and more professional studies are involved in the development of novel treatment plans and drug discovery. The current treatment plan for MDR Gram-negative bacteria, which are the most critical "superbugs", often involves using antibiotics, like polymyxin B and colistin. These antibiotics are thought to be the last resort and they are reserved for critically ill patients with MDR bacterial infections[7]. In the case of carbapenem-resistant *A. baumannii*, polymyxin and other old or novel antibiotics are administered as the first-line treatment option and different methods of drug delivery are used concomitantly, such as inhalation and intravenous infusion of polymyxin[7]. In addition, MDR treatment can be more effective during early-stage infection so rapid diagnostics that aim to narrow the antimicrobial susceptibility profile are essential[7].

The need for novel antimicrobials continues to rise as AMR strains are continuously emerging, especially for those antibiotics of the last resort. Moreover, many drugs that are currently effective can be improved or have significant side effects, like neurotoxicity and nephrotoxicity of polymyxin[18]. Many different approaches target MDR bacteria, as in Table 2, while they are still in the basic and early stages of research.

One major category is peptide or peptide analogue antibiotics, which can be naturally or artificially synthesized. Darobactin and lugdunin are examples of cyclic natural peptide antibiotics that were discovered to be produced from bacterial symbionts in nematodes[19] and human nasal bacterial commensals (*Staphylococcus lugdunensis*)[19–21], respectively. However, the former is ribosomal synthesized and post-translationally modified and inhibits outer membrane formation, while the latter is formed by non-ribosomal peptide synthetases and interferes with bacterial membrane potential[19–21]. Inspired by natural peptides, researchers developed synthetic compounds that mimic natural antimicrobial peptides named chimeric peptidomimetic antibiotics[22]. They contain a β-hairpin peptide macrocycle, which is also found in natural antibiotics like polymyxin and colistin[22]. Another research developed two synthetic peptide-mimicking antibiotics called Pep16 and Pep19, which are cyclic heptapseudopeptides composed of seven pseudo amino acids that structurally resemble amino acids[23]. It was reported to be effective against several MDR Gram-negative and Gram-positive bacteria with minimal resistance[23].

With the advancement in CRISPR technologies, modules called antibacterial drones were made to directly act to hinder bacterial viability, growth or AMR mechanisms[24]. Among the recently discovered drugs, halicin was repurposed by applying machine learning to screen through more than 100 million drugs and was a preclinical thiadiazole-containing drug for treating diabetes[25,26]. Furthermore, cloning phage peptidoglycan hydrolases called endolysin allows the construction of recombinant endolysin and its derivatives, which leads to another effective approach to target MDR Gram-positive bacteria[27]. Bacteriophage therapy is different from traditional antimicrobial molecules, as they can proliferate within bacteria, allowing "auto" dosing within the patients[28]. The other advantages of using bacteriophages also include low toxicity, low chances of causing microbiome dysbiosis, high bacterial susceptibility, and biofilm clearance potential[28]. Nonetheless, the current research on this therapy is bottlenecked by several difficulties: (1) do not have many choices that can be used, as some bacteriophages can be inefficient at killing or carrying virulent genes[29]. (2) too high specificity with a narrow host spectrum, as even different strains of bacteria might require different bacteriophages to eliminate[29]. (3) ethical issues since they are biological agents with the ability to reproduce inside the patient[29].

Social Influences of AMR

The presence of AMR has resulted in severe infections with longer treatment duration. From the patient's perspective, the treatment against resistant bacterial infections can be time-consuming and ineffective, which may not only result in higher therapeutic costs but also more likely to develop into life-threatening infections[30]. Additionally, the increasing trend of AMR bacterial infections can be detrimental to the healthcare systems,

Table 2. The summary of novel therapeutic approaches for treating MDR bacteria.

Novel Drugs	Modes of Actions	Target Bacteria	References
Darobactin	Inhibiting the crucial protein (BamA*) for assembly and folding outer membrane proteins.	Gram-negative	[19]
Lugdunin	Dissipating membrane potential and stimulating skin cells to produce antimicrobial peptides.	Gram-positive	[20, 21]
Chimaeric peptidomimetic antibiotics	Binding BamA and lipopolysaccharides and disrupting the outer membrane formation.	Gram-negative	[22]
Cyclic heptapseudopeptide	Inducing bacterial membrane permeability and disruption.	None	[23]
Antibacterial drones	CRISPR-Cas9† or CRISPR-dCas9† complexes with bactericidal or virulence-blocking properties respectively.	None	[24]
Halicin	Disrupting electrochemical gradients and pH across bacterial membranes.	None	[25, 26]
Recombinant endolysin	Acting as peptidoglycan hydrolases to lyse bacteria.	None	[27]
Bacteriophages	Infecting and lysing bacteria upon lytic replication cycle.	None	[28, 29]

* BamA, β-barrel assembly machinery A.
† CRISPR-(d)Cas9: Clustered regularly interspaced short palindromic repeats and (deactivated) CRISPRassociated protein 9.

since this will require longer hospitalization periods and occupy medical workers and various important facilities and equipment like intensive care units. More importantly, when trying to contain nosocomial outbreaks, all contaminated sectors of a hospital will be secured and closed. This also reduces the maximum hospital capacity, further burdening the system. Lastly, by having more people die of infections and the overloading of hospitals, the economy can be impacted. According to the Centers for Disease Control, the estimated annual cost due to AMR is 55 billion dollars[30]. Therefore, the control and prevention of AMR are equally important, and the restrictions and policies about the use of antibiotics need to be established and strictly followed by both developed and developing countries. Also, the concept of AMR and other relevant knowledge is worth spreading across different countries for more prudent use of antibiotics.

Conclusion

The emergence of "superbugs" is a detrimental consequence of many factors, including human factors (e.g. antibiotic misuse) and natural factors (e.g. genetic mutations, gene transfer). AMR Gram-positive and Gram-negative bacteria have developed different resistance mechanisms and are detrimental to human society and the economy. Hence, the development of novel therapeutic approaches is vital and should keep up with the pace of the occurrence of MDR bacteria. Meanwhile, all antibiotics must be used with caution, despite being one of the most important medicines in human history.

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