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Chromosomal mutations involved in antibiotic resistance in *Staphylococcus aureus*

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1. ABSTRACT

Staphylococcus aureus is an important pathogen involved in infections in both the community and hospital setting. Strains that are resistant to multiple classes of antibiotics, particularly methicillin-resistant strains (MRSA), are prevalent in nosocomial infections and are associated with high morbidity and mortality rates. Such antibiotic-resistant strains limit the therapeutic options and place a burden on the health care system. In the hospital setting, horizontal gene transfer plays an important role in disseminating antibiotic resistant determinants among *S. aureus*. However, resistance to all known classes of antibiotics have been attributed to genes found within the *S. aureus* chromosome or to due to mutation as a result of selection pressure. Spontaneous mutations, in particular, are pivotal in the emergence of novel resistances. Consequently, newer drugs with better activity and/or antibacterial agents with novel targets need to be developed to combat and control the further spread of antibiotic resistance.

2. INTRODUCTION

Staphylococcus aureus is a ubiquitous commensal and frequent pathogen of humans and animals, causing various types of pyogenic infections. *S. aureus* has a propensity to develop resistance to new and different classes of antibiotics especially after the implementation of these antibiotics in clinical practice (Table 1) (1-2). Antibiotic resistance can arise in *S. aureus* (Table 2) from horizontal gene transfer and spontaneous mutation. Although horizontal gene transfer plays an important role in antibiotic resistance, chromosomally-encoded genes and mutations that affect their expression and function are important in conferring resistance to a wide variety of antibiotics particularly towards novel antibiotics. Modifications within the antibiotic target or binding site, due to missense mutations, are commonly associated with the development of antibiotic resistance due to antibiotic repulsion or steric hindrance.

Table 1. Seminal events in the emergence of antimicrobial resistance in *Staphylococcus aureus*

Date	Event	Country
1936	Sulphonamides introduced into clinical use	Germany
1940s	Penicillin used in humans	UK, USA, Australia
1943	Penicillinase detected in *S. aureus*	UK
1949	Tetracycline released	USA
1950	Aminoglycosides released	USA
1950s	Penicillin resistance common in hospital strains of *S. aureus*	UK, USA
1952	Macrolides released	USA
1956	Vancomycin resistance occurs *in vitro* on serial passage	France
1957	Methicillin released	UK
1961	MRSA first reported	UK
1960s	Increasing reports of MRSA, multiple hospital outbreaks	UK, USA, Australia
1962	Streptogramins and quinolones released	USA
1966	First MRSA isolated in Australia	Australia
1969	Gentamicin resistance detected	UK
1970s	Penicillin resistance common in community *S. aureus*	USA
1976	Outbreak of gentamicin-resistant MRSA	Australia, UK
Late 1970s	Multiple MRSA outbreaks throughout the world	Worldwide
1981	Extensive outbreaks of gentamicin-resistant MRSA	Ireland, USA, Australia
1980s	MRSA becomes endemic in hospitals	Australia, USA
1990s	MRSA endemic in most hospitals throughout the world	Worldwide
1990s	Community-acquired MRSA emerges	USA, New Zealand, Australia
1990s	Emergence of epidemic MRSA ("EMRSA") strains	UK
1990s	Emergence of gentamicin-susceptible MRSA strains	France
1992	MRSA strains found with teicoplanin MICs 8-16 mg L^{-1}	France
1992	*In vitro* transfer of *van*A gene complex from VRE to *S. aureus*.	UK
1996	VISA (*S. aureus* Mu50) first described	Japan
1997	hVISA (*S. aureus* Mu3) first described	Japan
1998	VISA strains found elsewhere	USA
1999	Q-D released	USA
Early 2000s	Q-D resistance demonstrated in isolates from retrospective large multi-center studies	Worldwide
2000	Linezolid released	USA
2001	Linezolid resistance detected	USA
2001	hVISA strains isolated in Melbourne	Australia
2002	MRSA strains with high level vancomycin resistance (VRSA) where *van*A gene complex from vancomycin-resistant enterococcus entered *S. aureus* via Tn*1546*	USA
2003	Daptomycin released	USA
2005	Daptomycin resistance observed in VISA isolate from an endocarditis patient	USA
2005	Tigecycline released	USA

Table 2. Antibiotic resistance mechanisms of chromosomally-encoded genes and mutations in *S. aureus*

Antibiotic	Mechanism of resistance			Genes responsible	Origin[a]	Cellular mechanism
	Target modification	Drug inactivation	Decreased accumulation			
Cell Wall Active Drugs						
Beta-lactams						
Penicillin		Y		*bla*ZIR	Acquired/ intrinsic	Inducible production of penicillinase
Antistaphylococcal beta-lactams (e.g., methicillin)	Y			SCC*mec* (*mec*AIR)	Acquired	Inducible production of modified PBP (PBP2a)
Glycopeptides						
Vancomycin (high-level)	Y			*van*A	Acquired	Production of altered peptidoglycan terminal precursor D-Ala-D-Lac with low affinity for vancomycin
Vancomycin (intermediate)	Y			cell wall biosynthesis genes	Intrinsic	Thickened cell wall causing "affinity trapping" of vancomycin
Lipoglycopeptides	Y			cell wall biosynthesis genes	Intrinsic	Alteration of cell surface charge, thickened cell wall
Nucleic Acid Synthesis Inhibitors						
Quinolones	Y			*grl*A, *gyr*A	Intrinsic	Topoisomerase IV and DNA gyrase mutations causing decreased affinity to quinolones
			Y	*nor*A	Intrinsic	Mutations causing overexpression of NorA efflux pump
Coumarins	Y			*gyr*B	Intrinsic	GyrB mutations causing decreased affinity to novobiocin
Sulfamethoxazole	Y			*dps*A	Intrinsic	DHPS mutations causing decreased affinity to sulfamethoxazole
	Y			*sul*A	Intrinsic	Increased PABA production
Trimethoprim	Y			*dfr*	Intrinsic	DHFR mutations causing decreased affinity to trimethoprim
Rifampicin	Y			*rpo*B	Intrinsic	RNA polymerase beta-subunit mutations causing decreased affinity to rifampicin

Protein Synthesis Inhibitors						
Macrolides, Lincosamides, Streptogramins	Y			*erm*A, *ermC*	Acquired	Inducible ribosome methylation
	Y			*erm*B	Acquired	Constitutive ribosome methylation
	Y			*Rrl*	Intrinsic	Ribosomal mutations causing decreased affinity to MLS
	Y			*rpl*V, *rpl*D	Intrinsic	Mutations causing structural changes of ribosomal proteins that line the peptide exit tunnel
Oxazolidinones	Y			*rrl*	Intrinsic	Ribosomal mutations causing decreased affinity to oxazolidinones
Aminoglycosides		Y		*aac*, *aad*, *aph*	Acquired	Modification of aminoglycosides
Tetracyclines	Y			*tet*A(M)	Acquired	Inducible production of proteins that protect the ribosomal tetracycline-binding site from tetracyclines
			Y	*tet*A(K), *tet*A(L)	Acquired	Expression of efflux pumps
Glycylcyclines			Y	*mep*AR	Intrinisic	Mutations in *mep*R causing derepressed expression of MepA efflux pump
Mupirocin	Y			*ile*S	Intrinsic	IleS mutations in causing decreased affinity to mupirocin
Fusidic acid	Y			*fus*A	Intrinsic	EF-G mutations causing decreased affinity to fusidic acid

Adapted with permission from (161), [a]Acquired genes are present on mobile genetic elements which have integrated into the chromosome.

3. RESISTANCE TO CELL WALL ACTIVE AGENTS

Antibiotic classes such as the beta-lactams (*i.e.,* penicillins, cephalosporins, monobactams, carbapenems) and the glycopeptides prevent the biosynthesis of peptidoglycan, a crucial component of the bacterial cell wall. In *S. aureus*, peptidoglycan biosynthesis relies on the activity of four penicillin-binding proteins (PBP1-4) which are enzymes that have dual transglycosylase and transpeptidase (TPase) activities and are involved in peptidoglycan synthesis and cross-linking, respectively (3). The instability of the weakened cell wall caused by beta-lactams and glycopeptides binding to the TPase domain of PBPs or the target site of PBPs, respectively, results in bacterial cell death during cell division.

3.1. Beta-lactams

S. aureus can be resistant to beta-lactams via one of two general mechanisms: production of beta-lactamases, enzymes which hydrolyze the beta-lactam ring; and/or production of altered PBPs. Beta-lactamases are inducible in the majority of *S. aureus* strains (4) and are encoded by the *bla*ZIR operon. *bla*Z, which codes for the beta-lactamase, is regulated by genes include *bla*I, *bla*R1, and *bla*R2 (5-6). *bla*ZIR1 are typically found on large plasmids, but *bla*R2 is always chromosomal (4, 7). *bla*Z can also be found on the chromosome (*e.g., S. aureus* NCTC9789) (8) and can be translocated, along with other chromosomal loci by transposons (9-10).

To combat staphylococcal beta-lactamases, methicillin, a penicillin with a side chain modification resistant to beta-lactamase activity, was developed. However, two years after the introduction of methicillin, the first methicillin-resistant *S. aureus* (MRSA) isolate was reported (11). MRSA typically possess a 30-50 kb staphylococcal cassette chromosome *mec* (SCC*mec*) that has integrated into the chromosome (12). Within SCC*mec* is the *mec* operon which contains the *mec*A gene required to confer methicillin resistance (13). *mec*A encodes PBP2A, an alternative PBP with a lower affinity for beta-lactams compared to the wild-type PBPs (*i.e.,* PBP1-4) (14). As a result, the *S. aureus* strain becomes resistant to all beta-lactam antibiotics except the new anti-MRSA cephalosporins (15-16). Interestingly, *mec*A could be derived from a fusion product of the upstream region of *bla*Z with a wild-type PBP gene (17).

Also within the *mec* operon are the regulatory *mec*I and *mec*R1 genes which exhibit strong suppression of *mec*A in the absence of beta-lactams making methicillin resistance an inducible phenotype (18-19). Similarities in molecular organization, function and regulation between *mec*IR1 and *bla*IR1 allows *mecA* expression to be controlled by both sets of regulatory genes whereby mutations affecting beta-lactamase induction also effect methicillin resistance (20-21). Thus, the absence of *bla*I and *bla*R1 (22), insertional inactivation of *mec*I and *mec*R1 (18, 23) or point mutations/deletions inactivating *mec*I contribute to PBP2A being constitutively expressed at high levels (24). The interactions between *mec*I, *mec*R1, *bla*I, and *bla*R1 on the expression of methicillin resistance are complex, particularly the cascade from the detection of extracellular beta-lactam to the production of PBP2A, and remain to be elucidated in staphylococci (22).

Isolates obtained prior to 1970 mostly have deletions of the penicillin-binding domain of *mec*R1 and the complete downstream *mec*I (24-25). Strains isolated since 1980 usually have intact regulatory genes but demonstrate polymorphisms in *mec*I and mutations in the *mec*A promoter (26). *mec*IR1 may also be truncated or absent due to the insertion of insertion sequences (*e.g.*, IS*1182*, IS*26*, IS*431*) (27). Further deletions, rearrangements, and recombination events commonly occur between *mec*A and IS*431* (14), a common staphylococcal insertion sequence associated with various resistance determinants. Such mutations likely reflect the selective pressure of beta-lactams for mutants lacking strong repressor activity, so that the amount of PBP2A produced will confer a survival advantage.

Some *S. aureus* strains have raised methicillin MICs but do not possess *mec*A. Point mutations in the penicillin-binding domains of PBP1, 2 or 4 decreases their affinity for beta-lactams resulting in raised methicillin

MICs (28). Alternatively, a variety of point mutations in PBP2 and 4 (29-30) can result in altered PBPs that bind penicillin more slowly and release penicillin more rapidly compared to wild-type PBPs (31). Over expression of PBPs, especially PBP4, may also cause a minor rise in methicillin MIC (32).

3.2. Glycopeptides

Glycopeptides form complexes with the peptidyl-D-Ala-D-Ala terminus of peptidoglycan precursors at the outer surface of the cell membrane, leading to inhibition of transglycosylation and transpeptidation steps in cell wall synthesis (33). The main clinical glycopeptides include teicoplanin and vancomycin, which is regarded as "drug of choice" to treat infections with MRSA (14).

3.2.1. Vancomycin

High-level vancomycin-resistant *S. aureus* (VRSA; MIC ≥32 g L^{-1}) is extremely rare (34-35) and is caused by the acquisition of a plasmid-borne transposon (Tn*1546*) from vancomycin-resistant enterococci (VRE) which has the ability to integrate into the staphylococcal chromosome (36). Within Tn*1546* is the *van*A gene which encodes a peptidoglycan precursor with alternative C-terminal residues (D-Ala-D-Lac) to which vancomycin cannot bind (37).

Vancomycin-intermediate *S. aureus* (VISA; MIC 4-8 mg L^{-1}) is more common and can be characterized by the absence of *van* genes, slower growth, pleomorphic colonial morphologies, thickened cell walls on electron microscopy, reduced susceptibility to lysostaphin, decreased autolysis and have alterations in cell wall metabolism (38-39). There is also a subset of VISA in which only a subpopulation of bacterial cells exhibits the resistance and is termed heterogeneous VISA (hVISA) (40).

VISA strains were shown to have an altered peptidoglycan precursor terminus (D-Alanyl-D-Ala instead of D-Ala-D-Ala) which vancomycin is able to bind to but does not inhibit transglycosylation and transpeptidation. The combination of bound vancomycin and the thicker cell wall prevents vancomycin from penetrating deeper into the cell wall and thus raising the vancomycin MIC of the strain. This phenomenon is referred to as the "affinity trapping" hypothesis (40-41). Acquisition of the VISA phenotype appears to be a multistep process involving multiple pathways. Many of these gene mutations described to date, associated with the VISA phenotype, are involved with cell wall synthesis, involving at least the *vra*SR and *wal*KR operons (38, 42). However, not all hVISA/VISA strains demonstrate these mutations. A comparative genomic whole genome sequencing study of the prototype hVISA and VISA isolates Mu3 and Mu50, respectively, identified missense mutations in the *gra*R locus of Mu50 (43). Introduction of the mutant *gra*R into Mu3 and in a VSSA strain conferred a VISA phenotype only in Mu3 suggesting that additional mutations are required for a VISA phenotype.

3.2.2. Teicoplanin

While vancomycin resistance is typically associated with teicoplanin resistance, teicoplanin resistance is not always accompanied by vancomycin resistance. The mechanism of teicoplanin resistance may be multi-factorial especially in VISA strains. However, mutations involving genes encoding the anti-sigma factor RsbW and transcription factor SigB were found involved in decreased teicoplanin susceptibility (44).

3.2.3. Lipopeptides

Daptomycin, a lipopeptide drug derived from glycopeptides, penetrates the cell wall leading to depolarisation and cell death (45). Reduced susceptibility to daptomycin (MIC >1 mg L^{-1}) can be generated by exposure to either vancomycin or daptomycin. A possible explanation for this observation is the selection of a thickened cell wall, as in hVISA/VISA isolates, which acts as a diffusion barrier for daptomycin. However, not all hVISA/VISA isolates are resistant to daptomycin and not all daptomycin-resistant MRSA isolates have thickened cell walls (46).

Genetic studies, mainly on laboratory generated isolates, have found genetic inconsistencies between non-susceptible isolates (47) suggesting that daptomycin resistance may be the result of multiple mechanisms as opposed to a single mutation. However, a recent study found a missense mutation at codon 621 (A621E) in the *rpo*B gene of laboratory strain *S. aureus* 10*3d1 conferring heterogeneous cross-resistance to vancomycin and daptomycin (48). Mutations in *rpo*B, which encodes the beta-subunit of RNA polymerase, are often associated with rifampicin resistance (see below) but no rifampicin resistance phenotype was observed in this strain (48). The A621E mutation was also associated with cell wall thickening and decreased cell surface charge (a daptomycin resistance mechanism) often observed in hVISA strains (48). Although, microarray analysis of 10*3d1, compared to its vancomycin/daptomycin-sensitive parent strain and isogenic strains containing the wild-type allele, showed numerous transcriptional changes in genes involved with cell wall metabolism (48), further studies are required to ascertain the role of this *rpo*B mutation in relation to heterogeneous vancomycin/daptomycin cross-resistance.

4. RESISTANCE TO NUCLEIC ACID SYNTHESIS INHIBITORS

4.1. Quinolones

A large number of quinolones exist, putatively divided into first-generation (*e.g.*, nalidixic acid, oxolinic acid), second-generation (*e.g.*, norfloxacin, ciprofloxacin), third-generation (*e.g.*, levofloxacin, sparfloxacin) and fourth-generation (*e.g.*, moxifloxacin, gatifloxacin) agents with improved activity/spectrum in each successive generation (49-50). Surveys of resistance over time have shown resistance to quinolones has increased since the introduction of second generation quinolones into clinical use (51-52) with 60-90% of MRSA worldwide currently being resistant to commonly used quinolones (*e.g.*, ciprofloxacin, levofloxacin) (35, 53-54).

Quinolones inhibit the action of the type II topoisomerases (*e.g.,* DNA gyrase and topoisomerase IV),

enzymes involved in DNA replication and segregation, by binding to and stabilising enzyme-DNA complexes and promoting the cleavage of DNA resulting in cell death (55-56). DNA gyrase has A and B subunits, encoded by *gyr*A and *gyr*B respectively (57). Topoisomerase IV is also composed of A and B subunits, encoded by *grl*A and *grl*B, and exhibit homology to GyrA and GyrB respectively (58). All quinolones are active against both DNA gyrase and topoisomerase IV, but differ in their relative activities against these enzymes. While most quinolones primarily target topoisomerase IV in *S. aureus*, nalidixic acid preferentially targets DNA gyrase (55).

Quinolone-resistant mutants are readily selected in the laboratory (56). Serial passaging results in the accumulation of multiple mutations and high-level resistance (59). Missense mutations observed in both *grl*A and *gyr*A occur within the quinolone resistance determining region of the two genes which encompasses codons 67-140 (60). Most low-level quinolone resistance in *S. aureus* is associated with a mutation at codons 80 (S80Y) or 84 (E84K) of *grl*A (61-62). A pharmacokinetic study suggested that strains with g*rl*A mutations are more prone to acquire secondary mutations and develop high-level quinolone resistance (63). Thus mutations in *grl*A are often seen prior to additional mutations in *gyr*A (62).

Mutations in *grl*B and *gyr*B are rare and play a minimal role in quinolone resistance (64-65). However, one study observed a novel mutation (G→A) 13 bp downstream of a putative ribosomal binding site of *grl*B in a clinical strain grown *in vitro* in the presence of premafloxacin, a quinolone in veterinary use (66). This mutation was associated with low-level quinolone resistance due to decreased *grl*B and *grl*A expression as both genes are in an operon under the control of the *grlB* promoter (66). A loss of fitness was observed when the G→A mutation was introduced in the quinolone-sensitive parent strain but not in the *in vitro*-derived mutants probably due to compensatory increases in *gyr*AB and *top*B (encoding topoisomerase III) expression observed in these mutants (66).

Quinolone resistance can also independently emerge by the overexpression of efflux pump genes *nor*A, *nor*B or *nor*C. NorA is the most studied efflux pump in *S. aureus* and exports hydrophilic quinolones (*e.g.*, norfloxacin, ciprofloxacin) and lipophilic, monocationic substances (*e.g.,* antiseptics and ethidium bromide) (67). Overexpression of *nor*A can augment the level of hydrophilic quinolone resistance in strains that already possess mutations in *grl* or *gyr* genes (67-68). Increased *nor*A expression levels can be associated with singe nucleotide mutations in and around the -10 promoter motif, particularly at a position 89 bp upstream of the transcriptional start codon (T-89G) (67, 69). The T-89G mutation is predicted to increase mRNA stability by generating an additional hairpin structure in the *nor*A leader mRNA (70). Furthermore, insertions downstream of the -10 promoter motif can also have upregulatory effects (71). Interestingly, the ability of antiseptics to select for *nor*A promoter mutations suggests that *nor*A overexpression is a response to chemical factors in the environment rather than quinolone selection by itself (69).

4.2. Coumarins

Coumarins (*e.g.,* novobiocin) competitively inhibit the ATP hydrolytic activity of GyrB (72). Mutations in *gyr*B, resulting in amino acid substitutions (*e.g.,* I102S and R144I) in the ATP-binding site have been associated with high-level coumarin resistance (65). While mutations in the coumarin-binding region of topoisomerase IV (*e.g.,* S80F) do not effect novobiocin resistance (73), mutations in the A subunit (A116P/E) or B subunit (N470D) confer coumarin hypersensitivity and increased quinolone resistance levels by altering the catalytic activity of the enzyme (65, 74-75).

4.3. Folate Synthesis Inhibitors

Purine and thymine synthesis relies on the production of tetrahydrofolate, the physiologically active form of folic acid (76). Dihydropteroate synthase (DHPS) utilises *p*-aminobenzoic acid (PABA) to form precursors involved in the synthesis of dihydrofolate (77). Dihydrofolate is subsequently converted to tetrahydrofolate by dihydrofolate reductase (DHFR) (78-79). Sulfamethoxazole (a PABA structural analogue) and trimethoprim are antibiotics that bind to and inhibit the activity of DHPS and DHFR, respectively, thus preventing tetrahydrofolate synthesis and ultimately preventing DNA synthesis leading to cell death (78). Due to the synergistic activity of both of these antibiotics, they are usually administered in combination as a drug called cotrimoxazole. However, despite the efficacy of cotrimoxazole, resistance rates in MRSA vary from 16-66% worldwide (53-54).

Sulphonamide resistance was reported in *S. aureus* soon after the introduction of these agents. A study of clinical isolates has shown that sulfamethoxazole resistance in *S. aureus* is complex and is associated with a variety of missense mutations in *dps*A which encodes DHPS (77). Despite crystallographic studies of DHPS, no amino acid changes have been determined to be the underlying resistance mutation (77). In some instances, strains expressing the chromosomal gene *sul*A have been shown to overproduce PABA and therefore outcompete sulfamethoxazole allowing DHPS activity to continue (33, 80-81).

Trimethoprim resistance in *S. aureus* is due to mutations in *dfr*A which encodes DHFR (82). Low-level (MIC 16 mg L^{-1}) trimethoprim resistance is occasionally due to overproduction of DHFR (83). However, a single mutation in DHFR (F98Y) is the primary mechanism for conferring low-level trimethoprim resistance due to a conformational change in the trimethoprim binding pocket resulting in inefficient trimethoprim binding (84). Additional mutations in the promoter region and within the protein (*e.g.*, H30N, H149R) can confer intermediate-high levels of resistance (79, 85).

4.4. Rifamycins

Rifampicin, the most commonly used rifamycin, is a bactericidal drug with typically very low MICs (<0.05 mg L^{-1}) (14) and has resistance rates varying between 5-45% in MRSA worldwide (54). Rifampicin prevents the

initiation of transcription by interacting with the RNA polymerase beta-subunit, encoded by the *rpo*B gene (33). Rifampicin resistance is the result of decreased rifampicin affinity for RNA polymerase beta-subunit due to mutations in two regions; cluster I (AA 471-495) and/or cluster II (AA 515-530) (86-88). The most common mutations occur in cluster I (*e.g.*, H481N) and confer low level rifampicin resistance (MIC 2-4 mg L^{-1}) (89-90). High level resistance (MIC ≥128 mg L^{-1}) generally requires additional mutations (*e.g.*, L466S, G468K, A477T, I527L, S529L) but other rifampicin resistance mechanisms may exist as studies described in clinical and *in vitro*-derived rifampicin-resistant *S. aureus* possessing only a single mutation in cluster I (88, 90-91).

5. RESISTANCE TO PROTEIN SYNTHESIS INHIBITORS

In bacteria, proteins are translated in the 70S ribosome which consists of two subunits; 50S and 30S. The 50S subunit further consists of the 23S RNA subunit (encoded by *rrl*), the 5S RNA subunit (encoded by *rrf*) and other small proteins (*e.g.,* L22). The 30S subunit consists of the 16S RNA subunit as well as small proteins. Antibiotics such as the macrolides, lincosamides, streptogramins and oxazolidinones all inhibit protein synthesis by strongly interacting with domain V of the 23S RNA subunit while aminoglycosides and fusidic acid, a steroidal antibiotic, target the 30S subunit.

5.1. Macrolides, lincosamides & streptogramins

Macrolides, lincosamides and streptogramin (MLS) antibiotics are bacteriostatic agents that inhibit protein synthesis (33). Macrolides, comprised of 14- (M_{14}; *e.g.,* erythromycin), 15- (M_{15}; *e.g.*, azithromycin) or 16- (M_{16}; *e.g,* spiramycin) membered ring structures, act by preventing 50S subunit assembly, preventing the peptidyl transferase reaction or obstructing the polypeptide exit tunnel of the 50S subunit causing premature release of peptidyl-tRNA during elongation (92-93). The lincosamides (L; *e.g.* clindamycin) and the streptogramins (consisting of components A (S_A; *e.g.*, dalfopristin) and B (S_B; *e.g.*, quinupristin) which act synergistically) are structurally distinct antibiotic groups compared to the macrolides but share similar modes of action and target sites with the macrolides (94). While erythromycin resistance rates are particularly high among MRSA strains worldwide (75-95%) (54), quinupristin-dalfopristin (Q-D) remains effective with very low levels of resistance seen in large surveys (95-97).

The adenine residue A2058 (*Escherichia coli* numbering) within the target site of the 23S RNA subunit (domain V) plays an important role MLS_B resistance (98). In staphylococci, methylases, encoded by genes (*e.g., erm*A-C) present in mobile genetic elements are able to integrate into the chromosome (99-100). These enzymes methylate A2058 causing a conformational change of the rRNA preventing MLS_B binding (98). S_A, however, is not affected by the A2058 epimutation (94). Although *erm*A and *erm*C confer inducible resistance to M_{14-15} in the presence of M_{14-15} only (94), $M_{16}LS_B$ exposure can select for strains having mutations (*e.g.,* deletions, tandem duplications, point mutations and disruption by IS*256*) in the regulatory units of the methylase genes converting an ErmA or ErmC-producing strain from being inducibly-resistant to constitutively resistant as seen in ErmB-producing strains (99, 101). In contrast to inducible strains, constitutively resistant strains express additional resistances (*i.e.,* $M_{14-16}LS_B$).

Nucleotide substitutions at A2058 (*e.g.,* A2058G/T) in *rrl* genes also confer resistance to MLS_B. However, resistance is difficult to develop in this method as there are 5-6 *rrl* alleles present in the staphylococcal genome (102); thus the effects of one mutated *rrl* gene can be overcome by the remaining wild-type *rrl* alleles. Interestingly, S_A resistance was observed in a clinical isolate with the A2058G mutation and a concurrent deletion in the *rpl*V gene which encodes the L22 protein (103). The L22 protein lines a portion of the peptide exit tunnel of the 50S ribosomal subunit near the MLS_B target site. Although amino acid duplications within L22 have also been associated with varying levels of MLS_B resistance, deletions in L22 can also widen the peptide exit tunnel allowing protein synthesis to remain active (104-105). *rpl*V mutants have also been shown to contain an insert from part of the *rpl*B gene which is approximately 790 bp upstream of the insertion site (106). A non-reciprocal recombination event is believed to have transferred a section of *rpl*B to *rpl*V between homologous sequences in both genes (106). It is proposed that these insertions may reduce antibiotic binding due to 50S surface property alterations or structural perturbations of the 23S subunit (104). However, the *rpl*B-*rpl*V mutation imparted a fitness cost as the doubling times of mutants, in nutrient-rich media, were 3-4 times slower than the sensitive parent strain (106).

Amino acid changes in the L4 protein (*e.g.,* G69A and T70P), encoded by *rpl*D, have also been associated with MLS_B resistance in two *S. aureus* isolates independently isolated from different cystic fibrosis patients (107). It is predicted that MLS_B resistance is caused by structural changes in the peptide exit tunnel as the mutations in L4 are present between two α-helices which are important in binding to the 23S RNA subunit (107). Interestingly, higher level resistance, including S_A, was observed in one of these strains which possessed an additional R168S mutation in L4 (107).

5.2. Oxazolidinones

The oxazolidinones (*e.g.,* linezolid, eprezolid) have bacteriostatic activity and are the only fully synthetic class of antibiotics in clinical use. Oxazolidinones bind to the 23S RNA subunit and prevents the peptidyl transfer reaction causing a premature release of aminoacyl-tRNA (108).

Although linezolid resistance is rare (35), point mutations in the *rrl* alleles (within the domain V region) are the predominant mechanism for linezolid resistance. There appears to be a positive correlation between the level of linezolid resistance and the number of mutated *rrl* alleles.

In a clinical report, a linezolid-susceptible MRSA (MIC 8 mg L^{-1}), with a G2576T mutation in 2/6 *rrl* alleles, became linezolid-resistant (MIC 32 mg L^{-1}) after the mutation developed in a further 3 alleles (109). The G2576T also confers cross resistance to Q-D and chloramphenicol, another type of protein synthesis inhibitor (110).

The presence of identical mutations in each *rrl* gene suggests that *rec*A-dependent recombination is involved in gene conversion of wild-type alleles (111). An *in vitro* study showed that *rec*A-deficient *S. aureus* mutants developed different mutations in the *rrl* alleles and required longer linezolid exposure to develop resistance (111). Although mutations such as G2576T can be stable even after 15 passages in antibiotic-free media (112) they may impart a biological cost to the bacterium and may explain the reversion of mutated *rrl* alleles to wild-type alleles observed in some strains (113). Provided a bacterium has at least one wild-type *rrl* allele, *rec*A can also be involved with the reversion of mutated *rrl* alleles to wild-type alleles after prolonged linezolid-free periods and thus confer linezolid susceptibility (113).

5.3. Aminoglycosides

Aminoglycosides (*e.g.,* gentamicin, netilmicin, and tobramycin) inhibit protein synthesis by binding to the 30S ribosomal subunit and inhibiting protein synthesis (114). However, these aminoglycosides are not useful clinically as single agents due to the excessive toxicity if therapeutic levels are achieved, and due to the propensity for resistance to readily emerge (115). The major mechanism of resistance in staphylococci is aminoglycoside modification by cellular enzymes (aminoglycoside acetyltransferases (*aac*), adenyltransferases (*aad*), and phosphotransferases (*aph*)) that reduce their ribosomal binding affinity (116).

Most aminoglycoside modifying enzyme genes are located on mobile genetic elements. pCL4, a 35.5 kb highly conjugative plasmid conferring resistance to multiple aminoglycosides (gentamicin, tobramycin, kanamycin, amikacin, astromicin, and arbekacin), contains an *aac*A/*aph*D gene complex that is able to integrate into the chromosome at multiple sites (117). Other notable mobile elements carrying aminoglycoside modifying enzymes that integrate into the chromosome include Tn*5405* with *aph*A3 and *aad*E (118) and SCC*mec* with *aad*D conferring tobramycin resistance to a large proportion of MRSA strains (119-120).

5.4. Tetracycline antibiotics

Tetracyclines are bacteriostatic agents that inhibit protein synthesis by binding to the peptidyltransferase center (PTC) within the 70S ribosome and causing premature aminoacyl-tRNA release (121). The tetracyclines encompass a commonly used group of antibiotics which include tetracycline, doxycycline and minocycline. Resistance to the tetracyclines can result from ribosomal protection whereby inducible chromosomally-encoded proteins (*e.g.,* TetA(M)) interact with the tetracycline target site preventing tetracycline binding while allowing protein synthesis to continue (122). Alternatively, the TetA(K) or TetA(L) efflux pumps, encoded by genes found in both the chromosome and on plasmids, confer inducible resistance to the tetracyclines but not semisynthetic analogues such as minocycline (33, 123-124) to which >98% of MRSA strains are susceptible to, worldwide (35).

Tigecycline, the first and only glycylcycline in clinical use, is a tetracycline-derivative having a minocycline backbone. Tigecycline resistance is rare due to the presence of a glyclamido side chain that allows evasion of conventional tetracycline resistance mechanisms (*i.e.*, efflux and ribosomal protection) providing increased binding and activity over the tetracyclines (125). However, tigecycline is a substrate of MepA, a multidrug and toxin efflux pump encoded by *mep*A (126). An *in vitro* study found mutations (*e.g.,* single nucleotide mutation or deletion) resulting in the formation of a premature stop codon in *mep*R, which represses *mepA* expression, in *S. aureus* strains Mu3 and N315 after passaging in media containing increasing concentrations of tigecycline for 16 days (126). Although *mep*A was overexpressed in these strains, only low-level tigecycline resistance was observed suggesting other mechanisms must be involved to confer high-level resistance (126).

5.5. Pseudominic Acid

Mupirocin (pseudomonic acid A) is effective for the topical treatment of *S. aureus* infections (14). Mupirocin inhibits protein synthesis by acting as an isoleucine analogue binding to isoleucyl-tRNA synthetase, IleS (127). tRNA synthetases catalyse the formation of aminoacyl-tRNA whereby amino acids are charged to their respective tRNA for peptide formation in ribosomes.

High-level mupirocin resistance has emerged in strains possessing a plasmid-encoded *mup*A gene which codes for a novel IleS which is not affected my mupirocin (128). Low-level mupirocin resistance (MIC 8-64 mg L^{-1}), however, is associated with single amino acid changes (*e.g.*, V588F, G593V or V631F) in IleS from both clinical strains and *in vitro*-derived mutants (129-130). These mutations occur near the binding pocket (Rossman fold) of IleS and cause steric hindrance and conformational changes preventing mupirocin binding. Extended mupirocin resistance was observed in *in vitro* derived mutants with a combination of these mutations but came at a fitness cost compared to single mutants in both *in vivo* and *in vitro* models (129). Although double mutants may revert to a mupirocin-susceptible phenotype, revertants still retain the double mutations but acquire other intra- and extra-genic mutations which affect the conformation of IleS allowing mupirocin to remain active (129). As topical concentrations (20,000 mg L^{-1}) of mupirocin are well above the MICs of strains with low-level resistance, clinical failure is not common (14).

5.6. Steroids

Fusidic acid is a steroid-based antibiotic with high activity against *S. aureus* including MRSA strains (131). Fusidic acid prevents the release of elongation factor G (EF-G) which binds to the ribosome and catalyzes translocation during protein synthesis (132). Resistance to

fusidic acid may be chromosomally or plasmid encoded. Chromosomal resistance is associated with missense mutations in *fus*A, which encodes EF-G, that results in a decreased affinity for fusidic acid (133). While numerous EF-G mutations have been associated with low-intermediate fusidic acid resistance (134-135), mutations within domain III (*e.g.,* H457Y or L461K) confer high-level resistance (136-137). Other EF-G mutations have been observed, especially in clinical strains, but are believed to be compensatory mutations (*e.g.,* S416F) as *in vitro* mutants with single mutations (*e.g.,* L461K) have reduced fitness (134-135). Naturally resistant subpopulations exist at rates of 10^6-10^7 and are rapidly selected by exposure to fusidic acid alone (138). However, such mutants grow more slowly than wild-type strains, exhibit a reduced virulence and revert to susceptibility on removal of fusidic acid (139).

6. SMALL COLONY VARIANTS OF *S. AUREUS*

Small colony variants (SCVs) of *S. aureus* appear as small, pale or colourless, slow-growing colonies on agar plates, often resembling (and can be mistaken for) coagulase-negative staphylococcal species (140). They are important intracellular pathogens (141), particularly in biofilms (142), which is likely to explain why certain staphylococcal infections such as infected bioprostheses are refractory to standard antibiotic treatment (*e.g.,* beta-lactams, vancomycin) which do not have intracellular activity (143-144). Most SCVs have defective electron transport chains resulting in the inability to take up cationic antibiotics (145). The SCV phenotype is inducible by exposure to antibiotics such as beta-lactams (146), gentamicin (147), quinolones (148) and cotrimoxazole (145).

7. CONCLUSION & FUTURE PERSPECTIVE

Staphylococcal infections, particularly those caused by MRSA strains are important as they are typically resistant to an additional 3-6 antibiotics, all belonging to different classes (54). Although antibiotic resistance can emerge by horizontal gene transfer and the spread of resistant clones, the independent acquisition of different mutations in chromosomally-encoded genes, arising from a single strain, plays an important role in developing resistance particularly to novel antibiotics (68). New cephalosporins in clinical trials such as ceftobiprole and ceftaroline have good inhibitory activities towards PBP2A in MRSA strains (149-151). However, an *in vitro* study has already generated ceftobiprole-resistant mutants in a laboratory strain of *S. aureus* (COLnex) cloned with a plasmid bearing the *mec*A gene only (independent of SCC*mec*) (152). Spontaneous mutations in *mec*A that developed after prolonged exposure to ceftobiprole were shown to be responsible for ceftobiprole resistance (MIC 128 mg L^{-1}). Additionally, transformation of plasmids with the *mec*A mutant alleles into the same plasmid-naive COLnex parent strain conferred an equivalent level of resistance (152).

Other novel antibiotics include the semisynthetic glycolipopeptides (*e.g.,* oritavancin, telavancin) which combine the activities of the glycopeptide and lipopeptide antibiotics. In addition to cell wall depolarisation, the lipid side chains of glycolipopeptides allow the drug to concentrate in the cell membrane allowing easier access to their target site (peptidyl-D-Ala-D-Ala terminus of peptidoglycan precursors) (153). Furthermore, both oritavancin and telavancin demonstrate activity against VRSA (154-155). Telavancin has now been approved for use in the United States for the treatment of complicated skin and skin structure infections (156).

Advances in molecular biology provide an opportunity to develop novel strategies in combating antibiotic-resistant bacteria. Antisense agents are oligonucleotides which can target the expression of particular genes such as those responsible for antibiotic resistance. This principle has successfully been used to restore vancomycin susceptibility in a VanA-producing *Enterococcus faecalis* isolate (157). However, there are issues in delivering antisense oligonucleotides into the bacterial cell. Research into modified nucleic acids and attachment to cell-permeabilizing peptides has improved the stability and uptake into the cytoplasm of the antisense agents (158).

Whole genome sequencing approaches may also yield novel targets. Recently, toxin-antitoxin (TA) systems, usually found on plasmids as a maintenance mechanism, have been described on the chromosomes of clinically-relevant genera including the enterococci, lactobacilli and staphylococci (159-160). The role of such chromosomally-encoded TA systems remains to be elucidated; however, if they are required for chromosomal maintenance within dividing cells, they may prove to be an important target for new antibacterial agents.

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Abbreviations: DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; EF-G, elongation factor G; hVISA, heterogeneous vancomycin-intermediate *S. aureus*; MIC, minimum inhibitory concentration; M_xLS_y, macrolide (subscript number indicates ring composition), lincosamide, streptogrammin (subscript indicates component); MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; PABA, *p*-aminobenzoic acid; PBP, penicillin-binding protein; PTC, peptidyltransferase center; SCC*mec*, staphylococcal cassette chromosome *mec*; SCV, small colony variant; TPase, transpeptidase; VISA, vancomycin-intermediate *S. aureus*; VRE, vancomycin-resistant enterococci; VRSA, vancomycin-resistant *S. aureus*; VSSA, vancomycin-sensitive *S. aureus*

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