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Review Article

REVIEW ARTICLE

Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects

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One sentence summary: *Staphylococcus aureus* has become resistant to all antibiotics used to combat infection through acquisition of resistance mechanisms acquired by horizontal transfer and by chromosomal mutations. The current dearth of treatment options might be overcome by new discoveries and synergistic combinations

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ABSTRACT

The major targets for antibiotics in staphylococci are (i) the cell envelope, (ii) the ribosome and (iii) nucleic acids. Several novel targets emerged from recent targeted drug discovery programmes including the ClpP protease and FtsZ from the cell division machinery. Resistance can either develop by horizontal transfer of resistance determinants encoded by mobile genetic elements viz plasmids, transposons and the staphylococcal cassette chromosome or by mutations in chromosomal genes. Horizontally acquired resistance can occur by one of the following mechanisms: (i) enzymatic drug modification and inactivation, (ii) enzymatic modification of the drug binding site, (iii) drug efflux, (iv) bypass mechanisms involving acquisition of a novel drug-resistant target, (v) displacement of the drug to protect the target. Acquisition of resistance by mutation can result from (i) alteration of the drug target that prevents the inhibitor from binding, (ii) derepression of chromosomally encoded multidrug resistance efflux pumps and (iii) multiple stepwise mutations that alter the structure and composition of the cell wall and/or membrane to reduce drug access to its target. This review focuses on development of resistance to currently used antibiotics and examines future prospects for new antibiotics and informed use of drug combinations.

Keywords: *Staphylococcus aureus*; antibiotic resistance; horizontal gene transfer; new drugs; cell envelope; resistome

INTRODUCTION

Conventional antibiotics are natural products synthesised by microorganisms that act against other microbes (Walsh 2016). A few entirely synthetic molecules have been developed and are also referred to here as antibiotics. Chemical modifications to the original antibiotic molecules have been made to increase potency, to improve solubility and pharmacokinetics and to evade resistance mechanisms. This principle was first applied empirically to modify sulphanilamide and achieved great success with modifications to β-lactams. Nowadays, modifications also can be tailored based on knowledge of the structure of the drug bound to the active site.

The development of resistance to many antibiotics by *S. aureus* has involved acquisition of determinants by horizontal gene transfer of mobile genetic elements (Jensen and Lyon 2009). These determinants may have evolved in antibiotic producers to protect them from potentially inhibitory molecules, or in their competitors. Analysis of the soil resistome shows that bacteria that express resistance to antibiotics are widespread (Nesme and Simonet 2015).

Resistance also can emerge by mutations that alter the drug binding sites on molecular targets and by increasing expression of endogenous efflux pumps. The development of resistance by mutation can in principle be reduced by using combinations of inhibitors that target different sites or for two or more mutations

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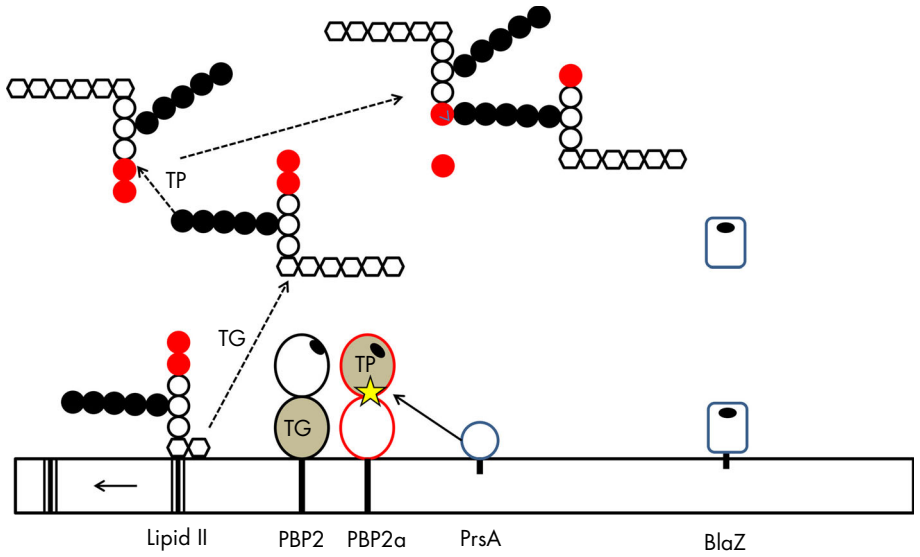

Figure 1. Resistance to β-lactam antibiotics. The figure summarises in schematic form the proteins that are involved in synthesis of peptidoglycan in wild-type *S. aureus* and MRSA. The active transglycosylase (TG) domain of PBP2 is indicated. The TP of PBP2 and PBP2a of MRSA have catalytic serines at their active sites (black elipses). The TP domain of PBP2a is buried. The serine -OH forms an acyl enzyme intermediate with the fourth amino acid in the stem peptide (D-Ala4), cleaving the peptide bond between D-Ala4 and D-Ala5 (red circles). The intermediate is attacked by the side chain NH_2 group of Gly5 in the cross-bridge peptide (black circles) of an adjacent peptidoglycan chain. This results in regeneration of the serine active site and formation of an isopeptide bond between D-Ala4 on one peptidoglycan chain with Gly5 of an adjacent molecule and formation of the final cross-linkage in PG. The figure shows the housekeeping PBP2 and the PBP2a of MRSA along with the lipoprotein chaperone PrsA, the activity of which is inhibited by the increased level of lysyl phosphatidyl glycerol in the membrane in mutants that are resistant to daptomycin (Fig. 2). The shaded parts of the PBPs indicate domains that are active in peptidoglycan biosynthesis in MRSA exposed to β-lactams. The yellow star on PBP2a indicates the allosteric site which promotes opening of the buried active site serine in the TP domain. The lipid moiety of lipoproteins is indicated by the short back rod. The membrane binding domain of PBPs is indicated by the extended black rod. The C55-isoprenol of lipid II Is depicted by the triple black rod. Attached to the lipid is the disaccharide MurNAc GlcNAc and the stem peptide terminating in two D-Ala residues and the pentapeptide glycine cross-bridge peptide. β-lactamase (BlaZ) with its active site serine is shown.

to be required for resistance to pass the MIC breakpoint. Resistance to vancomycin requires up to six mutations in different genes that result in remodelling of the cell envelope to reduce drug access to the lethal target.

This review is organised into sections based on inhibitors of major cellular targets viz the cell envelope, protein synthesis and nucleic acid synthesis, and focusses on those drugs that are currently used in treating infection in humans and/or are used in animal husbandry. After briefly considering their mechanisms of action, resistance mechanisms will then be discussed.

Very few antibiotics that represent novel chemical classes have been introduced in the past 30 years (Silver 2011). The prospects for badly-needed new drugs to combat staphylococcal infections with novel mechanisms of action will be assessed, focussing on those that are in pre-clinical and early commercial development.

TEXT BOX

MIC breakpoint

'A breakpoint is a chosen concentration (mg L^{-1}) of an antibiotic which defines whether a species of bacteria is susceptible or resistant to the antibiotic. If the MIC is less than or equal to the susceptibility breakpoint the bacteria is considered susceptible to the antibiotic. If the MIC is greater than this value the bacteria is considered intermediate or resistant to the antibiotic' according to the British Society for Antimicrobial Chemotherapy Resistance Surveillance Project. The European Committee on Antimicrobial Susceptibility Testing provides MIC breakpoints for all antibiotics in clinical usage.

ANTIBIOTICS THAT TARGET THE CELL ENVELOPE

β-Lactam antibiotics

Penicillin-resistant strains of *Staphylococcus aureus* emerged shortly after the introduction of the antibiotic in the early 1940s (Lowy 2003; Peacock and Paterson 2015; Walsh 2016). They expressed a β-lactamase that hydrolysed the critical β-lactam bond and destroyed the drug's antibacterial activity. Substitutions of the natural aminoadipoyl chain of penicillin with bulkier moieties created semisynthetic variants that were not substrates for β-lactamase. Methicillin was the first but had the disadvantage of being acid labile. It was superceded by the acid stable isoxazoyl penicillin oxacillin. Shortly after the introduction of methicillin resistance to it was detected and the methicillin-resistant *S. aureus* (MRSA) has stuck even though the term methicillin is no longer used.

Mechanism of action of and resistance to penicillin

The major inhibitory target for β-lactam antibiotics in *S. aureus* is the bifunctional transglycolylase-transpeptidase PBP2 (Giesbrecht *et al.* 1998; Walsh 2016). The transglycosylase domain of the enzyme is responsible for transferring the disaccharide pentapeptide building block of peptidoglycan from membrane-bound lipid II to growing polysaccharide chains while the transpeptidase (TP) domain cross-links the glycine cross-bridge of the fourth D-alanine of an adjacent chain (Fig. 1).

β-Lactam antibiotics bind at the active site serine of the TP in PBP2 by acting as structural analogues of D-Ala4-D-Ala5. The β-lactam bond is broken and a penicilloyl-O-serine intermediate is formed. The difference between this intermediate and the

peptidoglycan acyl enzyme intermediate is that the former is extremely stable, taking 1–4 h for the addition of H_20 to regenerate the active site Ser and release the penicilloic acid product. This compares to the milliseconds for the natural reaction to be completed. In essence, the active site of the TP enzyme is blocked and peptidoglycan biosynthesis ceases. Hence, it is possible to label the TP with ^{14}C penicillin and to detect the protein by SDS-PAGE and autoradiography (Walsh 2016). The bactericidal effect is more complex than simply inhibiting PBP activity. The drug induces toxic malfunctioning of the wall biosynthetic apparatus which involves a continuing cycle of synthesis and autolysis (Cho, Uehara and Bernhardt 2014).

The *S. aureus* β-lactamase responsible for resistance to penicillin is a typical serine β-lactamase (BlaZ) that forms the same type of acyl enzyme intermediate as the TP of PBP2 (Massova and Mobashery 1998; Lowy 2003). The catalytic difference between BlaZ and TP is the kinetics of deacylation. With BlaZ, the addition of water is rapid resulting in regeneration of the active site serine and release of the ring-opened penicilloic acid, a hydrolytic degradation product with no inhibitory activity.

The β-lactamase structural gene *blaZ* gene is carried by the transposon Tn552 or Tn552-like elements (Jensen and Lyon 2009). The transposon is either located on a large plasmid, the prototype of which is pI524, or is integrated into the bacterial chromosome. Expression of the enzyme is inducible, being controlled by the BlaI repressor and the BlaR sensor (Zhang *et al.* 2001; Lowy 2003). The enzyme itself is a lipoprotein that is partly located on the outer face of the cytoplasmic membrane where it is strategically located to protect PBP2s while some is released into the surrounding medium (Fig. 1) (Nielsen, Caulfield and Lampen 1981).

Mechanism of resistance to methicillin and oxacillin

The mechanistic basis of resistance to methicillin and oxacillin (Fisher and Mobashery 2016) is through acquisition of a gene that encodes a homologue of the PBP2 called PBP2a (Hartman and Tomasz 1984; Peacock and Paterson 2015) or PBP2' (Utsui and Yokota 1985) which is not susceptible to drug action. This is because the active site serine of the TP of PBP2a is located in a deep pocket which is not accessible to β-lactams (Lim and Strynadka 2002). The enzyme can therefore take over PG biosynthesis if the housekeeping PBP2 TP is inactivated. In fact, the PBP2 transglycosylase activity is required for peptidoglycan biosynthesis because the PBP2a moiety is non-functional (Pinho, de Lencastre and Tomasz 2001). Thus, PG biosynthesis is a cooperative effort between the two proteins when the TP of PBP2 is inactivated (Fig. 1).

When an MRSA strain is grown in the presence of β-lactams, the PG formed is poorly cross-linked. One of the consequences of this is that the peptidoglycan has stronger proinflammatory effects which could contribute to pathology during infection if the MRSA strain is exposed to β-lactams (Muller *et al.* 2015). The altered PG structure might also account for the inability of the global accessory gene regulator (Agr) to be induced in some MRSA strains (Rudkin *et al.* 2012).

Expression of methicillin resistance

PBP2a is encoded by the *mecA* gene which is located within a family of distinct but related staphylococcal chromosome cassette (SCC) elements (Jensen and Lyon 2009; Malachowa and DeLeo 2010; Peacock and Paterson 2015; Liu *et al.* 2016). However, a distinct PBP2a called MecC with only 63% residue identity to MecA was discovered recently. It occurs predominantly in a single lineage of MRSA in Europe (Paterson, Harrison and Holmes 2014). Distinct MRSA strains are endemic to particular geographical regions while some strains have spread globally (DeLeo *et al.* 2010; Uhlemann *et al.* 2014). Originally, MRSA were confined to hospitals (hospital-associated MRSA). These strains are resistant to multiple antibiotics, usually have large SCC *mec* elements and have sacrified virulence for high levels of resistance to β-lactams (Chambers and Deleo 2009; Rudkin *et al.* 2012). These strains frequently cause wound infection and systemic infection resulting from bacteraemia.

In the past 15 years, community-associated MRSA strains have emerged which cause serious skin and soft tissue infections (SSSTI) in otherwise healthy individuals (Chambers and Deleo 2009; DeLeo *et al.* 2010). These strains carry a small SCC*mec* cassette (Malachowa and DeLeo 2010), are not resistant to multiple antibiotics, have the ability to survive on human skin (which enhances transmission and infectivity) and have enhanced virulence (DeLeo *et al.* 2010; Thurlow *et al.* 2013). They cause SSSTIs that often require hospitalisation. CA-MRSA typically express lower levels of resistance to β-lactams (Rudkin *et al.* 2012).

In the prototype MRSA strains, the *mecA* gene is only expressed following induction by exposure to the drug. It is under the control of MecIR regulatory proteins which are homologous to the BlaIR proteins that regulate BlaZ expression (McKinney *et al.* 2001; Peacock and Paterson 2015; Fisher and Mobashery 2016). The *mecA* gene is inefficiently induced following exposure to β-lactam antibiotics (Jensen and Lyon 2009). In many MRSA strains, the *mecIR* genes have been truncated by insertion sequences which in principle should result in constitutive expression of *mecA*. However, the β-lactamase regulators BlaI and BlaR can also repress *mecA* expression. Thus, the nature of the expression of PBP2a can vary from strain to strain depending on the presence of functional Mec and Bla regulators. The structure of PG in MRSA may vary depending on whether they have been exposed to antibiotic in strains where one or both regulators are intact.

Most natural MRSA isolates express resistance to β-lactams heterogeneously (Peacock and Paterson 2015). This means that in a culture derived from a single colony the majority of cells express a low level of resistance while only a minority express resistance at a high level (Finan *et al.* 2002). Some isolates naturally express high-level resistance homogeneously but these are in the minority. The conversion from heterogeneous to homogeneous high-level resistance is the result of chromosomal mutations that directly or indirectly increase the transcription of the *mecA* gene and increase levels of PBP2a. In a model system involving introduction of a plasmid-borne *mecA* gene into a susceptible laboratory strain, a mutation in the *relA* gene triggered conversion from heterogeneous to homogeneous methicillin resistance (Kim *et al.* 2013). The *relA* mutation resulted in a non-functional RelA protein, constitutive (p)ppGpp expression and induction of the stringent response. In clinical isolates, mutations in several different genes are associated with the development of homogeneous resistance, including *relA,* as well as and *rpoB* that encodes the β-subunit of RNA polymerase (Dordel *et al.* 2014).

New β-lactams with activity against MRSA

Over the past 50 or more years, a large family of β-lactam antibiotics has been developed. Different β-lactam natural products were discovered viz cephalosporins, carbapenems, monobactams and clavams (Walsh 2016). Many semisynthetic derivatives were produced which had broader spectra of activity, improved pharmacokinetics and importantly insensitivity to the rapidly evolving β-lactamases of Gram-negative bacteria which

could hydrolyse the newer molecules. However, only the fifth generation of semisynthetic cephalosporins, ceftaroline fosamil and ceftobiprole, exhibited significant inhibitory activity against the PBP2a of MRSA. Only the former has approval to treat *S. aureus* infections (SSSTIs), while both are used to treat community-acquired streptococcal pneumonia (Saravolatz, Stein and Johnson 2011).

Ceftaroline has a novel mechanism of action (Otero *et al.* 2013; Fisher and Mobashery 2016). The structural and functional attributes of the substituents have been described (Laudano 2011). X-ray crystallography revealed that two moles of the drug bound to PBP2a of MRSA. One inactivated the active site serine while the other bound to an allosteric binding site located 60 Å away. Binding to the second site introduced a conformational change that opened up the deep active site pocket allowing drug access. Not unexpectedly clinical usage of ceftaroline has selected for strains with resistance to the drug with mutations that cause amino acid substitutions both close to the active site and at the allosteric site of PBP2a that presumably interfere with drug binding (Long *et al.* 2014; Lahiri and Alm 2016; Schaumburg *et al.* 2016).

Vancomycin and other glycopeptides

Vancomycin is a glycopeptide antibiotic that is widely used to treat serious infections caused by MRSA strains in hospital patients. It binds to the dipeptide D-Ala4-D-Ala5 of lipid II and prevents transglycosylation and transpeptidation catalysed by PBP2 and PBP2a and antagonises peptidoglycan remodelling (Zeng *et al.* 2016).

Several semisynthetic lipoglycopeptides that are related to vancomycin have been approved for treating acute bacterial SSSTIs. Oritavancin and televancin cause membrane damage in addition to inhibiting peptidoglycan biosynthesis and are rapidly bactericidal towards MSSA, MRSA and VISA strains (see below) including cells in the stationary phase of growth (Crotty *et al.* 2016; Zeng *et al.* 2016) and thus might be active against persisters (see below). The increased potency is attributed to the lipophilic substitution and the enhanced formation of drug dimers.

Enterococci have acquired the ability to express resistance to high levels of vancomycin. The *van* genes are located on mobile genetic elements and encode inducible enzymes that take over the biosynthesis of peptidoglycan precursors resulting in a lipid II molecule with D-lactate replacing D-Ala5 (Courvalin 2006; Zeng *et al.* 2016). This structure has a much reduced affinity for vancomycin but can be used as a substrate for transpeptidation by PBP2 in the final stage of wall biosynthesis.

It had been widely feared that MRSA would acquire vancomycin (Van) resistance determinants from enterococci resulting in serious invasive infections and SSSTIs becoming untreatable by vancomycin. Indeed, there have been sporadic reports of vancomycin-resistant MRSA but these strains have not spread and have not become established in the hospital environment (Courvalin 2006; Gardete and Tomasz 2014). The reason(s) for this are not entirely clear. Following introduction of the *vanA* determinant into the model homogeneous high-level methicillin-resistant strain COL, it was shown that the MRSA PBP2a could not utilise lipid II with D-Ala-D-lactate. Two naturally occurring VRSA strains had a very long lag phase before they could start growing in an inhibitory concentration of vancomycin *in vitro*. Finally, the plasmid carrying the *van* genes was genetically unstable. Thus, a combination of factors conspired to reduce the fitness of VRSA.

VISA

A common form of treatment failure with vancomycin is due to strains that emerge during prolonged treatment by acquiring multiple mutations in chromosomal genes that affect cell wall biosynthesis and homeostasis. These variants are called vancomycin-intermediate *Staphylococcus aureus* (VISA) and have an MIC of 4–8 μg ml^{-1} while vancomycin-susceptible *S. aureus* (VSSA) have an MIC of $\leq$2 μg ml^{-1} (Howden *et al.* 2010; Gardete and Tomasz 2014). Intermediate in the evolution of VISA are hetero-VISA (h-VISA) strains, variants where the majority of cells in the population have an MIC of 2 μg ml^{-1} or less and are thus defined as sensitive but they contain a subpopulation of cells with an MIC of 4–8 μg ml^{-1}. In essence, the subpopulation comprises single step mutants that will be selected at the final step in the pathway to insensitivity to therapeutic concentrations. The VISA subpopulation is often slow growing and is sometimes difficult to detect in a diagnostic laboratory by conventional E-test even if incubated for 48 h.

The development of h-VISA and VISA is a multistep process that can occur by many different pathways. The proportion of insensitive cells in different h-VISAs can vary. Nevertheless, the underlying mechanistic basis of insensitivity is an increase in thickness and altered architecture of the cell wall (Howden *et al.* 2010; Gardete and Tomasz 2014) (Fig. 2). The increased thickness results in the drug having to travel further to meet lipid II, its lethal target on the outer face of the cytoplasmic membrane in the division septum where cell wall biosynthesis is active. A reduction in cross-linking in peptidoglycan provides an abundance of D-Ala -D-Ala false targets which sequester the drug, preventing diffusion to the membrane (Fig. 2). In addition, large amounts of the bulky glycopeptide bound to the outer layers of the enlarged cell wall act to clog drug diffusion. In some VISA strains, an increase in the level of D-Alanine substitution on teichoic acids alters the charge of the cell envelope which acts to repel the positively charged glycopeptides.

Whole genome sequencing (WGS) of strains isolated from patients following prolonged treatment has provided insights into the complex and heterogeneous nature of the pathways to resistance. In general, it is believed that the initial step occurs in one of several global regulators that directly or indirectly alter cell wall biosynthesis and cell physiology (Howden *et al.* 2010; Gardete and Tomasz 2014; Howden, Peleg and Stinear 2014; Zeng *et al.* 2016). A succession of further mutations gives small increments in resistance leading to the h-VISA state.

The first h-VISA and VISA strains to be characterised were Mu3 and Mu50, respectively, from ST5. Since then several strains from different genetic backgrounds have been isolated from infected patients and subjected to WGS. The association of non-synonymous SNPs in candidate genes with the evolution of drug insensitivity has been strengthened by analysing the loci in panels of independently isolated VISAs. However, to prove that the mutations cause the phenotypic changes requires painstaking genetic analysis involving allelic exchange to restore the mutation in the VISA isolate to wild type and/or to introduce mutations successively into a VSSA background.

Recently, the VISA phenotype of Mu50 was recreated in a naïve ST5 vancomycin-sensitive background by successively introducing the mutations in six genes from Mu50 that were suspected of being required for resistance (Katayama *et al.* 2016). The first mutation affected the VraSR two-component signal transduction (TCST) system. This led to constitutive expression of the response regulator VraR and elevated expression of the ~40 genes in the cell wall stimulon including several involved in cell wall biosynthesis. A second mutation in the MsrR

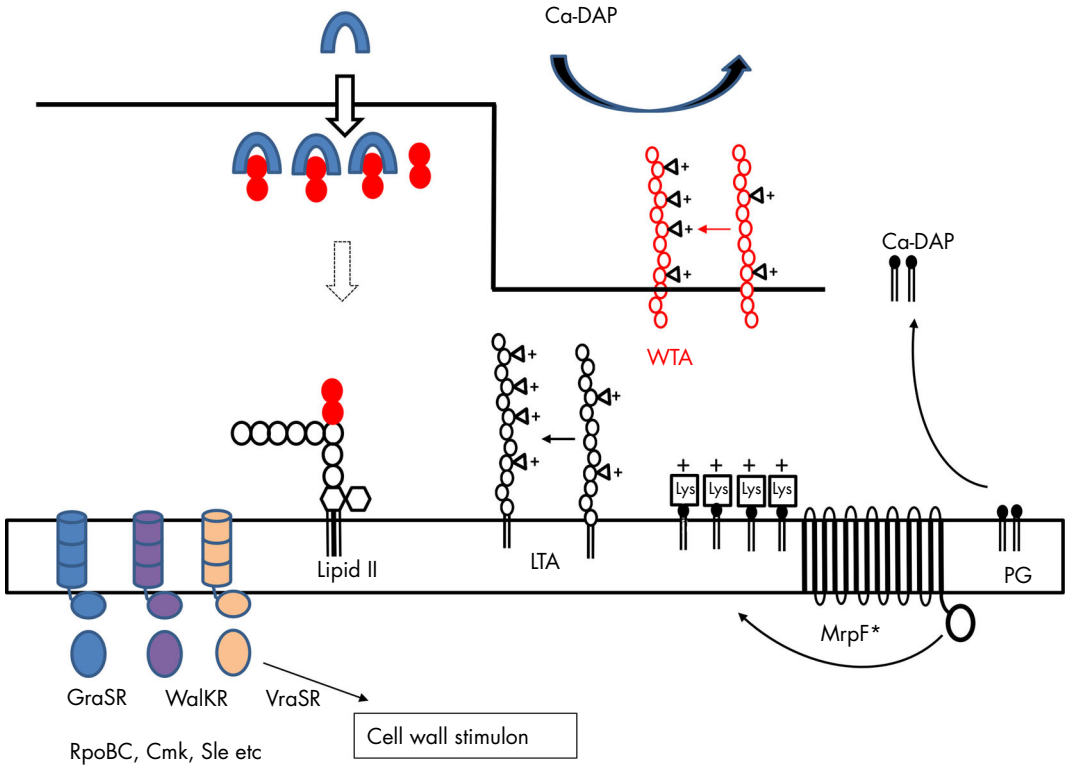

Figure 2. Resistance to vancomycin (VISA) and daptomycin. The membrane-associated MprF protein controls the level of lysyl-phosphatidyl glycerol (Lys-PG) by MrpF* mutants. Daptomycin (Ca-DAP) can be sequestered by free PG and can be repelled by the increased positive charge associated with D-alanylation (triangles) of WTA and LTA, red and black circles representing poly-ribitol and poly-glycerol, respectively. VISA strains have a thicker cell wall with increased levels of uncross-linked PG (red circles, D-Ala) providing false targets and reducing diffusion of the antibiotic (blue half circle). Some frequently altered TCST systems and cytoplasmic genes that occur in VISA strains are indicated.

which controls addition of the secondary wall polymers capsular polysaccharide and wall teichoic acid (WTA) to peptidoglycan led to a strain with features of h-VISA. Two further mutations led to a VISA phenotype, one in *graR* encoding the response regulator of the TCST GraSR and the other in the RNA polymerase B subunit that functions globally by slowing growth rate and reducing autolysis. Although phenotypically VISA, the characteristic increased cell wall thickness was only induced on exposure to vancomycin. Two further mutations in *fdh2* and *sle1* of Mu50 resulted in a reduction in murein hydrolyase activity of the Sle1 protein which in turn reduced autolytic activity. This created a strain that was phenotypically indistinguishable from Mu50.

An earlier study with the h-VISA strain Mu3 where 45 independent single step VISA mutants were selected and analysed by WGS revealed that mutations in many different genes can lead to the same phenotype (Matsuo *et al.* 2013). The fact that so many genes were involved explains the high mutation frequency of the VISA subpopulation in an h-VISA culture. Several mutated genes had been identified in earlier studies (e.g. *rpoB, rpoC* and *walK*) while previously unrecognised mutations in *cmk* affecting cytidylate kinase were found in 6/45 isolates. Similar *cmk* mutations were subsequently found in clinical VISA strains. Reversal of the *cmk* mutation to wild type by allelic exchange converted the VISA strain back to h-VISA. This mutation likely results in an increase in cellular UTP which possibly facilitates an increase in the peptidoglycan precursor UDP-GlcNac.

Another detailed study focussing on CC8 ST239 clinical VSSA-VISA pairs in Australia identified mutations affecting the essential TCST system WalKR as being crucial in the VISA phenotype in that genetic background (Howden, Peleg and Stinear 2014). Strains were analysed by WGS and the *walKR* mutations were analysed genetically by bidirectional allelic exchange. *walKR* mutations were found in 6/8 VISA strains from different genetic backgrounds. It is unclear why *walKR* mutations feature so widely in these backgrounds but rarely in VISA strains isolated from Mu3. The *walKR* mutations affect different functional domains of the proteins and likely result in reduced activity. Transcriptional profiling revealed reductions in autolysin *atl* gene expression and changes in expression of genes involved in central metabolism. One possible consequence is increased pyrimidine synthesis resulting in increased availability of UDP-GlcNac.

Daptomycin

Daptomycin (Dap) is a cyclic peptide antibiotic with a decanoyl fatty acid side chain. It is active against a variety of Gram-positive bacteria and is licenced to treat *S. aureus* bacteraemia and endocarditis. Daptomycin is now a mainstay of anti-MRSA therapy (Arbeit *et al.* 2004). The native molecule is anionic. Calcium is absolutely required for the anti-bacterial activity of the drug and forms a complex here called Ca-DAP (Arbeit *et al.* 2004; Bayer, Schneider and Sahl 2013; Miller, Bayer and Arias 2016). The Ca-Dap complex behaves as a cationic peptide in its interaction with the cell. First, Ca-Dap oligomerises to form micelles which penetrate the cell wall and insert into the cytoplasmic membrane by binding to negatively charged phosphatidylglycerol (PG) head groups. This induces strain into the lipid bilayer which results in depolarisation, permeabilisation and leakage of ions, notably K^+, and ultimately cell death.

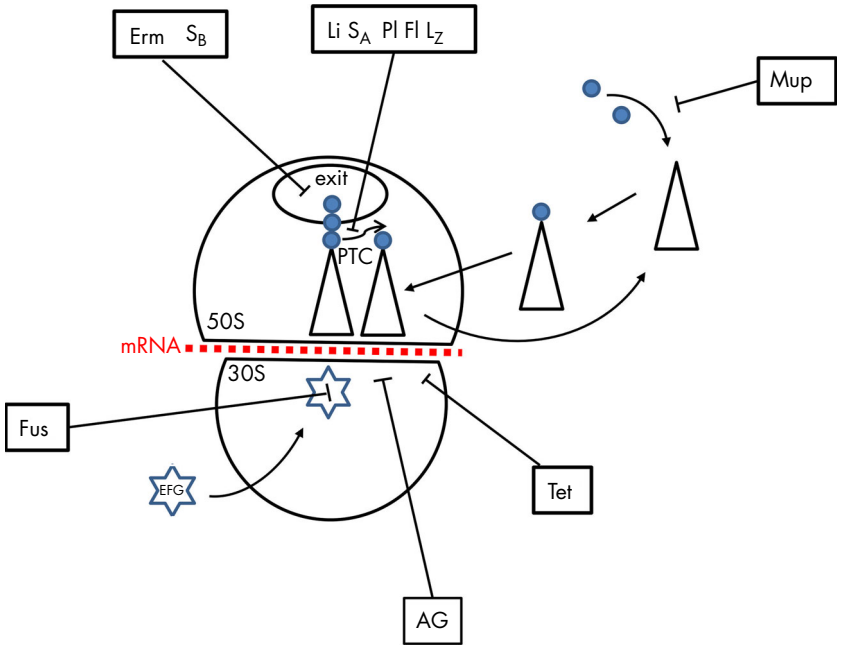

Figure 3. Antibiotics that inhibit protein biosynthesis. Drugs that bind the 50S subunit either block the polypeptide exit tunnel bind by binding at overlapping sites (Erm, erythromycin and S_B, streptogramin B) or interfering with amino acyl tRNA binding at the PTC (Li, lincomycin; S_A, streptogramin A; Pl, pleuromutilin; Fl, florphenicol; Lz, linezolid). Tet binds to the 30S subunit close to the decoding centre and causes aa-tRNA (amino acids, blue circles; tRNA, triangles) to dissociate from the A site. Aminoglycosides (AG) cause misreading of mRNA. Fusidic acid (Fus) binds EF-G (star) and blocks ribosome translocation following peptide bond formation and transfer of peptidyl tRNA from the P site to aa-tRNA at the A site. Mupirocin (Mup) inhibits isoleucyl tRNA synthetase.

Daptomycin resistance (Dap^r) results from mutations in genes that activate the defences of the bacterium against damage to the cell envelope including host cationic antimicrobial peptides (Bayer, Schneider and Sahl 2013; Miller, Bayer and Arias 2016). Resistance arises during prolonged therapy of infections that involve a high density of bacteria, such as endocarditis. Prior treatment of the patient with vancomycin is associated with a more rapid in-therapy development of Dap^r because of overlapping resistance mechanisms (see below and Fig. 2). In essence, to resist daptomycin the bacteria must prevent the drug from reaching the cytoplasmic membrane and/or interfere with penetration of the drug into the membrane. Several mutations occur in different genes which can result the MIC passing the susceptibility breakpoint.

Cytoplasmic membrane changes

If susceptible cells are subjected to prolonged exposure to the drug in increasing concentrations during *in vitro* passage, the most usual first mutational changes occur in the multiple peptide resistance factor (*mrpF*) gene (Bayer, Schneider and Sahl 2013). The MrpF protein is an integral membrane protein that adds a positively charged lysine residue to PG forming lysyl-phosphatidylglycerol (L-PG) (Ernst *et al.* 2009; Ernst and Peschel 2011) (Fig. 2). This is synthesised in the cytosol and is then translocated to the outer face of the membrane. The *mrpF* mutations associated with Dap^r occur in hotspots. They result in gain-of-function changes in the protein which increase the level of L-PG in the membrane. This increases the charge of the outer face of the membrane which repels the Ca-Dap as well as reducing the amount of negatively charged PG needed for drug binding to initiate membrane damage.

Cardiolipin may have a protective effect against Dap. Dap^r is sometimes associated with mutations in cardiolipin synthase which might change the ratio of PG to cardiolipin. It has also been noted that changes to the fluidity of the membrane and increased rigidity due to higher levels of staphyloxanthin pigment can contribute to a reduction in Ca-Dap binding. This has been discussed in detail by Bayer, Schneider and Sahl (2013).

Mutants of *S. aureus* that are defective in the global regulator Agr often emerge during invasive infections. Agr mutants are less susceptible to daptomycin because phospholipids are released that bind to and neutralise the antibiotic before it reaches its target in the membrane (Pader *et al.* 2016). Much less lipid is released by Agr$^+$ organisms. In addition, phenol soluble modulins that are secreted exclusively by Agr$^+$ organisms block the phospholipid–daptomycin interaction.

MRSA strains that have acquired resistance to daptomycin paradoxically become sensitive to β-lactam antibiotics. Indeed, combinations of daptomycin and β-lactams act synergistically on MRSA (the so-called see-saw effect) and have been used successfully to treat persistent infections caused by Dap^r MRSA (Dhand *et al.* 2011; Dhand and Sakoulas 2014; Sakoulas *et al.* 2014). The molecular basis of β-lactam sensitivity in Dap^r MRSA is associated with *mrpF* gain-of-function mutations which increase the level of L-PG in the membrane at the expense of the substrate for anchoring the lipoprotein chaperone PrsA on the outer face of the cytoplasmic membrane (Renzoni *et al.* 2016) (Fig. 1). PrsA is needed for correct localisation and stability of PBP2a. The lack of PBP2a despite normal levels of its mRNA explains how cells become sensitive to β-lactams that target PBP2a. β-Lactam antibiotics such as imipenem that target PBP1 have a more pronounced synergistic effect than those that target PBP2, PBP3 or PB4. Exposure to the combination increased *pbpA* gene (encoding PBP1) transcription and resulted in a more rapid bactericidal activity (Berti *et al.* 2015).

Relationship between VISA and daptomycin

There is overlap in the pathways leading to the VISA and Dap^r phenotypes. Indeed, a survey of VISA strains showed than many had MICs to Dap that exceeded the susceptibility breakpoint

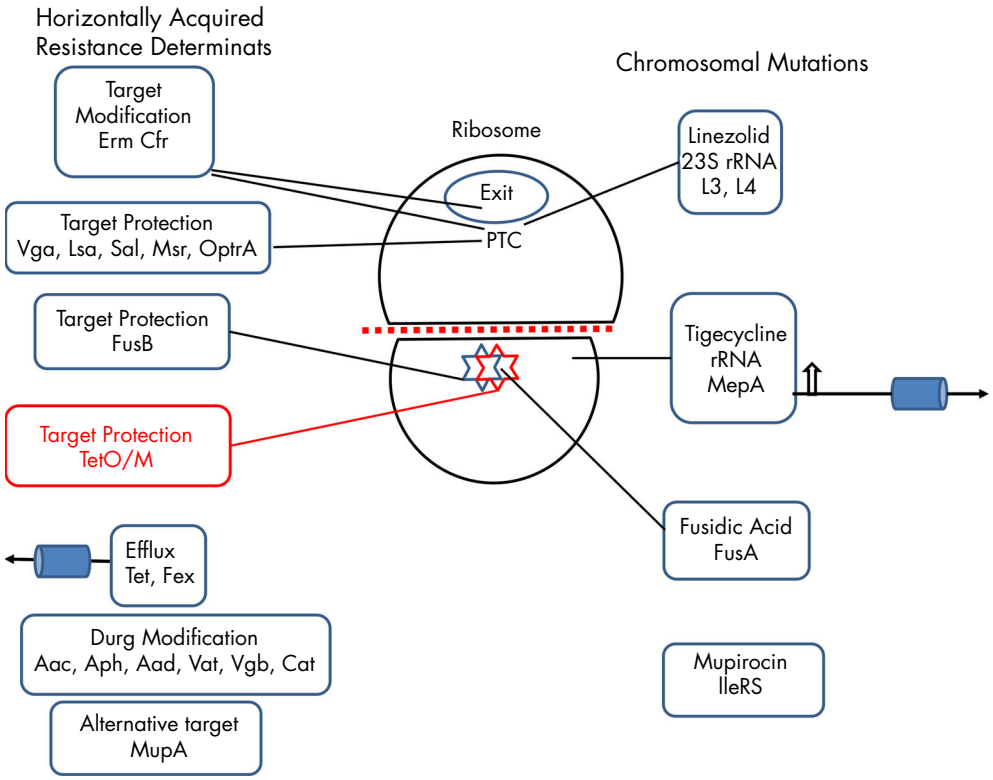

Figure 4. Summary of mechanisms of resistance to antibiotics that target protein biosynthesis. Resistance mechanisms are organised according to whether they have been either acquired by horizontal transfer or by mutations in chromosomal genes. The resistance determinants are described in the text. TetO/M is an EF-G-like molecule that displaces Tet from its ribosomal binding site. FusB binds to EF-G displacing bound drug allowing translocation to occur.

even though they had not been exposed to the drug (Allington and Rivey 2001; Howden *et al.* 2010, 2011). Mutations in genes that contribute to VISA such as *walKR*, *vraSR*, *rpoC* and *dtl* also occur in Dap strains. Thus changes that cause an increase in cell wall thickness and increased positive charge needed for the VISA phenotype will reduce accessibility to Ca-Dap as well as vancomycin.

ANTIBIOTICS THAT INHIBIT PROTEIN SYNTHESIS AT THE 30S SUBUNIT

Many different antibiotics interfere with protein biosynthesis by binding to the ribosome or by inhibiting cytoplasmic proteins that contribute to the process of translation. The binding sites of antibiotics that target the ribosome cluster together at two major sites, one in the 50S subunit around the peptidyl transferase centre (PTC) and the polypeptide exit tunnel, and the other on the 30S subunit at the A site where incoming aminoacyl-tRNA binds (Fig. 3). Only those drugs that are used to combat *Staphylococcus aureus* infections in humans or farm animals will be considered here. X-ray crystallography of ribosomes in complex with antibiotics has revealed important details of drug binding sites and has provided mechanistic explanations for their inhibitory action (Wilson 2009, 2014; Arenz and Wilson 2016; Walsh 2016). Studies have revealed that all antibiotics that target ribosomes bind to rRNA (16S or 23S). Only one class (oxazolidinones) are synthetic molecules. Of the drugs that target the 30S subunit, only the tetracyclines and the aminoglycosides gentamicin and neomycin are clinically relevant for treating staphylococcal infections. Of the molecules that bind the 50S subunit oxazolidinones, pleuromutilins, macrolides, lincosamides and streptogramins are used clinically or in veterinary medicine. Cytoplasmic protein targets are elongation factor G (fusidic acid inhibits translocation) and isoleucyl tRNA synthetase (mupirocin inhibits charging of Ile-tRNA; Thomas *et al.* 2010). Resistance mechanisms are summarised in Fig. 4

Tetracyclines

Tetracyclines (Tet) have been widely used for many decades to treat a variety of infections. A third-generation semisynthetic derivative tigecycline was approved in 2005 for treating infections caused by multidrug-resistant pathogens including MRSA. Tigecycline is considerably more potent than tetracycline and is able to function in bacteria expressing resistance to tetracycline.

Tetracyclines bind to the 30S subunit close to the site where the codon in mRNA is recognised by the anticodon in incoming amino acyl (aa) tRNA (Wilson 2009; Nguyen *et al.* 2014). A complex between Tet and Mg^{2+} forms bonds with six different residues in the 16S rRNA. Stable binding of aa-tRNA is disrupted and it dissociates.

Tigecycline has two substitutions on ring D of the Tet nucleus. The first is present in the second-generation molecule minocycline. In addition, it has a bulky butylglycylamido substitution at position C9 on ring 4 that is responsible for increased potency and along with the substitution in minocycline which renders the molecule impervious to existing Tet resistance mechanisms. The enhanced potency of tigecycline is due to a 10- to 100-fold higher affinity for its binding site on the 30S subunit which results 10- to 30-fold reductions in IC_{50} in *in vitro* translation experiments (Nguyen *et al.* 2014). Crystallisation showed that tigecyline binds to the same site as Tet with the C9

side chain substitution forming additional bonds and increasing the steric overlap with the anticodon loop in aa-tRNA (Jenner *et al.* 2013; Nguyen *et al.* 2014).

Resistance to tetracyclines: efflux

In staphylococci, two related Tet efflux pumps with 14 transmembrane helices called TetA(K) and TetA(L) have been described. TetK is encoded by the small multicopy plasmid pT181 and is integrated within the chromosomal SCCmecIII cassette of MRSA strains (Jensen and Lyon 2009). They are both members of the major facilitator superfamily (MFS) transporters with 14 transmembrane domains (the majority of MFS transporters including the archetypal TetA protein of Gram-negative bacteria have 12) (Chopra and Roberts 2001). The Tet efflux proteins exchange a proton for a tetracycline molecule against a concentration gradient (Piddock 2006). The molecular basis of efflux has been deduced by molecular modelling based of the structures of the YajR or the LacY transporters of *Escherichia coli* (Nguyen *et al.* 2014). The bulky substitutions on ring D of tigecycline prevent the molecule from gaining access to drug binding site in the efflux protein with the result that bacteria expressing TetK or TetL confer little or no resistance (Testa *et al.* 1993; Chopra and Roberts 2001).

Resistance to tetracyclines: ribosomal protection

The TetO/M determinants are typically encoded on chromosomally located conjugative transposons such as Tn*916* and Tn*1545* (Jensen and Lyon 2009). The resistance gene encodes a protein with GTPase activity which has significant similarity to EF-G (Donhofer *et al.* 2012). However, the TetO/M GTPase cannot function as an elongation factor. The TetO/M protein binds to the EF-G binding site on the ribosome in the post-translocation state and dislodges bound Tet from the A site (Connell *et al.* 2003a,b). The high efficiency of this mechanism allows translation to continue in the presence of otherwise inhibitory Tet concentrations. The TetO/M determinant has little effect on the efficacy of tigecycline.

Experimental evolution and potential to develop resistance to tigecycline

There have been very few reports of tigecycline resistance in clinical isolates of *S. aureus*. This is likely due to controlled drug usage but prolonged exposure will undoubtedly result in clinically significant resistance emerging. Selection of tigecyline resistance in *S. aureus* as a result of serial passage of bacterial cultures in increasing drug concentrations has been reported. Exposing MRSA strains N315 and Mu3 resulted in mutants with 16- to 32-fold increased MICs. In both strains, mutations in a repressor gene resulted in increased transcription of a gene encoding a previously uncharacterised multidrug and toxin extrusion family transporter called MepA (McAleese *et al.* 2005). Strain Mu3 expresses TetM resistance. Drug exposure also resulted in a mutant with increased *tetM* transcription but this did not alter the MIC to tigecycline.

Another study focussed on resistance due to mutations in the *rpsJ* gene that encodes ribosomal protein S10 (Beabout *et al.* 2015). Mutants were selected in several different *S. aureus* strains including MRSA131. The mechanistic explanation for resistance is unclear but most likely results from a conformational change in the tigecycline binding site that reduces the drug's access. What is unclear is why two different studies resulted in mutations in different loci. Would prolonged selection result in mutations in both loci? It is also not clear if either the tetracycline efflux pumps or the ribosomal protection proteins can be altered by mutations to generate resistance to tigecycline.

Aminoglycosides

The aminoglycoside neomycin is used topically to prevent or treat skin infections and in nasal ointments as an alternative to mupirocin for decolonisation of carriers. Gentamicin was introduced in the 1970s to combat serious nosocomial infections caused by *S. aureus* but its usage was compromised by the emergence of high-level resistance encoded by mobile genetic elements (Jensen and Lyon 2009). The aminoglycosides are the only ribosome targeting antibiotics that are bactericidal. This is due to their unique mechanism of action in causing misreading during translation. The error rate in translation is increased from <1 in 1000 to around 1 in 100 so that every average sized protein has several incorrect amino acids (Walsh 2016). It was proposed that the lethal event stems from faulty membrane proteins causing membrane damage from which cells cannot recover (Davis, Chen and Tai 1986).

There are several aminoglycoside binding sites close to the decoding centre (Wilson 2009; Wilson 2014). Normally only the correct codon–anticodon pairing is recognised and the bound tRNA is stabilised by two unpaired 16S rRNA bases (A1492 and A1493) 'flipping' their orientation. When an aminoglycoside is bound, non-cognate pairing is tolerated, particularly at base 1 of the codon such that codon–anticodon pairings with one mismatch occur, and the flipping of the unpaired bases triggered despite incorrect pairings.

Aminoglycoside modification

Resistance to aminoglycosides in clinical strains of *S. aureus* is due to acquisition of cytoplasmic aminoglycoside modifying enzymes encoded by mobile genetic elements (Jensen and Lyon 2009). Recent reviews describe the diversity of aminoglycoside modifying enzymes (Wright 1999; Ramirez and Tolmasky 2010). Drug modification prevents ribosome binding. Gentamicin and neomycin resistance are conferred by a bifunctional acetyltransferase-phosphotransferase (*aacA-aphD*) encoded by Tn*4001* while neomycin resistance is due to a phosphotransferase (*aphA*) encoded by Tn5405) or an adenyltransferase (*aadD*) specified by plasmid pUB110. The plasmid is integrated within the SCCmecII cassette that occurs in some MRSA strains.

ANTIBIOTICS THAT INHIBIT PROTEIN BIOSYNTHESIS AT THE 50S SUBUNIT

Several different classes of antibiotic bind close to or at the PTC in the 50S ribosome subunit (Wilson 2009, 2014; Arenz and Wilson 2016; Walsh 2016). Some molecules block the aminoacyl end of aa-tRNA or peptidyl-tRNA and prevent peptide bond formation (Fig. 3). Others block the polypeptide exit tunnel and prevent elongation of the nascent polypeptide chain. Many of the antibiotic binding sites overlap and there are similarities in the drug's mechanisms of inhibition. Linezolid, florfenicol, clindamycin, pleuromutilins, streptogramins and macrolides are used to combat staphylococcal infections in man and animals and will be discussed together. The review of resistance mechanisms will focus on linezolid and Synercid, the two drugs most relevant to combating MRSA. Because several resistance mechanisms inhibit the action of more than one different drug class, these will be discussed after the properties of the each of antibiotics have been described.

Linezolid

Linezolid is an oxazolidinone drug that was approved in 2000 for difficult-to-treat nosocomial infections caused by MRSA (Brickner *et al.* 2008). It is noteworthy that linezolid is the only completely synthetic antibiotic to act at the ribosome and it is the first new chemical class of antibiotic to be introduced in the 20 years between 1980 and 2000. The binding site for linezolid is in the PTC. It overlaps the binding site for chloramphenicol and clindamycin (Fig. 3) and interferes with amino acyl moiety of aa-tRNA stopping peptidyl transferase and peptide bond formation (Wilson 2009, 2014; Long and Vester 2012). A second generation oxazolidinone, tedizolid, which has 4- to 6-fold enhanced potency, was approved in 2014 for treating SSSTIs under the GAIN act (Locke *et al.* 2014; Crotty *et al.* 2016). It binds to the same site but there are more contacts between the drug and the target which increases binding affinity.

Chloramphenicol and florfenciol

The binding site for linezolid closely overlaps that of chloramphenicol and presumably compounds with related structures (Wilson 2009, 2014). Chloramphenicol is only used topically to treat conjunctivitis, but a fluorinated derivative of thiamphenicol called florfenicol is used in veterinary medicine (Schwarz *et al.* 2016). Presumably florfenicol has the same mechanism of action as chloramphenicol by interfering with the aminoacyl end of aa-tRNA and inhibiting peptidyltransferase.

Lincosamides and pleuromutilins

Lincomycin and clindamycin have important therapeutic roles in infections caused by methicillin-sensitive *S. aureus* (MSSA) and in individuals with penicillin hypersensitivity (Rayner and Munckhof 2005). They are also widely used in veterinary medicine. Another lincosamide pirlimycin has verterinary usage (Schwarz *et al.* 2016). Retapamulin is a pleuromutilin that is indicated as a topical antibiotic to treat skin infections caused by *S. aureus* and *Streptococcus pyogenes*. It has been tested as a nasal decolonisation agent and has potential for topical usage to reduce the *S. aureus* burden in atopic dermatitis. Other pleuromutilins are used in veterinary medicine (Schwarz *et al.* 2016). The binding site for retapamulin overlaps both the aa-tRNA and the peptidyl-tRNA binding sites in the PTC in a similar fashion to clindamycin and streptogramin A (Fig. 3) (Wilson 2009, 2014; Walsh 2016).

Macrolides

Erythromycin is a macrolide that blocks the polypeptide exit tunnel adjacent to the PTC (Fig. 3). Its binding site partially overlaps that of the quinupristin B component of Synercid (see below) (Wilson 2009, 2014). Today macrolides are not widely used to combat staphylococcal infections in the developed world but do have a role in MSSA infections (Rayner and Munckhof 2005). The semisynthetic macrolides clarithromycin, azithromycin and telithromycin are used clinically to treat infections caused by bacteria other than *S. aureus*. Thus, commensal staphylococci in humans will be exposed to macrolides and this may contribute to erythromycin resistance being commonly encountered in clinical isolates.

Streptogramins

Pristinamycin is a streptogramin antibiotic that has been used in Europe since the 1970s to treat *S. aureus* infections. It is composed of two molecules PIA and PIIA which act synergistically. Synercid is composed of semisynthetic derivatives of the pristinamycin molecules called dalfopristin A (derived from PIIA) and quinupristin B (derived from PIA). Its improved solubility allows it to be administered intravenously. It was introduced in 1999 to treat infections caused by MRSA (Allington and Rivey 2001). Another streptogramin antibiotic virginiamycin is used as a feed additive for intensively reared farm animals in the USA—it has been banned in Europe (Hershberger *et al.* 2004).

Dalfopristin A binds at the PTC where it interferes with binding of aa-tRNA and inhibits peptide bond formation in a similar fashion to clindamycin (Fig. 3) (Wilson 2009, 2014; Walsh 2016). Quinupristin B binds next to its partner at the beginning of the polypeptide exit tunnel where it blocks short peptidyl tRNA chains entering the tunnel at the beginning of polypeptide translation. This causes release of peptidyl tRNA. Dalfopristin A acts synergistically with quinupristin B by altering the conformation of rRNA and creating an additional H bond for the B component allowing it to bind more tightly.

Mechanisms of resistance

Resistance to streptogramins

Although Synercid has been available since 1999 to combat MRSA, there are few reports of clinically significant resistance. However, a number of different horizontally acquired resistance determinants affect either streptogramin A or streptogramin B molecules but no single determinant can compromise both (Fig. 4, Table 1). Resistance to pristinamycin and virginiamycin was first reported in France and involved combinations of resistance determinants (Allignet and El Solh 1999; Haroche *et al.* 2003). Faecal enterococci from intensively reared animals in the USA also harbour streptogramin resistance determinants (Hershberger *et al.* 2004) which have potential to transfer into *S. aureus*. There have been reports recently of resistance to Synercid in livestock-associated *S. aureus* from ST9 in China (Yu *et al.* 2014) and ST398 in Europe (Argudin *et al.* 2011), and ST22 MRSA infecting humans (Shore *et al.* 2016).

The archetypal ribosomal target modification mechanism MLS_B (macrolide lincosamide streptogramin B) is a S-adenyl methionine-dependent methyltransferase that dimethylates the amino group of A2058 of 23S rRNA (Weisblum 1995). This blocks antibiotics in the peptide exit tunnel including quinupristin B. However, the dalfopristin A component is still be able to bind and a second mechanism would be required to elevate the MIC sufficiently to confer clinical resistance. Several orthologues of Erm methylases occur in *S. aureus* and other Gram-positive bacteria (Roberts *et al.* 1999).

Several mechanisms that affect pristinamycins have the potential to compromise the effectiveness of Synercid. Determinants also occur in animal strains where virginiamycin, now banned in Europe, is used as a feed additive. Virginiamycin B lyases (Vgb) linearises the cyclic type B molecule causing its inactivation (Mukhtar *et al.* 2001). The virginiamycin acetyltransferases (Vat) O-acetylate the hydroxyl group at position O8 of streptogramin A molecules (Allignet *et al.* 1993).

Resistance to pristinamycins is also conferred by a family of proteins called ABC-F that have two ATP binding cassette (ABC) domains (Sharkey, Edwards and O'Neill 2016; Wilson 2016). This group comprises (i) the lincosamide, streptogramin A (LS_A) and lincosamide, streptogramin A, pleuromutilin (LS_AP) determinants Vga, Lsa and Sal; (ii) the macrolide, streptogramin B (MS_B) determinant Msr; and (iii) the oxazolidinone, phenicol determinant OptrA (see below). They were originally thought to provide the driving force for a drug efflux mechanism by

Table 1. Summary of resistance mechanisms to drugs binding to 50S subunit acquired by horizontal gene transfer or by mutation.

			Drug binding order in exit tunnel and PTC						
Mechanism		Spectrum	Macrolide	Streptogramin B	Lincosamide	Streptogramin A	Pleuromutilin	Florfenicol	Linezolid
rRNA methyltransferase	Erm	MLS_B	R	R	R				
rRNA methyltransferase	Cfr	$PhLOPS_A$			R	R	R	R	R
Ribosomal protection ABC-F	Vga	LS_A			R	R			
	Lsa, Sal	LS_AP			R	R	R		
	Msr	MS_B	R	R					
	OptrA	OPh						R	R
Efflux	Fex	Ph						R	
Drug modification	Vgb	S_B		R					
Drug modification	Vat	S_A				R			
Target site mutation: 23SrRNA, ribosomal proteins L3 L4									R
Target site modification Increased expression RluC: A2504 pseudouridylation Loss of function: RlmN A2503 methyltransferase									R

associating with unknown transmembrane proteins. Decreased drug accumulation occurs in strains harbouring these determinants. Upon closer examination, this can be explained if the drugs were prevented from binding to the ribosome which would inhibit the 'sink' effect of multiple intracellular binding sites and reduce drug uptake.

It is now clear that ABC-F resistance determinants confer resistance by protecting the ribosome from the drug. This was convincingly demonstrated by showing that Vga and Lsa allowed *in vitro* translation to continue in the presence of inhibitors (Sharkey, Edwards and O'Neill 2016). It is proposed that the protein binds close to the PTC altering its conformation and protects the target either by displacing drug molecules that have bound or by preventing binding. Molecular details will depend on structural analysis of the ABC-F proteins in complex with the ribosome.

In conclusion, although resistance to Synercid in clinical isolates is rarely reported there is potential for the determinants of resistance to quinupristin and dalfopristin to combine and confer complete resistance (Table 1). Multidrug-resistant livestock-associated strains can colonise and cause infections in humans who come into contact with farm animals. Resistance can also be transferred into HA- and CA-MRSA strains.

Resistance to linezolid by Cfr rRNA methyltransferase

Resistance to linezolid in clinical isolates is still quite rare but there have been several reports recently of acquisition of plasmids that encode the Cfr resistance determinant by MRSA, including the pandemic ST22-MRSA-IV clone (Shore *et al.* 2016). Cfr stands for chloramphenicol and florfenicol resistance, the phenotypes with which the determinant was associated when first detected (Long *et al.* 2006). Originally, Cfr was restricted to staphylococci of animal origin where it was probably selected for by usage of florfenicol in veterinary medicine. The resistance spectrum has expanded to $PhLOPS_A$ resistance (phenicol, lincosamide, oxazolidinone, pleuromutilin, streptogramin A) (Table 1). The Cfr protein is a methyltransferase that methylates C-8 of A2503 in 23S rRNA (Kehrenberg *et al.* 2005). This base is in close proximity to the overlapping binding sites for these drugs and its methylation prevents the molecules from binding. It appears that a resistance mechanism that could compromise important treatments for serious human infections was originally selected in staphylococci of animal origin to combat florfenicol, a drug that has never been used in human medicine. To emphasise this point Cfr resistance is often accompanied by the florfenicol exporter Fex, a member of the 14 transmembrane segment MFS (see resistance to tetracycline) (Kehrenberg and Schwarz 2006; Kehrenberg *et al.* 2006; Shore *et al.* 2016). Chloramphenicol resistance once commonly found in *S. aureus* is due to enzymatic modification by chloramphenicol acetyltransferase (Foster 1983).

Resistance to linezolid conferred by OptrA

Resistance to linezolid is also specified by the oxazolidinine and phenicol transferable resistance determinant OptrA. This is widely encountered in enterococci from human and animal sources in China (Huang *et al.* 2017). It is also found in coagulase-negative staphylococci but has not yet been reported in *S. aureus* although this is likely to only be a matter of time. Unlike Cfr, OptrA specifies resistance both to linezolid and to tidezolid. OptrA is phylogentically related to the two ATP binding site ABC-F proteins Vga, Lsa and Mcr (Wang *et al.* 2015) and almost certainly promotes resistance by displacing the drug and protecting the target, and not due to efflux as is often assumed (Sharkey, Edwards and O'Neill 2016). Like Cfr, OptrA was likely selected due to extensive usage of florfenicol in intensive animal farming in China.

Resistance to linezolid: Ribosomal mutants

Mutations in 23S rRNA genes that alter bases at or close to the linezolid binding site occur in several different bacteria (Long and Vester 2012). The relationship between mutations and resistance is complicated by multiple rRNA operons and the

possibility of the population of ribosomes being heterogeneous, with some containing mutated 23S rRNA and others the wild type. The resistance pattern encountered is dependent on the organism. Often the level of resistance is due to bases that are directly implicated in drug binding or are located close to the binding site

In staphylococci, the G2576U mutation is important. It has been reported in *S. aureus, S. epidermidis* and *S. haemolyticus*. There is a clear correlation between the MIC to linezolid and the number of mutated rRNA operons. U2500A and G2447U mutations have also been seen in clinical isolates of *S. aureus* (Long and Vester 2012; Locke *et al.* 2014).

There is a cost to fitness for carrying multiple copies of the G2576U mutation (Long and Vester 2012). Having one copy involves minimal cost but a progressive loss of fitness is incurred with increasing copies. It seems that prolonged exposure to the antibiotic selects for variants where one mutated copy of the rRNA gene provides the template for gene conversion to alter additional copies. Thus, there is a balance between fitness and resistance but in an immunocompromised patient the less robust but more highly resistant mutant is still able to cause infection.

Changes in expression of housekeeping enzymes involved in rRNA modifications have also been encountered in linezolid-resistant clinical isolates (Long and Vester 2012). Upregulation of an enzyme that causes pseudouridylation of U2504 confers resistance to several antibiotics that bind the PTC, including linezolid. Loss of activity of the RlmN methyltransferase targeting C-2 of A2503, the same base modified by the horizontally acquired Cfr methyltransferase, is associated with resistance (Long and Vester 2012).

Mutations causing amino acid substitutions in ribosomal proteins L3 and L4 have also been associated with linezolid resistance (Long and Vester 2012). However, it is less clear whether such variants can alone be responsible for clinically significant resistance because they usually occur in strains that have mutations affecting 23S rRNA. Both proteins have loops that extend close to the binding site for linezolid so it is feasible that changes contribute indirectly to its conformation. Mutations in the L3 and L4 protein genes should be isolated by allelic exchange to investigate their role directly.

The newer oxazolidinone tedilozid is active against strains expressing Cfr resistance because its binding is not occluded by methylation of A2503 (Locke *et al.* 2014). It is also active against linezolid-resistant mutants with chromosomal-encoded resistance affecting 23SrRNA and ribosomal proteins L3 and L4.

OTHER ANTIBIOTICS THAT INHIBIT PROTEIN SYNTHESIS

Fusidic acid

Fusidic acid is commonly used topically to treat *S. aureus* skin infections and to reduce skin colonisation in eczema patients. However, resistance rapidly emerges in a single step by mutation when the drug is used alone. A combination with rifampicin and fusidic acid is effective in combating difficult-to-treat MRSA infections and is a useful alternative to linezolid. It is worrying that widespread topical usage could compromise this activity (Howden and Grayson 2006).

Fusidic acid targets elongation factor G, a cytoplasmic protein which binds to the ribosome after peptide bond formation and transfer of the growing peptide chain to the aa-tRNA at the A site (Bodley *et al.* 1969; Fernandes 2016). Normally, the EF-G GTPase promotes translocation of the ribosome such that the peptidyl-tRNA returns to the P site and the deacylated tRNA is expelled from the E site. EF-G then detaches allowing the next round of translation to begin with an aa-tRNA molecule entering the vacant A site. The presence of fusidic acid prevents release of EF-G after the translocation event and blocks the next round of aa-tRNA binding (Bodley *et al.* 1969).

Resistance to fusidic acid

There has been a recent increase in resistance to fusidic acid, mainly involving plasmids expressing the FusB and FusC mechanisms (O'Neill and Chopra 2006). While *fusB* gene is plasmid encoded *fusC* is located within SCC elements (SCC*fus* and SCC*mec*) and is linked to the *tirS* gene which encodes the TirS protein that inhibits Toll-like receptor signalling and likely contributes to virulence (Askarian *et al.* 2014; Baines *et al.* 2016; Patot *et al.* 2017). Furthermore, *fusC* and *tirS* seem to be co-expressed because *tirS* expression is elevated in subinhibitory concentrations of fusidic acid (Patot *et al.* 2017).

FusA resistance is due to spontaneous chromosomal mutants affecting EF-G that prevent the drug from binding to its target (Nagaev *et al.* 2001; Fernandes 2016). FusB is a small ~25 kDa protein that binds EF-G with 1:1 stoichiometry. It binds to the EF-G fusidic acid complex on the stalled ribosome in the post-translocation state. This triggers a conformational change in EF-G promoting its release and allowing translation to proceed (Cox *et al.* 2012; Fernandes 2016; Tomlinson *et al.* 2016).

Mupirocin

The antibiotic mupirocin is widely used as a topical agent to reduce nasal carriage of MRSA by hospital patients and staff. It is also indicated as a topical agent to treat skin infections. The target for mupirocin is isoleucyl-tRNA synthetase (IleRS, Fig. 3) (Thomas *et al.* 2010). Low-level resistance to mupirocin is due to mutations that cause amino acid substitutions in the target that prevent the molecule from binding efficiently. High-level resistance is due to plasmid-borne MupA determinants that specifies an intrinsically insensitive IleRS enzyme that allows the sensitive target to be bypassed (Fig. 4).

ANTIBIOTICS THAT TARGET NUCLEIC ACIDS BIOSYNTHESIS

Topoisomerases

The fluoroquinolones are the most successful entirely synthetic antimicrobial drug class. The major breakthrough in the development of these drugs was the discovery that fluorination at position C6 enhanced the spectrum and potency of the prototype molecule nalidixic acid (Andriole 2005; Walsh 2016). There have been several generations of fluoroquinolones where molecules with additional substitutions have been introduced which have improved potency and pharmacokinetics.

The molecular targets for the fluoroquinolones in *Staphylococcus aureus* are (i) DNA gyrase that introduces negative supercoils into chromosomal DNA and (ii) topoisomerase IV which promotes chromosome decatenation following replication. Both enzymes are heterotetramers of A and B subunits, GyrA and GyrB in DNA gyrase, ParC and ParE in topoisomerase IV. The enzymes catalyse staggered double-stranded breaks. The 5′ end of each cleaved DNA strand forms a phosphotyrosine bond with the active site tyrosine in the two A subunits. A DNA strand is passed through the break, the gap is resealed and the

phosphodiester bond between the backbone deoxyriboses regenerated (Wang 1996; Walsh 2016). The fluoroquinolones form ternary complexes with Mg^{2+} and the A subunit of the topoisomerases at the intermediate covalent protein–DNA complex stage. This prevents resealing of the cleaved DNA strand which leads to double-stranded DNA breaks that cannot be repaired and results in a rapidly bactericidal effect.

Fluoroquinolones

Resistance to fluoroquinolones in clinical isolates of *S. aureus* involves both mutational changes to the topoisomerases that reduce drug binding efficiency and elevated expression of endogenous efflux pumps (Hooper 2002; Hooper and Jacoby 2015).

Resistance to fluoroquinolones – topoisomerase mutants

The most common types of mutational change result in amino acid substitutions in residues that constitute the drug binding site, the so-called quinolone resistance-determining region (Hooper and Jacoby 2015). In staphylococci, ParC is the topoisomerase with the greatest sensitivity and is therefore the primary target. DNA gyrase is less susceptible and is the secondary target. Staphylococci are very sensitive to fluoroquinolones so in order to develop resistance that exceeds the MIC breakpoint, changes in both enzymes are required. In general, a single amino acid substitution will elevate the MIC by 8- to 16-fold. The requirement for two mutational changes should have inhibited the emergence of resistance in the same way as using a combination of two antibiotics that act at different targets. However, if bacteria are exposed to low (subinibitory) concentrations of antibiotics resistance in the primary target could be selected (Andersson and Hughes 2014) allowing the MIC breakpoint to be achieved by a mutation affecting the second target. Furthermore, subinhibitory concentrations of quinolones induce the SOS response and elevate the mutation rate (Nagel *et al.* 2011).

Resistance to fluoroquinolones – efflux

Clinical isolates that are highly resistant to fluoroquinolones often overexpress chromosomally encoded efflux pumps (Hooper and Jacoby 2015). Three pumps can efflux fluoroquinolones, NorA handles the hydrophilic molecules norfloxacin and ciprofloxacin while NorB and NorC target hydrophobic drugs such as sparfloxacin and moxifloxacin (Yu, Grinius and Hooper 2002; Truong-Bolduc *et al.* 2005; Truong-Bolduc, Strahilevitz and Hooper 2006). The Nor proteins are members of the MFS and are thus related to the Tet efflux pump, an important difference between the two being the wide spectrum of substrates in the former and the very narrow spectrum of the latter. The increase in MIC determined by each transporter is between 4- and 8-fold depending on the affinity of the pump for a particular fluoroquinolone.

The expression of NorA, NorB and NorC is governed by complex interactions between regulatory proteins. The small transcriptional regulator MgrA is centrally involved (Truong-Bolduc *et al.* 2005). It can bind directly to the promoters of the *nor* genes depending on the degree of phosphorylation catalysed by kinase PknB (Truong-Bolduc, Ding and Hooper 2008; Truong-Bolduc and Hooper 2010). For example, phosphorylated MgrA results in reduced binding to the *norA* promoter and increased binding to *norB*.

Expression of transporters is influenced by environmental conditions such as the level of aeration, the pH and iron limitation, conditions that vary according to the site of infection (Chen *et al.* 2006; Truong-Bolduc *et al.* 2011, 2012). It is noteworthy that the increased expression of *norB* occurs in a murine abscess compared to laboratory growth conditions (Ding *et al.* 2008). This implies that susceptibility to fluoroquinolones might vary according to the site of infection.

Folic acid metabolism

Sulphonamides

Sulphanilamide is the active breakdown product of the red dye prontosil rubrum, a substance which had antibacterial activity *in vivo* but not *in vitro* (Lesch 2007). Many chemical variants were synthesised by modifying the N in sulphanilamide. Today, only sulfamethoxazole and sulfadiazine are used clinically, the former in combination with trimethoprim in a formulation called co-trimoxazole (Wormser, Keusch and Heel 1982) (see below) and the latter as the silver-sulfadiazine combination used prophylactically in wound and burns dressings.

Sulphonamides inhibit dihydropteroate synthase (DHPS) which condenses pteroate and p-aminobenzoic acid (pABA) to form dihydropteroate, a precursor of folic acid (Babaoglu *et al.* 2004; Walsh 2016). This is an essential enzyme in prokaryotes which, unlike mammals, synthesise folic acid *de novo*. The sulfonamide competes with pABA at the active site of the enzyme and also acts as an alternative substrate forming a dead-end pteroate-sulfonamide product (Walsh 2016). This shuts off a precursor of folic acid resulting in a slow acting, bacteristatic affect (Skold 2000).

Resistance to sulfamethoxazole (SMX) in clinical isolates results from amino acid substitutions in the chromosomally encoded DHPS which presumably prevent the drug from binding to the enzyme. The only substantive study on sulphonamide resistance in clinical isolates of *S. aureus* was performed two decades ago (Hampele *et al.* 1997). Having established that resistance to SMX was not plasmid encoded, the chromosomal genes encoding DHPS from nine resistant strains were sequenced. Resistance was associated with several different amino acid substitutions in DHPS.

Trimethoprim

The target of the 2,4-diaminopyridine trimethoprim is dihydrofolate reductase (DHFR). This enzyme is essential in both prokaryotes and eukaryotes but the bacterial enzyme differs sufficiently from the mammalian counterpart for it to be specifically inhibited (Hitchings and Burchall 1965). Indeed, trimethoprim has a 100 000-fold higher IC_{50} for the bacterial enzymes compared to the human enzyme.

The folate biosynthetic pathway delivers the product in the biologically inactive oxidised form dihydrofolate which is converted to tetrahydrofolate by the action of DHFR (Walsh 2016). The most important function of folic acid is to serve as a one carbon donor in the synthesis of deoxythymidylate monophosphate (dTMP) catalysed by thymidylate synthase (Carreras and Santi 1995). This requires N^5 N^{10} methylene tetrahydofolate that is generated by serine transhydroxymethylase, the second enzyme in the three enzyme cycle. Each mole of dTMP synthesised requires 1 mole of the reduced folate which is converted to dihydrofolate. This must be reduced again by DHFR. Thus, DHFR activity is required for the synthesis of DNA and inhibiting the enzyme stops DNA replication.

Trimethoprim is used clinically in combination with sulfamethoxazole in a formulation called co-trimoxazole where the drugs act synergistically (Wormser, Keusch and Heel 1982).

Neither sulfamethoxazole nor trimethoprim is used in monotherapy. In theory, emergence of resistance by mutation in the chromosomal genes encoding DHPS and DHFR would be impeded by using the combination.

The major clinical usage of co-trimoxazole against *S. aureus* is for empiric treatment of SSSTIs (Goldberg and Bishara 2012). It is a second-line drug for treating pulmonary exacerbations in cystic fibrosis patients. It has also been considered for treatment of bacteraemia and endocarditis. A group in Israel published a restrospective cohort study comparing the efficacy of co-trimoxazole with vancomycin and recently published the outcome of a randomised controlled trial where it 'did not reach inferiority' (Paul *et al.* 2015). Co-trimoxazole might have usage as a step-down treatment or where the strain infecting a patient with persistent bacteraemia has developed insensitivity to vancomycin.

Resistance to trimethoprim in clinical isolates occurs either by amino acid substitutions in the chromosomally encoded DHFR or by acquisition by horizontal gene transfer of genes that encode DHFR enzymes that are not susceptible to inhibition and that allow the blockade of the chromosomal DHFR to be bypassed.

The most common change in trimethoprim-resistant DHFR in *S. aureus* is a single amino acid substitution F98Y (Dale *et al.* 1997) in the DfrB resistance phenotype which confers intermediate resistance with an MIC $\leq$ 256 mg ml^{-1}. Three different horizontally acquired DHFRs that specify high-level resistance of $\geq$512 mg ml^{-1} have been reported. The first to be described was DfrA specified by Tn*4001* (Rouch *et al.* 1989). DfrK is found in livestock-associated staphylococci and is rarely encountered in human clinical isolates (Kadlec and Schwarz 2009). Until recently, the DfrG determinant (Sekiguchi *et al.* 2005) was mainly confined to livestock-associated staphylococci. Resistance to co-trimoxazole in Europe is rare (den Heijer *et al.* 2013). However, recent studies report that it is widespread in MSSA and MRSA in sub-Saharan Africa (Nurjadi *et al.* 2014) and in Asia (Nurjadi *et al.* 2015). In one study, 54% of *S. aureus* strains were resistant to trimethoprim and 94% of these strains carried dfrG. Moreover, European travellers returning from these regions and seeking treatment for SSSTIs are frequently infected by strains that are resistant to trimethoprim and carry DfrG. This is very worrying because spread of DfrG will compromise an effective empiric treatment for SSSTIs and may have consequences for the development of new antifolates.

Iclaprim

Iclaprim is a derivative of trimethoprim with a higher affinity for the chromosomally encoded DHFR (Oefner *et al.* 2009). It is >10-fold more potent that trimethoprim and is bactericidal at the MIC. It retains activity against DfrB mutant strains that are resistant to Tmp although the MIC increased from 0.03 to 2 μg ml^{-1}. However, no data were reported for the activity of iclaprim against strains expressing DfrA, DfrK or DfrG. Iclaprim was tested in a phase III trial for treatment of SSSTIs but was refused approval by the FDA in 2009 although it performed equally as well as the then standard-of-care treatment linezolid (Krievins *et al.* 2009). This trial was conducted and approval sought before the GAIN act of 2014 which set to lower the bar for clinical trials and allow easier approval of much needed new antibiotics. A UK-based company Motif Bio acquired the rights to iclaprim in 2014 and have proposed a new development plan with clinical trials for SSSTIs and for hospital-acquired pneumonia (http://www.motifbio.com/iclaprim/).

RNA polymerase

Rifampicin

Rifampicin is a broad spectrum bactericidal antibiotic that specifically targets prokaryotic RNA polymerases. It binds to the B subunit about 12 Å from the active site of the enzyme. It does not prevent RNA polymerase from binding to the promoter and initiating transcription (Campbell *et al.* 2001). However, transcription is blocked at the stage of the first ribonucleotide phosphodiester bond.

Monotherapy with rifampicin is problematic because of the rapid emergence of high-level resistant mutants in a single step. The mutations result in amino acid substitutions in and around the drug binding site which reduce affinity for the target (Aubry-Damon, Soussy and Courvalin 1998; Wichelhaus *et al.* 1999). Clinical usage of rifampicin is mainly confined to treatment of tuberculosis where it is one of the frontline drugs used in prolonged combination therapy.

Rifampicin has been considered for use as an adjunctive agent for treating bacteraemia and endocarditis caused by *S. aureus* where it would be administered in combination with a β-lactam (in MSSA) or glycopeptides (Russell *et al.* 2014). Several reviews of the medical literature indicate that rifampicin shows some promise but there are concerns about toxicity and unexpected drug interactions. The result of a randomised double-blind placebo-controlled trial that began in 2012 with 940 patients in 17 centres in the UK (Thwaites *et al.* 2012) has not as yet been published. This should establish whether rifampicin can still be considered useful to combat *S. aureus* infections.

NEW TARGETS, NEW DRUGS

In this section drugs in pre-clinical development that interfere with novel targets will be reviewed.

Fatty acid biosynthesis

The elongation of fatty acid chains in *S. aureus* is a cyclical pathway catalysed by a four enzyme cycle involving the fatty acid biosynthesis Fab proteins (FabG, FabZ, FabI and FabF). The fatty acid biosynthetic pathway is essential and is an important target for development of novel antimicrobial agents (Campbell and Cronan 2001; Parsons and Rock 2011; Yao and Rock 2016). A compound that was originally discovered by Glaxo Smith Kline targets FabI (Payne *et al.* 2002) is now called Debio1452. It is being developed commercially and entered a phase 2a clinical trial for SSSTIs in 2015 (Flamm *et al.* 2015). The antibiotic platensimycin inhibits FabF but it is unclear if it or other inhibitors of this enzyme will be developed further and progress towards clinical trials (Wang *et al.* 2006). In addition, the widely used biocide triclosan targets FabI (Schweizer 2001) and molecules derived from triclosan are being developed as antibiotics (Park *et al.* 2007).

Debio1452 (previously AFN1252) specifically inhibits the enoyl-acyl carrier protein reductase FabI in the elongation cycle of fatty acid biosynthesis (Payne *et al.* 2002; Kaplan *et al.* 2012; Flamm *et al.* 2015). In order to improve solubility and bioavailability including potential for oral administration, a prodrug of Debio1452 called Debio1450, previously AFN1720, is being developed. It has a very narrow spectrum of activity by specifically targeting staphylococci and has limited activity on other Gram-positive cocci including streptococci and enterococci. Many firmicutes do not have FabI but instead use FabK to reduce enoyl-ACP. One benefit of such a narrow activity spectrum is that there

would be little disturbance of the gut microbiome during treatment (Yao *et al.* 2016).

One concern about the potential clinical usage of AFN1452 is the development of resistant mutations in the chromosomal *fabI* gene (Payne *et al.* 2002). Several single amino acid substitution mutants of FabI have decreased susceptibility to the inhibitor so the potential for usage of the FabI inhibitor in monotherapy is likely to be compromised (Yao and Rock 2016).

Triclosan

Triclosan is a widely used biocide with multiple cellular targets when it is used at high concentrations (Russell 2004). At lower concentrations, it specifically targets FabI resulting in a stable ternary complex with NAD^+ (Heath *et al.* 1999). It is worrying that resistance to triclosan has developed in clinical isolates of *S. aureus*. Mutations that increase transcription of the *fabI* promoter have been reported. Presumably increased FabI protein levels reduce susceptibility by providing more drug target molecules to be inactivated. A significant proportion of triclosan resistance in *S. aureus* is due to *fabI* heterodiploidy (Furi *et al.* 2016). The *fabI* gene of *S. haemolyticus* has been mobilised by insertion sequences and transferred into *S. aureus*. It is unclear if the second FabI protein is less sensitive to triclosan, if it is expressed at high levels to titrate out the inhibitor or if it allows triclosan-resistant FabI variants to be selected without compromising the integrity of the native FabI enzyme. The potential to bypass a blockade of an essential biosynthetic process by horizontal acquisition of a second target also contributes to resistance to trimethoprim and mupirocin (Thomas *et al.* 2010). The development of triclosan resistance by such a process indicates that a similar mechanism could occur if AFN1452/AFN1450 is widely used to treat *S.aureus* infections. Anti-staphylococcal drugs derived from triclosan are in development (Park *et al.* 2007; Escaich *et al.* 2011) but may also be compromised by missence mutations in *fabI* causing resistance (Yao and Rock 2016).

FtsZ and cell division

The FtsZ protein has been investigated as a potential target for antibiotic action because of its essential role in cell division. The monomeric protein has GTPase activity which is utilised during polymerisation to form the Z ring at the mid-cell during the initiation of cell division and serves as a scaffold for recruitment and organisation of the septum (Adams and Errington 2009; Pinho, Kjos and Veening 2013). The FtsZ inhibitor PC190723 prevents cell division by disrupting FtsZ function and causes displacement of the Z ring (Haydon *et al.* 2008; Andreu *et al.* 2010).

Pre-clinical development of the original FtsZ inhibitor PC19073 was hindered by poor pharmacokinetics. Investigation of prodrugs with improved metabolic stability, better pharmacokinetics and *in vivo* efficacy has culminated in TXA707 (Kaul *et al.* 2015) which is currently undergoing pre-clinical commercial development by Taxis Pharmaceuticals. As with the original FtsZ inhibitor spontaneous resistant mutants occur in *S. aureus* populations at a frequency of about 1 in 10^{-8} which could limit clinical usage to drug combinations.

PC190723 acts synergistically with β-lactam antibiotics such as imipenen both *in vitro* and *in vivo* in an infection model with an MRSA strain (Tan *et al.* 2012). The mechanistic basis of the synergistic effect is not entirely clear but results in delocalisation of PBP2 which acts in concert with the MRSA PBP2a to synthesise peptidoglycan in the presence of β-lactams. Furthermore, the rate of mutation to resistance to PC190723 *in vitro* is reduced by a factor of 10. The mutants had reduced virulence and restored β-lactam susceptibility.

A ClpP protease inhibitor kills persisters

A small proportion of a susceptible population of *S. aureus* growing in exponential phase survive exposure to bactericidal antibiotics such as quinolones, aminoglycosides, rifampicin and β-lactams. These cells are only temporarily resistant because they regain sensitivity when re-cultured. Upon a second exposure to the drug, the population shows the same killing phenotype as the original. Thus, in any population of bacterial cells a small proportion are in a transiently insensitive state. The phenomenon of 'persisters' was first described by Bigger in early studies on the effect of penicillin on *S. aureus* (Bigger 1944).

In Gram-negative bacteria, several different toxin–antitoxin modules control entry into the persister state (Lewis 2010). The toxin molecules such as an mRNA endonuclease or the HipA protein kinase that acts on glutamyl aminoacyl tRNA synthetase are expressed constitutively along with a specific antitoxin that normally inhibits activity of the toxins (Page and Peti 2016). Occasionally, the antitoxin fails and the toxic enzyme is activated and stops growth. This is a bacteriostatic effect from which the cells can recover. Clinically important bactericidal antibiotics only act on cells that are actively growing.

In *S. aureus*, the mechanism controlling entry into the persister state is completely different to that of Gram-negative bacteria (Conlon *et al.* 2016). Unlike Gram-negative bacteria, stationary-phase *S. aureus* cells are not susceptible to bactericidal antibiotics. A small proportion of cells in any population of *S. aureus* have entered stationary phase early, a state that is characterised by depletion of ATP. Indeed, by artificially depleting cells of ATP the entire population will enter stationary phase and become persisters. Persister formation is achieved by an as yet uncharacterised mechanism (or mechanisms) but appears not to be a toxin–antitoxin system.

The acylpepsipeptide antibiotic ADEP4 can kill *S. aureus* cells that are in the persister state (Conlon *et al.* 2013). It activates the ClpP protease by dissociating the enzyme from its ATP-dependent chaperone allowing it to degrade intracellular proteins in an uncontrolled fashion.

A novel lipid II inhibitor

Teixobactin binds to the lipid moiety of lipid II and kills *S. aureus* by inhibiting peptidoglycan and WTA biosynthesis (Fig. 1) (Ling *et al.* 2015; Homma *et al.* 2016). Unlike RNA or protein the structure and composition of lipid II cannot be readily altered by point mutations. Teixobactin was discovered by a novel soil enrichment process with the iCHIP which allows previously unculturable soil bacteria to grow in the laboratory and their products to be tested for antibiotic activity. While mutational target site resistance is precluded, it is possible that resistance has developed in competitor soil bacteria, for example by enzymatic drug modification. It would be interesting to investigate the soil resistome (Nesme and Simonet 2015) for such activity.

Teichoic acid biosynthesis

Lipoteichoic acid (LTA) and WTA are cell wall polymers (Fig. 3) that are of fundamental importance to bacterial cell wall integrity. Despite similarities in structure, WTA and LTA are synthesised by different pathways with the former being a ribitol

phosphate polymer while the latter is composed of glycerol phosphate. Mutants defective in either WTA or LTA are defective in growth *in vitro* and *in vivo*. These properties suggest that enzymes involved in biosynthesis and export could be effective drug targets (Pasquina, Santa Maria and Walker 2013; Sewell and Brown 2014). Several inhibitors of WTA biosynthesis have been reported. Targocil inhibits the membrane-bound ABC transporter which exports WTA across the membrane (Swoboda *et al.* 2009). In combination with a β-lactam, a synergistic effect restoring efficacy of the β-lactam towards MRSA was reported (Wang *et al.* 2013). Inhibitors of the LtaS proteins involved in LTA biosynthesis have also been reported (Richter *et al.* 2013).

CONCLUSIONS AND FUTURE PROSPECTS

Since the late 1980s, there has been a void in the discovery of new chemical classes of antimicrobial drugs and their introduction to combat staphylococcal infections, the last being the lipopeptide daptomycin in 1987 (Silver 2011). Many factors have contributed to pharmaceutical companies stopping or curtailing their antibiotic discovery and development programmes. The rapid development of resistance will reduce usage, newer molecules are introduced only for specific indications following expensive clinical trials, the regulatory bar might have been set too high, and the big investment in target-based discovery programmes combined with structural biology did not yield the hoped-for breakthroughs (Zorzet 2014).

It is very clear that *Staphylococcus aureus* along with other bacteria has an extraordinary ability to develop resistance to any antibiotic to which it has been exposed. This was first revealed by the acquisition of β-lactamase on 'penicillinase plasmids' and the subsequent response to β-lactamase stable derivatives by acquisition SCCmec elements by MRSA. For many antibiotics, pre-existing highly efficient resistance mechanisms evolved in antibiotic producers or their competitors and were too easily acquired by pathogenic staphylococci by horizontal gene transfer of mobile genetic elements, for example, from organisms in soil (Nesme and Simonet 2015).

It is clear that better stewardship of new antibiotics will be required. In hindsight, it was a great folly to allow usage of molecules in veterinary medicine and animal husbandry that are the same as or closely related to those used in human medicine. The streptogramin virginiamycin is no longer used in Europe but earlier studies showed how easily resistance could develop with mechanisms that could impact on usage of Synercid to combat MRSA infections today. Even usage of a molecule in veterinary medicine that is not employed to treat humans can select a broad-spectrum resistance mechanism that gives cross-resistance. This occurred with the Cfr resistance determinant $PhLOPS_A$ which was most likely selected by florfenicol usage in animal husbandry but which confers resistance both to linezolid and to one component of Synercid. Allowing fusidic acid to be employed alone in an ointment for topical usage has compromised a useful combination therapy with rifampicin because of the rapid selection of resistance (Howden and Grayson 2006).

Recently, there has been some movement in the introduction of new molecules. The USA GAIN Act has encouraged small companies and academic groups to persue new discovery and development programmes and to bring products towards clinical trials. The resurrection of iclaprim, although not a new chemical class but rather a derivative of trimethoprim that emerged from structure based studies, is a case in point. Other promising molecules target fatty acid biosynthesis (FabI), the cell division protein FtsZ, a ClpP protease activator and the lipid A moiety of lipid II. The last example is teixobactin which, because of the nature of the target, resistance cannot easily develop by mutation. However, history tells us that even when resistance development should be difficult because the drug has more than one target (fluoroquinolones) or where the drugs are used in combinations (co-trimoxazole) the microbe finds ways to combat them.

There have been pronouncements that the end of the antibiotic era is nigh that have been widely publicised in the media. In certain infections, treatment options are indeed running out (e.g. ESKAPE pathogens including carpbapenem resistance in enterobacteria combined with colistin resistance and extreme drug-resistant *Mycobacterium tuberculosis*) (Aminov 2010; Pendleton, Gorman and Gilmore 2013; Seung, Keshavjee and Rich 2015). It is still possible to treat most *S. aureus* infections caused by MRSA if the infecting strain acquires resistance by switching drugs or employing different combinations, bearing in mind that persistent bacteraemia and infective endocarditis are notoriously difficult to treat because of underlying illnesses leading to an immunocompromised state, and the ability of bacteria to avoid antibiotics by forming biofilms incorporating persisters and SCVs. Given that the development of newer drugs is feasible, better stewardship should prolong their activity, and along with more informed usage of combinations should ensure the continued ability to treat many MRSA infections.

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