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Overview of antibiotics against *S. aureus*: mechanisms of action and adaptive resistance.

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Abstract

Since their discovery in 1928, antibiotics have played a significant role in advancing public health. However, overuse and dependency on antibiotics has allowed *S. aureus* to develop resistance using various mechanisms. Antibiotics can be categorized into three classes based on their activity, targeting cell wall synthesis, DNA replication, and protein synthesis. The goal of this work is to explain antibiotic mechanisms of action and the subsequent mechanisms of resistance to highlight the adaptability of *S. aureus*. The high adaptability of *S. aureus* poses a need for creating new therapeutics and approaches to counter the current global spread of resistant bacteria.

Keywords

Staphylococcus aureus, Antibiotics, Antibiotic resistance, Efflux pump, Persisters, CRISPR-Cas9, Adaptation, Biofilm, Selective pressure, Quorum sensing

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Introduction

Multidrug-resistant (MDR) bacteria are one of the most prominent threats facing Public Health. With the dependency and prevalence of antibiotics in the modern world, strains continue to evolve mechanisms to counteract antibiotics. The global increase in infections with drug-resistant strains has significantly degraded economic and clinical settings, leading to numerous efforts by biomedical scientists to address bacterial resistance. Throughout 2019, 1.27 million deaths were attributable to multidrug-resistant bacteria, highlighting the growing spread and impact of drug resistance on a global scale (1). With 23,000 fatal cases in the United States and 20-50 cases per 100,000 people globally yearly, *Staphylococcus aureus* is the leading strain responsible for prominent infections in nosocomial and community-associated settings (2). Present In human flora, *S. aureus* is a gram-positive bacteria that causes lethal conditions after entering the bloodstream (3).

Since the discovery of penicillin in 1928, the field of medicine has offered innovative solutions to increase the efficacy of antibiotics. Antibiotics have expanded into three classes which target various aspects of the bacterium to counter its growth. The largest of the classes are cell wall inhibiting antibiotics, which inhibit mechanisms critical to bacterial cell wall biosynthesis (4). Other antibiotics inhibit nucleic acid and protein synthesis (5, 6). While the different classes of antibiotics prove to be distinct in specificity, they all face the problem of emerging resistant strains. The various mechanisms of resistance developed by *S. aureus* and their implications are discussed in this paper in order to highlight and explain this global threat.

Cell wall inhibiting antibiotics

S. aureus is a strain of gram-positive bacteria with a cell wall composed of a thick layer of peptidoglycan (PG). PG is a network consisting of duplicating units of disaccharides with stem peptide chains that are polymerized and interlinked through covalent glycosidic and peptide bonds, respectively. Various penicillin-binding proteins (PBPs), synthesize the PG network (7). There are two main enzymatic reactions for synthesizing PG: transglycosylation, in which glycan chains are covalently linked together through glycosidic bonds, and transpeptidation, the formation of peptide bonds between adjacent glycans. PBPs can perform one or both of these reactions to synthesize the PG precursor material, which forms a network that envelopes the cell membrane and provides structural support (8). Since the bacterial cell wall is essential only to prokaryotes, the synthesis of PG in *S. aureus*, and in many bacteria, is the target of two large groups of antibiotics: β-Lactams and Glycopeptides.

β-Lactam antibiotics contain a 3-carbon and 1-nitrogen ring, referred to as the beta-lactam ring, that is highly reactive (4). Among the various forms of β-Lactam antibiotics, penicillin has the greatest clinical significance, frequently being used to treat staphylococcal infections (9). The primary mechanism of action of penicillin and other β-Lactam antibiotics is to interrupt bacterial cell-wall biosynthesis by covalently binding to PBPs (6).

β-Lactam antibiotics can prevent the reactions of PBPs because of the unique nature of the β-Lactam ring. This ring mimics the terminus peptides in PG, d-Ala–d-Ala moiety, binding PG synthesis machinery, thus inhibiting peptide bond formation by acting as a substrate for PBPs (Figure 1). The binding initiates as the active PBP site's serine residue attacks the β-Lactam ring's carbonyl, cleaving the ring to leave an inactive acyl-enzyme (7). Ultimately, the β-Lactam blocks the active site from binding to its intended substrates, acting as a competitive inhibitor to prevent PBPs from catalyzing the crosslinking of peptidoglycan layers (7).

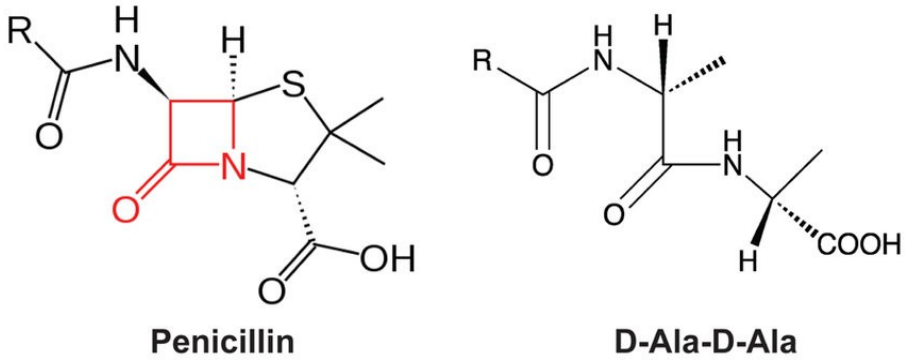

Figure 1: Adapted from reference 7.
The mimicry of β-lactam antibiotics and d-Ala–d-Ala. The lactam ring in penicillin is highlighted in red.

Although β-lactam antibiotics have shown the capability to inhibit critical functions of the cell, *S. aureus* has developed multiple mechanisms of resistance. Most prominent of them is the production of β-lactamases. These enzymes hydrolyze the four-membered β-lactam ring through an attack by the serine residue present in the active site. The transient covalent bond between the enzymatic serine hydroxyl group and the β-lactam carbonyl group leads to an inactive acyl PBP that slowly undergoes hydrolyzes to an inactive form of the antibiotic (10).

Since β-lactams have a functional dependency on the active sites of PBPs, cells have developed further resistance by altering PBP active sites. The modified PBPs are associated with the *mecA* gene: a significant sequence of nucleotides on the staphylococcal chromosomal cassette mec that provides varying degrees of resistance toward penicillin-like antibiotics. The *mecA* gene codes for PBP2A, the most successfully altered PBP because of a unique C-terminal domain known to have a transpeptidation function. Resistance is acquired because PBP2A has a lower efficiency of acylation, causing this extra PBP to have lower affinity for β-lactams. The acquisition of novel PBPs has proven to be a significant mechanism of resistance for methicillin-resistant *Staphylococcus aureus* (MRSA), strains of staph bacteria that have become difficult to treat. With β-Lactams being

a commonly used class to treat *S. aureus* infections, various resistance mechanisms have developed as a result of constant exposure over decades.

Efflux pumps represent unique transport mechanisms that allow organisms to regulate their internal environment and confer antibiotic resistance. Among a unique class of antibiotic known as sideromycins, cefiderocol (Fetroja®) contains a pyrrolidinium group on the C-3 side chain and a carboxypropanoxyimino group on the C-7 side chain which play an important role in improving transport of the antibiotic across the bacterial peptidoglycan membrane (11,12). Structure-activity relationship revealed that selected modifications of the C-7 and C-3 side chains directly contribute to the increased potency against drug-resistant gram-negative bacteria while simultaneously conferring stability against β-lactamases (Figure 2); a major concern for the previous generation of sideromycins. In addition to passive transport, active transport via iron transporters is utilized to deliver cefiderocol efficiently into the periplasmic space where PBPs are located, ultimately preventing the development of the peptidoglycan layer (12,13). With a similar mechanism to β-lactams, cefiderocol, an iron chelating antibiotic innovative cephalosporin drug that is taken up by bacterial cells through active transport, has highlighted the importance and significant role efflux pumps play in the regulation of bacteria.

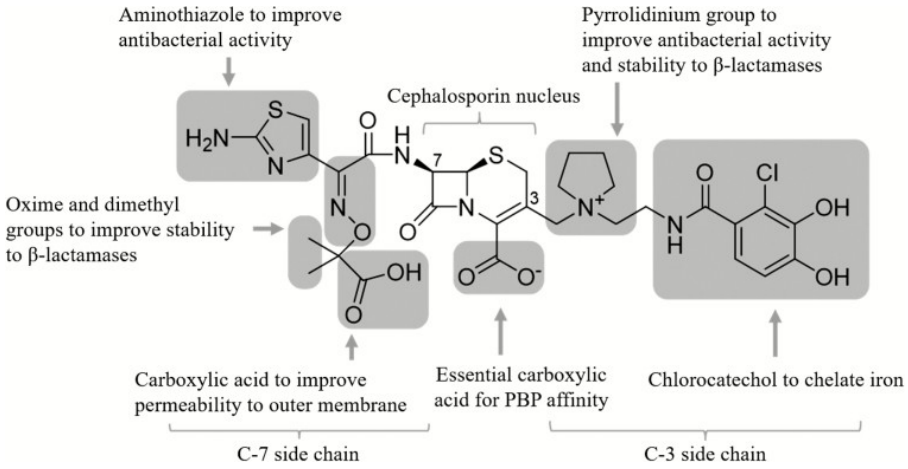

Figure 2: Adapted from reference 14.
Structure-activity relationships for cefiderocol.

Through its ability to obtain bacterial cell entry through iron-transport channels, Cefiderocol may decrease efflux pump upregulation or escape efflux pump activating mechanisms; a so-called 'Trojan-horse' strategy. Mutations in the bacterial iron uptake systems associated with cefiderocol uptake, specifically *fecI*, a gene regulating the ferric citrate transporter, offer resistance to the iron chelating antibiotic (14). However, given the essential role of iron in bacterial development, the ability of bacteria to actively transport essential nutrients suggests

that iron uptake pathways can be exploited for development of new drugs that can circumvent bacterial membrane impermeability and efflux pumps. Consequently, it remains difficult for bacteria to develop resistance to iron chelating antibiotics because essential nutrients such as iron are transported through the same pathway, thereby highlighting the effective ability of iron chelating antibiotics to exploit efflux pump inactivation dependencies.

Glycopeptide antibiotics are glycosylated tricyclic heptapeptides, the hallmark of which is a highly cross-linked core that includes polar uncharged asparagine residues that function to deter resistance (11, 15). Vancomycin, discovered in 1956, is the first member of the glycopeptide class among various generations, and vital for treatment of gram-positive bacterial infections caused by MRSA (11, 16). Since vancomycin's discovery, new glycopeptide antibiotics have been found exhibiting similar but distinct mechanisms used against *S. aureus* and other bacteria (15).

First-generation glycopeptides, including vancomycin and teicoplanin, inhibit cell wall biosynthesis by binding to the D-Ala–D-Ala moiety of lipid 2, the precursor material of PG in the cell wall (17). Vancomycin and several glycopeptides have been shown to dimerize in solution. Consequently, by forming non-covalent dimers, vancomycin and many related glycopeptides increase the avidity of ligand recognition by displaying two binding sites (18 - 20). Once bound, vancomycin blocks the transglycosylase step by sequestering the substrate, lipid 2, from the transglycosylation enzymes. Vancomycin also blocks transpeptidation by forming extensive hydrogen bonds with the peptide, sequestering the substrate of transpeptidation and reducing its affinity to bind. While vancomycin is an effective antibiotic with known mechanisms inhibiting the upstream process of cell wall synthesis, it is often only used as a last resort treatment (16).

While bacteria have found it challenging to resist vancomycin and other first-generation antibiotics, the extensive use of vancomycin led to the emergence of hetero-resistant vancomycin-intermediate *S. aureus* (hr-VISA) (21). The resistance involves nine gene clusters. The *vanC*, *vanE*, *vanG*, *vanL*, and *vanN* gene clusters aid in resistance by altering the d-Ala–d-Ala terminal of the cell wall precursor pentapeptide to d-Ala–d-Ser, while the *vanA*, *vanB*, *vanD*, and *vanM* encode for the replacement of the same with d-Ala–d-Lac (22,23). Van-A resistance is the only type detected in *S. aureus* to date. Specifically, the transposon Tn*1546* mediates Van-A resistance by altering the carboxyl terminus peptidoglycan precursor from D-alanine to D-lactate. This alteration is a combined effort between VanH, which reduces pyruvate to D-Lac for VanA to then form the D-Ala-D-Lac depsipeptide that replaces the D-Ala-D-Ala dipeptide. Furthermore, the dependency of glycopeptides on binding to the D-Ala-D-Ala moiety of lipid II is hindered by the effects of VanX and VanY, which hydrolyze the D-Ala-D-Ala formed by the host chromosomal D-Ala-D-Ala ligase (24, 25). Ultimately, Van genes change the D-ala-D-ala target, hindering glycopeptide interaction. Furthermore, by developing a thicker bacterial cell wall, VISA can increase the number of binding sites for

vancomycin, hence decreasing bacterial susceptibility (26).

Second generation glycopeptides are semi-synthetic lipoglycopeptide antibiotics effective against vancomycin-resistant strains (27, 28). The antibiotics of this generation, telavancin, oritavancin, and dalbavancin, contain a hydrophobic side chain to primarily anchor the membrane and the binding affinity to the pentapeptide termini (11, 29). Telavancin shares many similar properties to vancomycin as it is a semi-synthetic derivative. However, due to its lipophilic moiety and hydrophobic anchor, telavancin disrupts membrane potential and alterations in cellular permeability by depolarizing the gram-positive bacterial membrane (11, 29, 30). This modified mechanism allows for increased efficacy, compared to the first-generation glycopeptides, against MRSA and methicillin-susceptible *Staphylococcus aureus* (MSSA).

Glycopeptides have been extensively used for multiple generations to counteract bacterial infections. However, underlying aspects that have not been fully addressed make it difficult to use them effectively (11, 31). One of many challenges includes the intrinsic resistance of Gram-negative bacteria, which significantly hinders the abilities of glycopeptides. Due to an outer membrane, gram-negative bacteria inherently sustain a permeability barrier for large molecules such as glycopeptides. Innovative solutions have been developed to overcome permeability; for example, a lipophilic cationic vancomycin derivative has been developed to permeabilize the outer and inner membranes of the bacteria and depolarize the inner membrane (32, 33). Glycopeptides carry the potential to effectively inhibit bacteria in ways that are difficult for strains to adapt to. With ongoing research on the detailed mechanisms of resistance, the future may hold critical developments that will help glycopeptides reach their fullest potential.

DNA replication inhibiting antibiotics

DNA replication is the core mechanism responsible for developing and inheriting genetic material in all living organisms. Many bacteria, including *S. aureus*, contain one chromosome with one origin of replication. The process is initiated with the origin being recognized for helicase to cleave the hydrogen bonds of the double-stranded DNA to single-stranded DNA (34). Once helicase cleaves the Hydrogen bonds holding DNA, topoisomerases and gyrases are required to hold the single-stranded DNA, which is sterically strained as it unwinds (35, 36). While topoisomerase IV and DNA gyrase share similar structures and functions, vital physiological functions influence their susceptibility to quinolones. DNA gyrase depends on the hydrolysis of ATP to introduce negative supercoils into the DNA, while topoisomerase IV decatenates DNA for separation into daughter cells during DNA replication (37). However, both lead to the stabilization of replication forks, preventing the single-stranded DNA from rewinding into a double helix by coating the surrounding DNA with these single-stranded binding proteins (34, 36). The process of synthesizing the new DNA strand initiates as DNA polymerase III adds nucleotides to both the leading and lagging strands of DNA. These essential enzymes in the prokaryotic DNA replication process are

the targets of two groups of antibiotics: rifamycins and quinolones (35, 38).

Quinolones are a family of antibiotics containing a core structure similar to 4-quinolone, a 3-carboxylate and 4-carbonyl group essential for high-affinity antimicrobial binding (35). Among the various generations of quinolones, fluoroquinolones share a much broader spectrum of activity and clinical value due to their efficacy toward both Gram-negative and Gram-positive pathogens (39, 40). The quinolone class inhibits the DNA synthesis process by inhibiting the activity of topoisomerase IV and DNA gyrase, enzymes critical in regulating chromosomal supercoiling (41, 42). There has been significant research on the intricate mechanisms of action of quinolones.

Numerous studies have determined that quinolones' primary targets vary from species to species. For example, the primary target of quinolone in the *Escherichia coli (E. coli)* strain is gyrase, while that in *S. aureus* is topoisomerase IV (43 - 46). While the preference in determining the target remains unknown, the mechanism of action remains similar. Quinolones bind to the complexes of DNA with gyrase and topoisomerase IV at the active site between protein and DNA creating a concentration of cleavage complexes (Figure 3). This disruption ultimately destabilizes the DNA replication systems, leading to chromosomal breaks that depress and prevent the induction of the SOS DNA repair system. Quinolones bind DNA gyrase and topoisomerase IV and form a ternary cleavage complex with the enzyme and DNA strand, preventing strand rejoining (39, 44, 47 - 49).

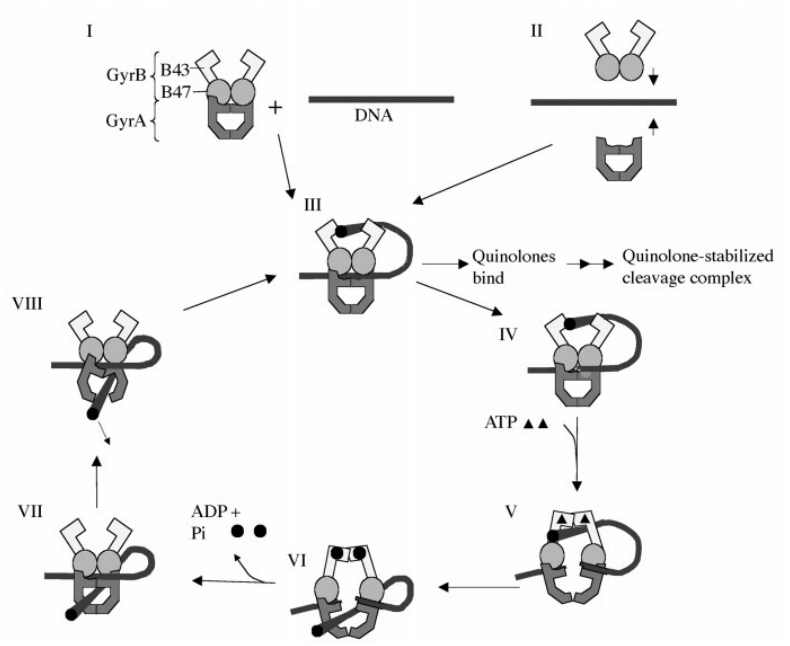

Figure 3: Adapted from reference 45.

Schematic illustration of the DNA gyrase supercoiling cycle, highlighting quinolone mechanism of action. Domains of DNA gyrase are shown in different shades of gray with the C-terminal domain of GyrA omitted.

The process by which susceptible strains become highly fluoroquinolone-resistant occurs in sequential steps (49). The first step is a mutation in the *gyrA* gene, which encodes for the A subunit of DNA gyrase.While a substitution in Ser-83 of the *gyrA* gene is sufficient to generate a level of resistance to nalidixic acid, acquisition of subsequent mutations, mainly amino acid Asp87-Asn and Thr83-Ile, is associated with greater quinolone resistance (50). The initial step in building resistance involves genetic mutations that alter the structure of DNA gyrase and topoisomerase IV, thereby reducing the quinolone binding affinity.

In addition to the point mutations in the *gyrA* gene, fluoroquinolone resistance can develop through the overexpression of efflux pumps. While *S. aureus* over-expresses NorA, NorB, NorC, and SdrM efflux pumps to build resistance, *E. coli* over-expresses the AcrAB efflux pump. The diverse use of efflux pumps between gram-positive and gram-negative strains of bacteria is due to the varying drug permeation. The cell wall of *S. aureus* and other gram-positive bacteria lacks an outer-membrane and, therefore, is more permeable to antibiotics than the gram-negative cell wall (50). Thus, to compensate for its permeable membrane, *S. aureus* expresses the NorA efflux pump to confer resistance to hydrophilic quinolones and overexpresses NorB and NorC to confer resistance to hydrophobic quinolones (50 - 52). Ultimately, these efflux systems are effective energy-dependent mechanisms that induce resistance by decreasing the cytosolic concentration of antibiotics (49, 52).

Rifamycins are broad-spectrum antibiotics derived from a soil bacterium capable of inducing bacterial cell death. Clinically approved rifamycins are commonly used to treat chronic staphylococcal infections and in combination therapies due to their potent activity against Gram-positive pathogens. (53, 54). The antibacterial action of rifamycin yields a long post-treatment effect due to its high-affinity binding to the DNA-dependent RNA polymerase of prokaryotes, leading to the inhibition of RNA synthesis of primers needed for DNA replication (53).

Rifamycins initiate the inhibition process by targeting the β-subunit of DNA-dependent RNA polymerase. The binding site is optimally structured within the RNA channel, allowing rifamycin to prevent the growth of the oligonucleotide chain that would serve as a primer for DNA replication. Rifamycin inhibits the elongating RNA strand from synthesizing an RNA primer product no longer than three nucleotides, which is insufficient for DNA replication (55, 56).

Rifamycin depends on its binding affinity to the β-subunit of RNA polymerase to inhibit the activity of the polymerase and stop growth. Thus, the alteration of RNA polymerase encoding genes has been the most effective mechanism of bacterial resistance (58). Specifically, missense mutations in the *rpoB* gene, a sequence of genetic material which encodes the β-subunit of RNA polymerase, were identified as etiological factors for rifamycin resistance (55, 57). The

accumulation of mutations in the *rpoB* gene leads to a decrease in hydrogen-bond interactions coupled with a decrease in van der Waals and desolvation energies, thereby hindering the binding affinity of rifamycin to the β-subunit of RNA polymerase (57, 58).

Protein inhibiting antibiotics

Proteins are responsible for the majority of cellular tasks and regulation, and their production is the target of a large class of antibiotics. The ribosome, one of the most sophisticated macromolecular machines of the cell, is responsible for translating messenger RNA sequences into functioning proteins (60). The ribosome in *S. aureus* comprises a 30S subunit, which primarily functions to decode the genetic information, and a 50S subunit that hosts the catalytic peptidyl transferase center (PTC) responsible for linking amino acids into peptides (59, 60). At the 30S subunit, aminoglycosides bind to the A site to prevent incoming aminoacyl-tRNA (61). Linezolid and other oxazolidinone antibiotics bind to the PTC and interfere with the aminoacyl moiety of aa-tRNA, ultimately deactivating peptidyl transferase and peptide bond formation (61, 62). By inhibiting the activities of ribosomes, linezolid and aminoglycoside antibiotics destabilize the growth and maintenance of bacterial cells (63).

Linezolid is a group of oxazolidinone antibiotics with activity against drug-resistant MRSA and other gram-positive bacteria (64). Linezolid prevents the synthesis of bacterial proteins by binding directly to the PTC of the 50S subunit with a potent morpholino group and fluoride atom (64, 66, 67). While Figure 4a depicts normal translation, the process of disruption highlighted in Figure 4b begins as the A and B sites of the PTC stack correspondingly for the nucleotides U2504 and U2506 to bind covalently to linezolid. Consequently, linezolid stabilizes the nucleobase U2585 in an orientation that is distinctly different from when the A and P-site tRNA ligands are bound, inducing a conformational change in the PTC and abrogating its enzymatic activity (65, 68, 69).

Linezolid's mechanism of action is similar to clindamycin, a toxic semisynthetic antibiotic that contains a lactone ring, a key distinction between macrolide antibiotics. Similarly, clindamycin inhibits bacterial protein synthesis by binding to the 23S of the 50S bacterial ribosome subunit. Clindamycin's three-dimensional structure closely resembles the 3’-ends of L-Pro-Met-tRNA and deacylated tRNA, allowing it to bind to the ribosomal subunit, impairing peptide chain initiation, and promoting disassociation of peptidyl-tRNA(70, 71). Ultimately, linezolid and clindamycin disrupt bacterial growth by targeting the PTC of the 50S ribosomal subunit to inhibit the initiation process of protein synthesis.

One of the critical advantages of linezolid over clindamycin and other protein synthesis inhibitors is its entirely synthetic structure (68). Until recently, there was no natural pool of intrinsically resistant genes due to the lack of a natural sturctural prototype. However, the chloramphenicol-florfenicol resistance (*cfr*) gene has been recently discovered as a horizontally transmissible resistance gene that modifies a specific rRNA nucleotide at the

binding site of the drug. The *cfr* gene significantly decreased the clinical efficacy of linezolid through its intricate mechanism (68). This mechanism is to add methyl groups at position A2503 of the 23S rRNA. By modifying and altering the identity of PTC nucleotides that overlap ribosomal binding sites, Cfr makes binding more difficult for protein-inhibiting antibiotics (69, 72). The nucleotide U2504, for example, plays a vital role in resistance to PTC antibiotics because it binds pleuromutilins, oxazolidinones, phenols, and lincosamides. Mutations in U2504 consequently alter the hydrogen-bonding of neighboring nucleotides, and because of the overlapping binding sites of the antibiotics, this causes resistance to more than a single antibiotic class of compounds (68, 73,74). Furthermore, the post-transcriptional conversion of U2504 to pseudouridine, a uridine isomer, decreases antibiotic interactions by altering the RNA conformational states and accessibility, and increases resistance to several antibiotics that target the 50S ribosomal subunit (68,75).

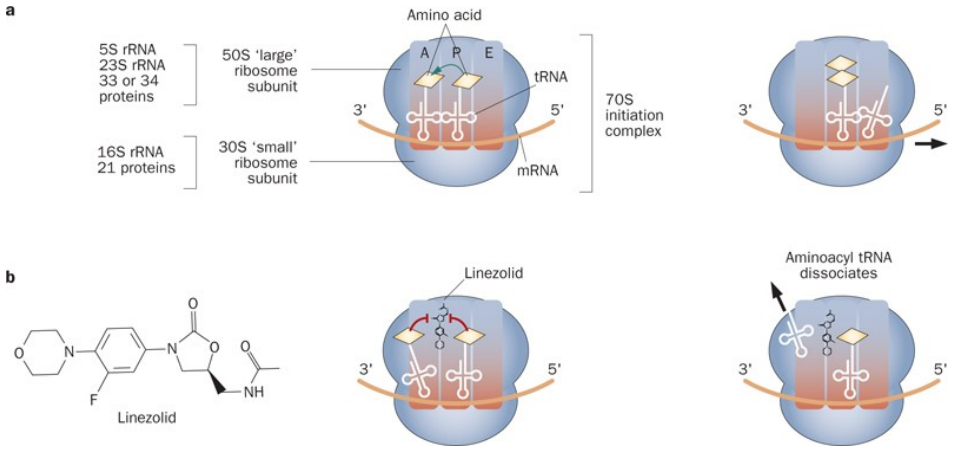

Figure 4: Adapted from reference 64.

a. Normal translation by which an initiator amino acid-tRNA complex is held by the P site of initiation complex.

b. Illustration of mechanism by which linezolid inhibits protein translation by the bacterial initiation complex. Linezolid binds to the nucleic residue of the peptidyltransferase center and prevents protein elongation.

The area of oxazolidinone and lincosamide research is an active field because of the emergence of MRSA and MDR Gram-positive *S. aureus.* Modifications of linezolid and its rings have been the first signs of development to circumvent resistance (76). Tedizolid, one of many novel oxazolidinones, has a broader spectrum of coverage and an increased activity against Gram-positive organisms compared to previous oxazolidinones due to its ring modifications. Specifically, Tedizolid possesses a modified side chain at the C-5 position of the oxazolidinone nucleus and an optimized C- and D-ring system that improves potency through additional binding interactions. These enhancements allow tedizolid to demonstrate activity against linezolid-resistant bacterial strains harboring

the horizontally transmissible *Cfr* gene (68,77). The recent acquisition of oxazolidinone and lincosamide resistant mechanisms indicates a growing threat that could multiply the risk of developing resistant strains.

Aminoglycosides are natural antibiotics derived from actinomycetes that are a cornerstone of antibacterial chemotherapy. They possess a core structure of amino sugars connected *via* glycosidic linkages to a dibasic aminocyclitol. While the antibacterial activity of aminoglycosides is directed against gram-negative bacteria, the addition of cell wall disruptive agents such as vancomycin allows aminoglycosides to produce a synergic effect (78, 79).

Aminoglycoside entry into bacterial cells involves three stages. The polycationic antibiotic first electrostatically binds to the negatively charged sites of the bacterial membrane, such as phospholipids. The displacement of magnesium ions allows the cations to stabilize and remove the lipid components of the membrane, ultimately disrupting the outer membrane (79). The aminoglycoside uptake is coupled by a slow, energy-dependent, electron-transport-mediated respiration process. Once the aminoglycoside molecules access the cytoplasm, protein synthesis and translation inhibition create harmful proteins that facilitate subsequent aminoglycoside entry (80 - 84).

Aminoglycosides utilize their core structure to bind with high affinity to the A-site on the 16S ribosomal RNA of the 30S subunit to inhibit protein synthesis. Consequently, the binding promotes mistranslation by inducing codon misreading when aminoacyl transfer RNA is delivered (80). This allows for error in protein synthesis, which ultimately leads to assembled polypeptides that are subsequently released to cause damage to the cell and its functions (85, 86). While the binding mechanism varies by chemical structure, all aminoglycosides produce prolonged post-antibiotic effects (PAE) that result from the antibiotic's binding with its target (87).

Aminoglycosides inhibit protein synthesis; however, there are many mechanisms that *S. aureus* and other strains of bacteria have developed to become resistant. Aminoglycoside-modifying enzymes (AME) commonly integrate among plasmids containing multiple resistance mechanisms. Acquired via horizontal gene transfer, AMEs are broadly categorized based on their ability to phosphorylate, acetylate, or adenylate amino or hydroxyl groups found at various positions around the aminoglycoside core (86, 88).

Aminoglycoside Acetyltransferases (AAC) comprise the largest group of AMEs and are known to acetylate aminoglycoside amino groups in an acetyl-CoA-dependent manner (85, 86). A frequently observed class found among *S. aureus* and other Gram-negative bacteria include the AAC(6')-1 enzyme that leads to resistance to different antibiotics such as amikacin, tobramycin, and netilmicin (88). The second largest group of AMEs is the aminoglycoside phosphotransferases (APHs), which catalyze the ATP-dependent phosphorylation of hydroxyl groups found on aminoglycosides. The modifying action of

APHs lowers the binding affinity to the target by decreasing the hydrogen bonding of aminoglycoside phosphorylated hydroxyl groups. Most APH enzymes belong to the APH(3') subfamily, which was discovered in *S. aureus*. The APH mechanism of action leads to kanamycin and neomycin resistance (86, 88, 89). The final group of AMEs consists of aminoglycoside nucleotidyltransferase (ANTs), enzymes primarily responsible for adding AMP from ATP donors to hydroxyl groups at various positions along the aminoglycoside. The most clinically relevant members of the group consist of ANT(2") and ANT(4'), which were first identified in *S. aureus* (80, 90). Ultimately, AMEs decrease the clinical efficacy of aminoglycosides due to their broad mechanism spectrum.

Over the last decade, there has been a resurgence in the use of pleuromutilins, a class of antibiotics that is distinct for its tricyclic scaffold with a glycolic ester moiety forming the side chain at position C14. Remarkable efforts have been made to achieve an optimal tricyclic core, with most modifications occurring at the glycolic side chain of pleuromutilin to optimize physicochemical characteristics such as solubility and antimicrobial activity (91). Among the numerous modified antibiotics of the pleuromutilin class, lefamulin has been the most successful.

Lefamulin and other pleuromutilins inhibit bacterial protein synthesis by binding to the PTC. The positioning is similar among pleuromutilins in that the tricyclic core is located in a pocket close to the A-site, while the C14 side chain extends toward the P-site to effectively hinder the 3’-end tRNA rotary motion [92]. The tricyclic core interacts with the A-site through hydrophobic interactions and hydrogen bonds with nucleotides G2505 or A2503 of the C11 site to prevent binding of incoming tRNA. Recent studies using *S. aureus* ribosomes have concluded that the C14 extension bonds with hydrogen bonds, specifically the amino acid groups of BC-3205 of lefamulin with the nucleotides U2506 and A2062, interfere with the flexible nucleotides U2585 and U2506 responsible for the rotary motion of interacting nucleotides, allowing for better fit of lefamulin in the pocket [93, 94].

Characterized by potent activity against many staphylococcal species, lefamulin, among other classes of other antibiotics, was the most active compound *in vitro* and its activity was unaffected by multidrug resistance. This can be attributed to its unique mode of action, one which involves binding to highly conserved ribosomal targets, implying a low probability of resistance development. However, pleuromutilins have partly overlapping interaction sites with oxazolidinones, lincosamides, phenicols, and streptogramins, hence increasing the probability of cross-resistance. Specifically, mutations and deletion in the *rplC* and *rplD* genes, which encode the 50S ribosomal proteins L3 and L4, cause alternating changes in the PTC and hinder effective positioning of the pleuromutilins in the pocket formed between nucleotides U2506 and G2505. Furthermore, the methyltransferase Cfr, an enzyme that is responsible for the methylation of nucleotide A2503 at 23S rRNA, can confer resistance (95). The nucleotide A2503 is among many that the tricyclic

pleuromutilin core depends upon for binding, and thus by methylating the nucleotide, methyltransferase Cfr effectively inhibits the binding of pleuromutilins.

In 1976, mupirocin was introduced as a promising drug against a wide spectrum of gram-positive bacteria. With an epoxide side chain structurally similar to that of isoleucine, mupirocin is able to effectively bind to the isoleucine-specific binding pocket of isoleucyl-tRNA synthetase, an enzyme responsible for promoting the conversion of isoleucine and tRNA to isoleucyl-tRNA. With the formation of the isoleucyl-tRNA synthetase blocked, the cellular levels of the isoleucine-charged transfer RNA are depleted, leading to the inhibition of bacterial protein and RNA synthesis (96).

The long term use of mupirocin has led to varying levels of resistance among *S. aureus* isolates. Low-level resistance occurs as a result of point mutations in the *ileS* gene, one that is responsible for expressing isoleucyl-tRNA synthetase. This mutation leads to a change in the amino acid configuration of the mupirocin binding site, most notably the Val-to-Phe change (97). The mechanism of high-level resistance is developed through the acquisition of a plasmid-mediated *mupA* or *ileS2* gene, both responsible for altering the chemical structure of isoleucyl-tRNA synthetase. It can be inferred that mupirocin is a prime candidate to succumb to bacterial resistance because of its dependent binding mechanism to isoleucyl-tRNA, an enzyme that can be altered in numerous ways to easily develop resistance (96).

Alternative mechanisms of resistance

The development of antibiotic resistance is mainly dependent upon genetic alterations. However, there exist unique subpopulations of bacterial cells that adopt unique phenotypes rather than mutated genotypes to counteract antibiotic pressure. Among them, the most significant are biofilms and persisters, which exhibit a metabolic state that influences their susceptibility to antibiotics.

The phenomenon of persistence is often induced by extracellular stress, commonly provided by bactericidal antibiotics. Persisters exist in a state of dormancy, adapting to survive under a phenotypic niche. This phenotype was first monitored among *S. aureus* in 1942, by Hobby et. al., who observed that ~1% of cells were not killed by penicillin and became persister cells (98). It was observed that these cells were nongrowing, unable to be killed by penicillin. In 1945, Chain and Duthie expanded upon the previous findings by confirming that penicillin did not completely kill *S. aureus* and longer treatments were necessary to kill the stationary-phase cells that were highly insensitive to penicillin. Antibiotics inhibit crucial cellular functions that inevitably, when destabilized, lead to cell death. Persisters exhibit tolerance to antibiotics because these cells do not perform cellular activities that antibiotics typically target (99). The phenotypic change allows the cell to remain tolerant to antibiotics in a dormant stage until the treatment has stopped, when the state of dormancy can be reversed and the cell can reactivate. While persisters do not promote resistance mechanisms through genetic changes

that block antibiotic activity, they phenotypically alter chemical processes that antibiotics rely upon to maintain longevity and tolerance. This phenomenon highlights the adaptability of *S. aureus* and the significance of phenotypic changes as a new means to counteract conventional antibiotics. Thus, it is imperative to continue to broaden the scope and understanding of persister resistant mechanisms developed by bacteria to effectively modify and develop antibiotics with alternative mechanisms of action.

S. aureus and other strains of bacteria often form biofilms in biological and on inert surfaces during infection. The development of biofilm is one of cooperative group behavior that is maintained by density-dependent chemical signals released by bacterial populations embedded in a self-produced extracellular matrix (100). Biofilm also emerges as a stress response to hostile environments. The stringent stress response is mediated by the alarmone guanosine tetraphosphate (ppGpp), a protein synthesized by the relA gene in *S. aureus*. This pathway results from nutrient limitation and allows *S. aureus* and other strains of bacteria to conserve cellular response (101,102). Thus, by modulating the ppGpp level, levels crucial to the susceptibility to cell-wall active antibiotics, the stringent response is the ultimate controlling factor of the phenotypic expression of oxacillin resistance in MRSA (Figure 5). Biofilm formation plays an important role in resistance, and numerous genetic controls have been identified to play a prominent role in biofilm formation. Resistance can be countered by targeting genetic controls to limit the transmission of antibiotic resistance (103).

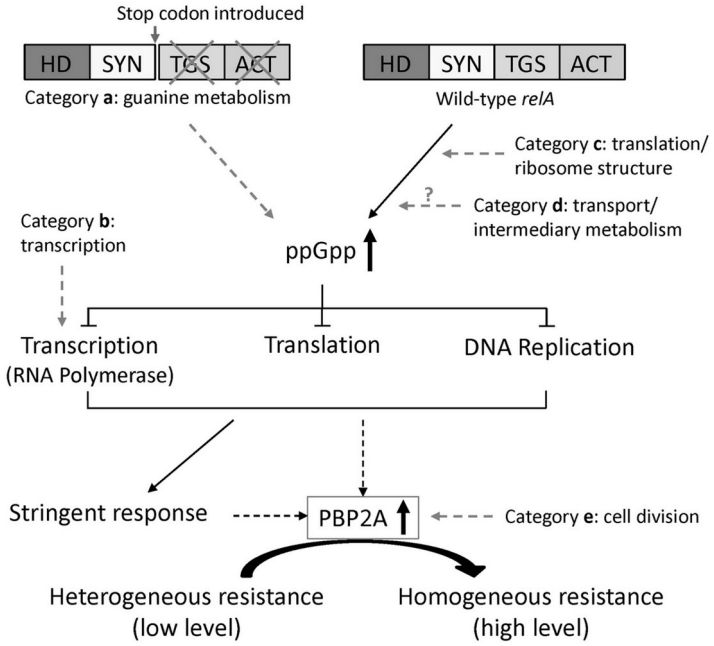

Figure 5: Adapted from reference 104.

Model for the postulated effect of the heterogeneous and homogeneous resistant mutations on the relA stress response.

The biofilm mode of growth also increases the adaptive ability of *S. aureus* to acquire and disperse plasmid-borne antibiotic resistance determinants by horizontal gene transfer. This can be reasoned by understanding the conditions for optimal biofilm growth. Conjugal transfer in *S. aureus* is optimal when the organism is applied to a surface, allowing for the biofilm formation that plays a role in the high frequencies of horizontal plasmid transfer. The close-to-cell contact occurring in the biofilm along with the stabilization of contacts between proximal bacteria is dependent on quorum sensing mechanisms, in which the accumulation of signaling molecules fosters plasmid transfer events by both conjugation and mobilization (104,105).

The levels of resistance against antibiotics are rapidly increasing worldwide and call for alternative methods to utilize current drugs more effectively. A promising strategy to precisely eliminate bacterial population involves the use of CRISPR-Cas9, an antimicrobial agent that can be programmed to cleave DNA sequences found in target bacteria. This mechanism is specified by the complementarity between a 20-nucleotide sequence present in a guide RNA and a target DNA sequence that is transmitted by the protospacer. Upon detecting the valid protospacer in the genome of the target bacterium, CRISPR-Cas9 induces a double-strand cut that is irreparable by the SOS DNA repair system, ultimately leading to cell apoptosis (106 - 108). This technology has opened new avenues in genome editing with potential in therapeutic applications. Bacteria already have CRISPR in their genomes to provide resistance to viruses. The key to enable this technology to effectively counter resistant strains of bacteria is to deliver the CRISPR-Cas9 system to all bacteria of the targeted resistant population. This has been demonstrated effectively in mice models as a group of scientists in a study modified the CRISPR-Cas9 system to be transferable. Then, the system was put inside harmless bacteria that were fed to mice. After four days, more than 99.9% of the targeted antibiotic resistant population was eliminated, highlighting the capability of the CRISPR-Cas9 mechanism to effectively transmit across bacteria and eliminate the targets (109). While the CRISPR-Cas9 mechanism offers an alternative method to counteract resistance bacteria, they display narrow host ranges as phage receptors on target surfaces can mutate to prevent binding, and environmental conditions can significantly limit their activity in the gut (110).

Combination therapy offers an alternative approach to countering resistant bacteria by increasing the target spectrum of targeted pathogens. Laboratory studies have found that the potency of drug mixes increases based on the specific combination (111). TXA709, a recently developed prodrug that is a derivative of TXY541, has proved to be an effective antistaphylococcal agent in combination with obsolete antibiotics. The mechanism initiates as TXA709 hydrolyzes to yield an active product known as TXA707. Measurement of time-dependent concentrations of TXA707 and PC109723, the active product of TXY541, revealed that TXA707 is associated with an

elimination time following i.v. administration of 6.5 times longer than the corresponding elimination time of PC109723. Furthermore, the bioavailability of TXA07 was found to be 95%, approximately 3.2 times greater than the corresponding value observed in the administration of TXY541 (112, 113). Evidently, TXA707 persists throughout the membrane for longer periods of time, having lasting impacts and a greater potency for binding to the Filamenting temperature-sensitive mutant Z bacterial cell division protein. By altering the C1 group on the pyridyl ring with a CF_3 functionality that is resistant to metabolic attack, TXA709 serves as an advanced hydrophobic group that holds greater potency for antistaphylococcal action. The success seen with this newly developed drug poses the possibility of a future in which obsolete antibiotics may be combined with other mutated bacterial proteins, like TXA709, to increase efficacy against resistant strains. Rather than relying on the pharmaceutical industry to develop new antibiotics in replacement of current ineffective antibiotics, TXA709 and other proteins allow for an efficient system to utilize current antibiotics in combination to counteract resistant strains of bacteria.

MRSA *Staphylococcus Aureus* Infections

There have been divergences genetically and phenotypically between infections acquired in a hospital and community acquired infections, specifically with MRSA. Generally, while bacteria associated with hospital-associated infections have been particularly adept at acquiring and maintaining antibiotic resistance, community-associated infections often have increased infectious activity. This is a result of the different selective pressures in these different environments and has significant impact in the clinic (114). Rates of MRSA infections had increased rapidly between 1990s and early 2000s, with cases of infection emerging in individuals who had no prior hospitalization. Consequently, MRSA diverged into two categories: community-associated MRSA (CA-MRSA) and hospital associated MRSA (HA-MRSA). There have been continued divergences with genetic and phenotypic distinctions that highlight the advanced adaptive ability of *S. aureus*.

Nosocomial settings involve persistent antibacterial treatment when compared to community settings, creating a need for bacteria to preferably evolve resistance, rather than transmissibility and virulence. A study on the epidemiological and antimicrobial susceptibility of MRSA from the Hospital of Naples corroborates this claim by showing that CA-MRSA strains were more susceptible to ciprofloxacin, tetracycline, and rifampicin, while harboring unique genes amplifying virulence (115). The Department of Microbiology of a tertiary hospital on the outskirts of Chennai, India, reported prevalence and antibiotic susceptibility patterns of MRSA isolates to compare drug resistance of MRSA between CA and HA infections. Among the 121 MRSA (27%) identified from Kirby Bauer disk diffusion method results, 91 HA-MRSA and 30 CA-MRSA were identified with a prevalence of 20% and 7% respectively. The resistance patterns of HA-MRSA were (10-30%) higher, for any given drug as compared to those of CA-MRSA (116). Phenotypically,

HA-MRSA was more capable of surviving a broader suite of various classes of antibiotics relative to CA-MRSA.

The molecular epidemiology of *S. aureus* successfully contributes to the emergence of strains with increased virulence specific to the local emphasis on antibacterial treatment. The most significant of these strains, USA300, rapidly spread to establish a domestic prominence in North America as a cause of CA-MRSA skin and tissue infections (117 - 119). Multiple mobile genetic elements (MGE) and core genome components have been identified as the factors responsible for the success of USA300. Specifically, the arginine-catabolic mobile element (ACME) associated with USA300 became prominent as a result of rapid clonal expansion. ACME serves as a key enzyme in the arginine catabolism pathway, which inhibits adaptive immune responses, hence improving pathogen survival (40). By increasing the expression of multiple genes within this pathway, ACME was hypothesized to increase the fitness of USA300 relative to other strains (120). While the epidemiological shift in MRSA cannot be fully attributed to a single genetic trait, the clonal expansion of CA-MRSA has shown a preceding influence on the acquisition of virulence on a global scale (Figure 6) (120,121).

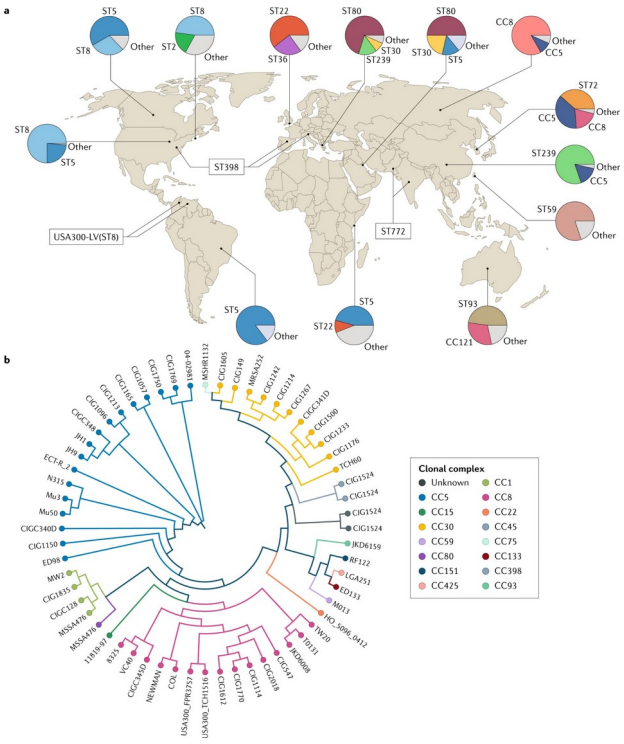

Figure 6: Adapted from reference 89.

a. Overview of regional strain diversity summarized from select studies performed in Africa, Asia, Australia, Europe, Middle East, North America, and South America.

b. Maximum likelihood SNP dendrogram for 60 *Staphylococcus aureus* isolates representing relationships between major clonal complexes.

Fey et.al. studied the genetic relatedness between CA-MRSA, HA-MRSA, and non menstrual toxic shock syndrome (nmTSS). It was observed, using gel electrophoresis, that 31 related CA-MRSA isolates produced staphylococcal enterotoxin, a toxin that, when produced, coincidently increases the virulence of *S. aureus.* Furthermore, all CA-MRSA isolates contained a type IV staphylococcal cassette chromosome mec (SCCmec) element. Antibiotic susceptibility patterns were different between the CA-MRSA and HA-MRSA isolates with HA-MRSA having significantly higher resistance to other antibiotics. (122). Interestingly none of the HA-MRSA isolates expressed any of these three superantigens vital for improved virulence, while they possessing increased resistance. It has become clear that CA-MRSA poses unique threats compared to HA-MRSA with a different SCC*mec* element that highlights the adaptive nature of *S. aureus* in evolving important mechanisms for different environments. This epidemiological shift highlights the impactful potential of CA-MRSA, which has adapted to increase infectious activity.

Emerging diseases have crossed species and geographical boundaries and have permeated almost every environmental niche. This has led to the interdependence among various microbes and the biological host, in which microbial communities act synergistically to benefit their host whilst simultaneously breeding resistant pathogenic determinants (123). Hedgehog surveys from Denmark and Sweden highlight the level of interdependence seen between MRSA and its host, an evolving relationship driven by natural selection in wildlife. The research found that the zoophilic dermatophyte *Trichophyton erinacei* infected hedgehogs in the pre-antibiotic era and produced β-lactam antibiotics. This finding highlights that methicillin resistance may have emerged as a co-evolutionary adaptation of *S. aureus* to the colonization of dermatophyte-infected hedgehogs (124, 125). This evolution of clinically significant antibiotic-resistant genes in wild animals demonstrates the presence of a natural selective environment in which MRSA isolates maintain an advantage over susceptible isolates. Studies from European countries have observed that MRSA is also present in domesticated animals, most prominently in dairy cows. The United States further contributes to the problem since 70% of all medically important antibiotics in the United States are sold for use in animals (126,127). This prominence suggests that the use of antibiotics in livestock has provided a selective advantage, which directly translates to the zoonotic transmission of *S. aureus* from domesticated species to humans.

A recent study cultivated *S. aureus* strains with varying susceptibility to daptomycin in a serum-rich medium to closely approximate *in vivo* conditions and bacterial ability to adapt to harsh conditions. Growth analysis and MIC testing determined that serum altered the metabolism in *S. aureus* as the strains exhibited altered amino acid biosynthesis and catabolism. Most importantly, all strains exhibited less sensitivity to daptomycin, suggesting that serum reduced the efficacy of the antibiotic. Including serum into the growth media to

simulate the milieu of the bloodstream served as an effective research method to discover viable and effective antibiotics specific to the setting. Furthermore, *S. aureus* strains ultimately proved their ability to survive and adapt to hostile host conditions by readily adjusting their metabolic activity, a critical consideration for designing effective drugs (128).

The emergence of antibiotic-resistant strains outside of clinical settings highlights and reinforces a major global concern: *S. aureus* is adaptable in all settings, clinical and beyond. Natural selection has proved capable of inducing microbial resistance against antibiotics they have not yet been exposed to. To move forward, actions such as subtyping of bacterial isolates, and molecular characterization of resistance genes will play an important role in understanding the development of naturally resistant strains. Ultimately, the management of antibiotic distribution and detailed antimicrobial usage data will help to retrace the impact of antibiotics in the natural environment, to distinguish areas of clinical and natural influence.

Conclusion

Overall, the innate adaptability of bacteria has allowed them to survive many harsh environments. *S. aureus* has extraordinary levels of adaptability, displayed through the development of the extensive variety of resistance mechanisms. The resistance developed can cross between multiple species through horizontal gene transfer, amplifying the selection for creation of novel sequences, and potential ease of access to resistance genes. Dependency on antibiotics has naturally selected resistant strains to various antibiotics on a global scale. Understanding how drug-resistant strains function may hold key insights for future research.

Since the discovery of antibiotics, society has overused antibiotics. This misuse has been the major contributing factor to resistance. Continuous exposure to various drug classes has selected MRSA and other resistant strains of bacteria to adapt to anti-infective conditions and develop resistance. Antibiotic drug development is a highly costly venture that is not very profitable for pharmaceutical companies. It could be argued that this lack of development has given resistant strains more time to adapt against the same antibiotics and potentially improve upon resistant mechanisms while doing so. In an environment lacking new antibiotics, MDR bacteria reflect the danger of this global crisis as they make existing antibiotics ineffective.

A multitude of research approaches is; or should; be pursued to overcome antibiotic-resistant S. aureus strains. 1] Restricting or excluding certain antibiotics in/from livestock feed may prevent the emergence of antibiotic-resistant bacteria so that zoonosis becomes less concerning. 2] Designing drugs to utilize the same bacterial import pathways as those used to import essential nutrients may make the drugs ‘invisible’ to efflux pumps – the so-called ‘Trojan horse’ approach. Alternatively, small molecules can be designed as efflux pump inhibitors so that some obsolete antibiotics may be again made effective. 3]

CRISPR-Cas9 with gRNA sequence(s) that is/are designed to bind to antibiotic-resistant gene(s) can be inserted into probiotic bacteria. In the gut, these probiotic bacteria transfer their genetic cargo to *S.aureus*, selectively killing the pathogenic bacteria. Methods to pursue this paradigm; not only in the gut; but also in the blood, must be discovered. 4] Drugs should be designed to interfere with bacterial biofilm production, persister metabolism, and with mechanisms that cause antibiotic resistance in the presence of serum. 5] If there exist mechanism(s) that create a need for bacteria to preferably evolve resistance (HA-MRSA), rather than transmissibility and virulence (CA-MRSA); they must be identified so that (relatively) benign, yet more transmissible bacteria may evolve at the expense of the completely antibiotic-resistant ones by the deliberate drug-focused application of selective pressure. This is a nascent research area that holds significant promise, especially with the advent of machine learning and artificial intelligence.

Fifteen years ago, Nobel laureate Joshua Lederberg wrote, "The future of humanity and microbes will likely evolve as… episodes of our wits vs. their genes (129)." The ongoing battle against MDR bacteria has shown the extraordinary ability of bacteria to adapt and overcome. Systematic collection and reporting national data on antibiotic resistance worldwide to monitor the emergence and mechanisms of resistant bacteria is a first step in understanding the problem. Proper antibiotic administration is also essential in controlling the global spread of resistant strains. By changing human behavior and aligning the economics of self-interest with the public interest, the effectiveness of existing antibiotics can be preserved. The most important step in this global crisis remains finding novel antibiotics and combinations. Novel economic approaches must be employed to match corporate and societal interests, specifically reestablishing adequate government funding to pharmaceutical entrepreneurs and start-ups. Under strong leadership, a coordinated effort between the corporate world and societal needs will reflect a practical approach to innovation and enable international harmonization of regulatory standards. Furthermore, continued research on effective combinations of both new and existing therapeutics to have multiple and variable selective pressures appears to be a feasible method moving forward.

Microbes have been adapting to changing conditions for billions of years. The long-term success of antibiotics depends on a multi-step approach where antibiotic resistance is monitored and maintained for continued efficacy. While we have currently managed with the same antibiotics for years now, reducing exposure is one area of improvement. Nevertheless, without developing new strategies and therapeutics, it will be challenging to effectively lower the mortality and morbidity rates of infections.

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