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A Genetic and Molecular Analysis of Antimicrobial Agent Resistance in Staphylococci

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The intention of this paper is to characterize staphylococcal resistance to antimicrobial agents on a genetic, molecular, and biochemical level using seminal studies from both microbiology and molecular epidemiology as primary references. This review is meant to be thorough, but by no means conclusive, comprehensive, or fully complete.

Discussed initially is "classical" resistance, mediated by the enzyme β-lactamase. This includes enzyme structure, classification, identification, mechanisms/kinetics of action, as well as the enzyme variants, extracellularity, and genetics of synthesis.

Secondly, resistance NOT mediate by β-lactams is covered, commonly known as methicillin resistance. Included are the origin and dissemination of the *mec*-resistance determinant, *mec*-associated DNA, and the core sequence of *mec* determinant, as well as the mec regulon, PBP' function, heterogeneity in expression of resistance and external factors affecting resistance. Also discussed in detail are chromosomal genes affecting resistance level including the *femAB* operon as well as *femC*, *femD*, *femE*, *femF*, and *llm* genes. In addition, a short summary of non *mecA*-mediated intrinsic resistance to β-lactams, BORSA and MODSA, is included.

Lastly, resistance to antimicrobial agents other than β-lactams is discussed. This is primarily limited to antibiotics, although resistance to organic

cations and inorganic ions as well as antiseptics and detergents has also been reported. Focused on are the most commonly known and used antibiotics, including aminoglycosides, chloramphenicol, macrolides, lincosamides, streptogramins, tetracyclines, trimethoprim and sulfonamides, fluoroquinolones, mupirocin, and vancomycin and teicoplanin.

I. RESISTANCE MEDIATED BY β-LACTAMASE

The first β-lactam antibiotic, penicillin, was discovered by Sir Alexander Fleming in 1929. It was first used therapeutically in 1941, and the penicillins, also known as the β-lactams, have since been used extensively and successfully as antibiotics in the treatment of a wide variety of bacterial infections. However, the first report that extracts from bacteria could destroy penicillin was published in 1940.[1] Strains of *S. aureus* soon emerged that were resistant to penicillin because they were able to produce the enzyme β-lactamase, called penicillinase until 1960, which converts the penicillin into an innocuous form by hydrolysis of the β-lactam ring. Today, this form of antimicrobial agent resistance is commonplace, particularly in strains isolated from hospital environments: there are reports that as many as 93 percent of isolates of *S. aureus* are resistant to β-lactamase-liable penicillins.[2]

To overcome the failure of β-lactam therapy, an intensive search has been underway for β-lactams that are not destroyed by this enzyme. This investigation was aided by the fact discovery that benzylpenicillin could be enzymatically deacylated to produce 6-aminopenicillinanic acid (6-APA). 6-APA was then

treated with various acyl halides to produce a variety of semisynthetic penicillins. Thus, it was possible to add bulky side chains to 6-APA to give, for example, methicillin, which is only slowly hydrolyzed by the staphylococcal β-lactamases, and cloxacillin, which is stable to acid and so can be given by mouth. Other β-lactams produced include the carbapenems, such as imipenem. Cephalosporins were also developed and some of these compounds such as cefazolin and cephalothin are effective against β-lactamase–producing *S. aureus*. An extensive search was also mounted for inhibitors of the enzyme that could act in synergy with a β-lactam antibiotic that would otherwise be destroyed.[3]

There has also been much research into the mechanisms by which the gram-positive staphylococci have evolved resistance to these antimicrobial agents. It is known that gram-negative bacteria produce, between them, a wide variety of β-lactamases. Evidence indicates that the introduction of modified β-lactams into therapy has resulted in the selection of gram-negative bacteria that produce mutant forms of β-lactamase able to hydrolyze the modified β-lactams. There is no compelling evidence, however, that *S. aureus* has followed this route, rather they have acquired a modified penicillin-binding protein that renders them intrinsically resistant to β-lactams such as methicillin, strains that are called MRSA. These will be discussed in detail following this first section, resistance mediated by β-lactamase, which includes accounts of the structure, mechanisms of action, and genetics of the staphylococcal enzyme.

There are two main systems for classifying the β-lactamases. One is based on amino acid sequence similarity and places the staphylococcal enzyme

in Group A, along with many other β-lactamases from both gram-positive and gram-negative bacteria.[4] The second system is based on the catalytic properties of the enzyme and placed staphylococcal β-lactamases in group 2a on criteria that include the ability to more rapidly hydrolyze penicillins than cephalsporins. Dendrograms have been constructed on the basis of sequence comparisons, and group 2a enzymes also appear as closely related in this system; consequently, the two distinct classification systems are seen to produce similar groupings.[5]

There are four main ways to estimate the activity of the β-lactamases. The first is useful, as it can be used with crude preparations and with whole colonies of bacteria. Iodine is used because it reacts with penicilloic acid, the compound resulting from the cleavage of the penicillin β-lactam ring, but not with the penicillin. Secondly, a pH change that results from the action of the enzyme can be observed, which is a good assay for purified enzyme and therefore for detailed kinetic studies. The third method uses nitrocefin as a substrate, and observes a color change. This method, however, is not a very good substrate for the staphylococcal β-lactamase. Finally, differences in the absorbance between the β-lactam and its hydrolysis products can be measured. These and other methods have been reviewed, but it is rare for laboratories in hospital or health care settings to need to carry out direct testing on staphylococci for β-lactamase production.[6]

Studies of the structure of β-lactamases effectively began with the determination of the amino acid sequence of the extracellular β-lactamase

purified from *S. aureus* PC1. This form of the enzyme consists of 257 amino acids with a molecular weight of 28.8 kDa. The sequence has been compared with that of several other staphylococcal β-lactamases and shown to possess four motifs that are characteristic of many enzymes interacting with β-lactams. The PC1 enzyme has been crystallized and the structure determined to 2.0 Å. All four common motifs are found at the presumed active site and their relative positions have been determined by x-ray diffraction studies. There is an active serine protease at position 70, and it is this serine that is thought to act as the acceptor residue during the reaction. From these crystal structures and other evidence, it is predicted that β-lactamases behave like other serine proteases, such as trypsin and chymotrypsin. In serine proteases, the oxygen of the substrate peptide bond is in contact with the main chain-NH-groups of the two amino acids, which, as part of the mechanism, help create an oxyanion hole. The active site-serine is in contact with a histidine that acts as a general base to group the serine's acylation by the –C=O group of the peptide bond. Therefore, the peptide bond is split and an acylated enzyme is formed as an intermediate. The histidine then acts as a general base to activate the water molecule that hydrolyzes the serine ester to regenerate the deacylated enzyme. The two peptide bonds are consequently released.[7]

The kinetics of action of β-lactamases have also been studied in depth. When discussing this, two of the most pertinent parameters of an enzyme are k_{cat} and k_{cat} / K_m of k_{cat} is a measure of the maximum number of times that an enzyme molecule catalyzes the reaction in a unit of time, and the Michaelis

constant (K_m) is the substrate concentration at which one-half of the enzyme molecules have substrate bound at any given time. Thus, the value of k_{cat} / K_m is the best measure to use in comparisons of the enzyme's ability to attack particular penicillins or cephalosporins – the higher the value, the more rapidly the enzyme is likely to destroy the substrate. These parameters have been determined for β-lactamases for many substrates, and are regularly used to type the variants of the enzyme, as will be discussed later.

Variants of staphylococcal β-lactamases have been classified into types A, B, C, and D on the basis of their interaction with an antiserum prepared against purified PC1 enzyme. Types A and C are generally produced in large amounts by *S. aureus* of phage-typing groups I and III, while type B is associated with phage group II isolates. Type D was discovered to be produced by strains also resistant to fusidic acid. The original antiserum made stimulated the activity of type A enzymes, but is was used up, and scientists have not been able to produce fresh antiserum that replicates the action of the original. However, the classification has been re-determined on the basis of the differing kinetic properties of the enzyme types. A compilation of kinetic data for the four variants taken from Rowland et al can be seen in figure 1. Type A can best be distinguished from types B and C by the large differences in the K_m when cefazolin is the substrate. The type A enzyme has a much higher k_{cat} for benzylpenicillin than that of type D. Rowland et al also found it is possible to make these distinctions even with whole cell preparations. There are differences

in types B and C, but they are not distinguishable by these parameters, it usually takes testing susceptibility to inhibition by tazobactum.[8]

The genes for several type A, one type C, and one type D β-lactamase have been sequenced. There are sequence differences between the type A's, but the only major change that could account for the serological and kinetic differences between types A and D is the substitution of an alanine at position 128 in the type D enzyme for the aspartic acid found at this position in type A. The sequencing of the gene for the type B enzyme is currently underway.[9] Most MRSA strains produce a b-lactamase. A survey of 27 epidemiologically distinct MRSA strains of *S. aureus* conducted during the 1960's found that all 27 produced type A β-lactamase.[10] Over 25 years later, a survey of 50 MRSA strains from cities in the United States revealed that 40 produced type A while the remaining 10 produced type C. No MRSA strain of *S. aureus* has been shown to produce either type B or type D. [11]

There is also work underway to discover whether mutations in the gene for β-lactamase that specify an enzyme that more efficiently hydrolyzes some of the newer β-lactams are selected for in the natural environment. There has been such selection found in the gram-negative β-lactamases, but none has been documented for the staphylococcal enzyme. Whether such selection is likely for the whole culture is debatable because, if the enzyme is entirely extracellular, and an individual bacterium would probably not be at significant selective advantage. In contrast, staphylococci with some cell-bound β-lactamase could be at an advantage.

There is no good evidence as of yet that β-lactams can pass across the staphylococcal membrane. They act at the membrane surface to inhibit the synthesis of mucopeptide, so there is no value to the organism in having a cytoplasmic β-lactamase. The enzyme is located either at the membrane or released into the medium. Inspection of the predicted amino acid sequence at the N-terminus of staphylococcal β-lactamase revealed that for the type A enzyme there is a leader peptide of 24 amino acids. East et al discovered that the isolate *S. aureus* 1071 releases very little, if any of its enzyme into an extracellular medium. They found a proline at position 22 of the leader peptide in place of the serine commonly found at this position for other β-lactamases. Changes they directed by site-directed mutagenesis of the serine at position 22 of the β-lactamase of another isolate, 3084, that produces a large amount of extracellular enzyme to a proline prevented the appearance of the extracellular enzyme.[12] The leader sequence also has a motif, including a cysteine, that is characteristic of a lipoprotein with a covalent bond to a glyceride, which is of interest because the nucleotide sequence of a signal peptidase II, which cleaves such lipoproteins, has been determined for a gene from *S. aureus*. If there is a lipoprotein from of β-lactamase in the cell membrane then it may have kinetics that differ from those of the free form of the enzyme.[13] The advantage of such a form to the bacterium could be that is has the potential to inactivate the antibiotic before it reaches the sensitive site at the membrane.

For most β-lactamase-producing staphylococcal, the enzyme is produced inducibly, but the type D enzyme is produced constitutively in one strain, but

inducibly in others. In early studies of the induction system, several regulatory genes were identified, including the *blaI*, the gene for a common repressor protein; *blaR1*, thought at that time to specify a second polypeptide that bound to the BlaI protein; and *blaR2*, thought to produce a protein that could, for example, result in the synthesis of an intracellular inducer such as a mucopeptide precursor. The structural gene *blaZ*, together with *blaI* and *blaRI*, is often found on the large plasmids whereas *blaR2* is always chromosomal. The site for the *blaZ* gene on the chromosome is between *purB* and *ilvB* and near *agr*. However, since there is evidence for transposons containing *blaZ* that may not have specific sites for insertion and since complete plasmids are capable of integrating into the chromosome, it is expected that the gene will be found in many different chromosomal locations. The *blaZ* gene was shown to be present on the chromosome of *S. aureus* PS80, but could not be transferred to a plasmid. This was the first indication that the gene was present on a transposon; since that time, several *blaZ*-containing transposons have been reported, including Tn*552*, Tn*4002*, Tn*3852*, and Tn*4201*. These are very similar to each other and probably have a common origin. The transposons have been shown to be active, in that both Tn*4201* and Tn*3852* have been shown to translocate – the former to a site near to, if not identical with, the site for *blaZ* mentioned above.[14]

The complete sequence of Tn*552* has been determined, allowing significant advances in the understanding of both the transposon and of the regulation of β-lactamase synthesis. There are six open reading frames, and functions can be attributed to all of them. The first ORF, orf480 or transposase,

contains the D,D(35)E motif found in several transposases as well as in the integrases of retroviruses. The protein has been purified and shown to bind the termini of Tn*552*. The second ORF, orf251, is probably an accessory transposition protein, important in the selection of target DNA, thought to act in a way analogous to the MuB protein of bacteriophage μ. The third ORF, BinL, is a recombinase thought to function by catalyzing recombination at the *resL* site within Tn*552*. The fouth ORF, BlaZ, is the β-lactamase, while, as mentioned earlier, the fifth is the BlaI, the classic repressor protein. The protein binds specifically to the *blaZ* operator region as shown by DNA footprinting and the protein protects two regions corresponding to the repeats found in the operator sequence. The protein shows 39 percent amino acid sequence identity to the BlaI protein of *B. lichenformis*, which has been shown to bind to the control region for expression of β-lactamases in that organism. The sixth and final orf is BlaR1, a putative signal transducer-sensor protein. It consists of 585 amino acids and has homology with the BlaR1's of *B. lichenformis* and methicillin-resistant *S. epidermidis*. There are five hydrophobic regions that could span the bacterial membrane, so that it is possible that the N-terminus is on the inside of the membrane and the C-terminus is on the outside. The C-terminal region has motifs found for β-lactam binding proteins.[15]

The inducer for β-lactam also binds to the C-terminal region of BlaR1, causing a signal to be passed, either directly or indirectly to the BlaI protein. The nature of this signal is not known and it is not known whether the BlaI is covalently modified or is modified by a noncovalent interaction with a protein or

small molecular weight effector. As a result of the signal the BlaI no longer binds to the operator region and so transcription of both *blaZ* and *blaR1/blaI* increases, giving elevated synthesis of the β-lactamase and the regulatory proteins.[16]

II. RESISTANCE NOT MEDIATED BY β-LACTAMASE (METHICILLIN REISTANCE)

The primary target of β-lactam antibiotics are the penicillin-binding proteins (PBP's), enzymes anchored in the cytoplasmic membrane involved in the last stages of peptidoglycan biosynthesis. They are responsible for the polymerization of peptide moieties of the peptidoglycan chains, which in *S. aureus* are cross-linked by a characteristic pentaglycyl side chain. Penicillin reduces the cross-linking of the peptidoglycan and inhibits new septum initiation. The effect of β-lactams in staphylococci is dose dependent, extending with increasing concentrations from growth inhibition, to lytic and nonlytic death. The lethal target of β-lactams has not yet been identified, and penicillin-induced death does not necessarily coordinate with bacteriolysis.[17]

Staphylococci have different means to become resistant to β-lactam antibiotics. They can do so either by mutation, or more efficiently and more clinically relevant, by acquisition of a foreign DNA element coding for methicillin resistance. The methicillin resistance determinant *mec* confers to the staphylococci an intrinsic resistance against all β-lactams, including carbapenems and cephalosporins. As stated in the introduction, the first methicillin resistant *S. aureus* (MRSA) containing the mec determinant were

isolated in 1960, shortly after the introduction of methicillin into clinical use. At that time, MRSA compromised less than 1 percent of all isolates, but they have since spread successfully all over the world.[18] MRSA reside mainly in environments in which there is a constant strong antibiotic pressure, such as hospitals. Once established, MRSA is difficult to control and eradicate.

A further property of MRSA is their tendency to accumulate unrelated resistance determinants and incorporate them into their genome. Their adaptability and ready response to antibiotic selection has led, in about 40 years, to the evolution of MRSA strains resistant to almost all commonly used antibiotics. MRSA have become a serious problem because of both their multiresitance, and their intrinsic resistance to β-lactam antibiotics, ruling out therapy with many currently used antibiotics. Another clinically less important class of staphylococci has appeared with borderline resistance to methicillin. Although these isolates can sometimes be mistaken in susceptibility tests for MRSA, they carry no *mec* determinant, are usually not multiresistant, and arise by mutation at additional non-*mec* loci and as a result of selection for resistance.[19]

The *mec* determinant resides on a DNA element of more than 30 kb that is absent in susceptible staphylococci. In all strains examined by scientists so far, this DNA element seems to integrate at a specific site in the *S. aureus* genome, between the *spa* (protein A) and *purA* (adenine requirement) genes found to be located on the *Sma*-I-G fragment of *S. aureus*. Neither the nature or origin of this element nor the mechanisms by which it is transferred are currently known. The

transient capture of the *mec* determinant on a plasmid by Trees and Landolo suggest that it resides on a transposon Tn*4291*.[20] It is believed that the early MRSA isolates descended from one single methicillin resistance clone, however since then, the *mec* determinant seems to have entered other phylogenetic lineages of *S. aureus.*

It is also possible that the *mec* determinant was acquired at different times by different strains and that additional mutations and deletions have served to further diversify the *mec*-associated DNA as well as the genome of the host. Clonal analysis of MRSA strains and of the *mec* determinant stemming from staphylococcal species other than *S. aureus*, mainly *S. epidermidis* and *S. haemolyticus*, supports the hypothesis that there may be a dissemination of the *mec* determinant by horizontal transfer, with the CoNS serving as the reservoir of the *mec* determinant for *S. aureus.* β-lactamases, which are transposon and plasmid encoded were rapidly and widely disseminated and are now widely present in about 80 percent of all staphylococci. In contrast, the *mec* determinant is still restricted to a few clonal lineages and seems to favor clonal over horizontal spread.[21]

The core sequences of the *mec* element consist of *mecA*, a gene coding for a low-affinity penicillin-binding protein, termed PBP2' or PBP2a, which is prerequisite of methicillin resistance, and a variable segment of 3' DNA that ends in an insertion sequence-like element, named IS*431*/*1*S*257.* The variable part codes for an open reading frame that has amino acid similarity with *E. coli* phosphodiesterase (UgpQ) and for a direct repeat unit (*dru*) containing 10

degenerate 40-bp repeats of unknown function. The length of the fragment between *mecA* and IS*431mec* is hypervariable, owing to a deletion within the *dru* element and postinsertional rearrangements between the IS element and the hypervariable region. This variability is utilized for epidemiological studies, using *Cla*I restriction site polymorphism of the *mec* determinant, or size variations after amplification of the *dru* element for molecular typing of MRSA.[22]

Another clinically important feature, and the likely reason for the frequent association of multiresistance with methicillin resistance, is the fact that *mec*-associated DNA acts as a trap for the integration of other unrelated resistance determinants in that chromosomal segment. IS*431mec* thereby serves as target for homologous recombination of other resistance determinants or plasmids flanked by similar IS elements. Transposon attachment sites found 5' to *mecA* are also responsible for the site-specification of transposon Tn*554*. Whereas the *mec* determinant of earlier clinical isolates was stable, in more recent isolates, which have accumulated more resistance determinants, and therefore contain several direct repeats of IS*431*/*1S257*, spontaneous deletions removing the *mec* determinant or part of it have been observed, sometimes even deleting nearby chromosomal genes.[23]

From the more than 30 kb of additional DNA contained in the element which is absent from susceptible strains, only a small part, namely the sequence carrying the *mecA* gene, is sufficient for producing methicillin resistance in susceptible strains. Its product is a low-affinity 76 kDa PBP2' that shows the characteristics of a membrane-bound PBP, with a putative transglycosylase

domain and the characteristic conserved sequences of a transpeptide. Its transglysosylase domain is sometimes degenerated, as most mutational events among different *mecA* genes that had no effect on the methicillin resistant phenotype were found in the putative transglycosylase domain. The highly conserved sequence similarity between the *mecA* genes of several unrelated methicillin-resistant *S. aureus* and *S. epidermidis* strains, suggest little time has passed since the *mecA* gene entered the staphylococci.[24]

As stated before, the *mecA* origin is unknown, but it is likely that the sequence stems from an organism with a high AT content, similar to staphylococci. It has been proposed that the *mecA* sequence arose by recombination between the promoter and N-terminal sequence of a penicillinase gene and the structural gene of a PBP of unknown origin. Upstream of *mecA*, some *mec* determinants contain a divergently transcribed regulatory element, holding the genes *mecR1* and *mecI*, separated from *mecA* by its promoter and operator. *mecI*, the repressor of *mecA*, has strong similarity to *BlaI*, the repressor of staphylococcal β-lactamase. *mecR1* is similar to, and has the same molecular organization, as *BlaR1*, which is involved in the induction of staphylococcal β-lactamase. It contains several hydrophobic transmembrane segments, an extracellular penicilloyl serine transferase motif, and a cytoplasmic Zn-peptidase motif.[25]

In the absence of *mecI*-*mecR1* and β-lactamase regulatory elements, there is a constitutive PBP2' production. Some strains that harbor a complete functional *mecI*-*mecR1* regulatory element are strongly repressed and produce

PBP2' only after induction. Induction is slow in these strains, and methicillin and oxacillin seem to be weak inducers. Consequently, methicillin resistance is established very slowly with full induction on methicillin-containing plates seen only after about 48 hours. Such strains may appear falsely susceptible in standard resistance tests. However, clinical strains that carry the sequences for *mecI* and *mecR1* produce PBP2' constitutively and in high amounts. They are usually found to have point mutations or deletions that inactivate the repressor.[26]

Because of their great similarity to MecR1 and MecI, the regulatory elements BlaR1 and BlaI of the staphylococcal β-lactamases can also regulate *mecA* transcription. Repression by BlaI is weaker than by MecI, but there is still some PBP2' produced in the uninduced strain, and induction by methicillin is as rapid as β-lactamase induction. Since staphylococcal β-lactamase and PBP2' share the same induction pathways, the putative chromosomal locus *blaR2* that is proposed to interfere with the *bla* regulon may also affect *mecA* regulation. The mechanisms of the induction cascade and its components, which lead from the extracellular β-lactam to the different factors involved in signal transduction to the final induction of either β-lactamase or PBP2', remain to be found in staphylococci.[27]

It is generally believed that PBP2', which has the binding characteristics of a normal PBP, acts as a transpeptidase, and that it takes over the functions of the resident PBP's of the cell when activated by high concentrations of β-lactams. Under normal growth conditions, in the absence of β-lactams, PBP2' does not seem to contribute to cell wall composition. Profound differences only

become apparent when the normal PBP's of the cells are inactivates by β-lactams and solely PBP2' is left functional. Whereas more than 60 percent of muropeptides in the wall of normal growing cells are cross-linked, cross-linking decreases to 15 percent in the presence of methicillin, suggesting that PBP2' is restricted to the formation of muropeptide dimers only. Nevertheless, it is still possible that PBP2' has an additional unknown role in resistance. Interestingly, the transfer of a resistant strain that constitutively produces PBP2' from a drug-free medium to a high concentration of methicillin induces a lag period in growth roughly proportional to the methicillin concentration in which it was placed. This was postulated to be related to the activation of a regulatory circuit that switches cell wall synthesis from the normal PBP's to PBP2'.[28]

Besides studying the PBP's of staphylococci, much research has gone into the heterogeneous expression characteristic of the methicillin-resistant strains. When an overnight culture of a MRSA strain is plated on increasing concentrations of methicillin or on any other β-lactamase resistant β-lactam and the colony-forming units are determined, only a small fraction of the total population is able to grow at much higher concentrations than the majority. The concentrations up to which most of the cells grow is commonly called the basal resistance level.[29] There have been observations that the population analysis profile is also growth phase dependent, which suggests that whatever changes are triggered by methicillin that allow resistance above the basal level are cell cycle dependent. In some strains, the highly resistant subpopulation will keep the high resistance once it has been formed, and descendents of this

subpopulation will remain resistant to those concentrations of methicillin to which they were selected. The stability of these subclones suggests that they may also be mutants. On the other hand, in some clinical isolates, the subclones return to their original resistance upon regrowth from a single colony in drug-free medium. This could be explained by assuming that the initial level of resistance was due to the induction of methicillin resistance and that induction was lost upon regrowth on drug-free medium, demonstrating the different mechanisms that may lead to high resistance. [30]

Clinical isolates carrying the *mec*-determinant vary widely in their basal resistance to methicillin (MIC's of less than 4 ug/ml to greater than 1,000 ug/ml), as well as in the ratio of cells able to develop high methicillin resistance. Since population profiles are a strain-specific property that can be reproduced under strictly controlled conditions, clinical isolates have been arbitrarily divided into four classes of expression by Tomaz et al, although it is not clear that each of these classes corresponds to a single *mec*/host background.[31] Despite many efforts such as this, there currently exists no satisfactory model that can explain the mechanisms governing heteroresistance. Rearrangements involving the mec determinant have been observed in strains after selection for high methicillin resistance, but they were shown not to be responsible for increased resistance.[32] Slow inducibility of *mecA* when controlled by a strong repressor, as in strains with *mecR1-mecI*, can lead to low resistance. It has also been shown that repression of *mecA* by the β-lactamase repressor turns a highly and homogeneously resistant MRSA into a heterogeneously resistant strain, when the corresponding

signal transducer, needed for induction (BlaRI) is inactivated.[33] This means that a certain threshold of PBP2' is needed for the expression of methicillin resistance. However, in strains with constitutive PBP2' synthesis, which vastly overproduce this protein, the same amount of PBP2' is present whether the cells are heterogeneously or homogeneously resistant. A number of studies have been unable to establish a correlation between the amount of PBP2' production and resistance levels, suggesting that other genes cooperate with PBP2'. [34]

In contrast, a series of experiments showed that a chromosomal gene(s), termed chr*, was responsible for the expression of high level resistance, and that neither mecA nor any of the additional mec-associated DNA was involved. A similar conclusion, namely that the chromosomal background of the staphylococcal strain plays an important role in determining resistance levels, was drawn from another experiment. Homogeneous high-level methicillin resistance was abolished by insertional activation of *mecA*. Methicillin resistance could be restored to its original high resistance level by reintroducing *mecA* even though its gene came from a low-level heterogeneously resistant strain. The functions of genes involved in high-level methicillin resistance such as *chr** remain to be identified, however a chromosomal segment that may mediate high-level resistance was recently cloned. Characterization of genes such as this may help clarify the differential expression of resistance. [35]

External factors, such as temperature, pH, osmolarity, light, divalent cations, chelating agents, and anaerobiosis can also affect methicillin resistance. Lowering the temperature and increasing NaCl concentrations enhances

methicillin resistance, and these conditions are routinely employed in the detection of methicillin resistance in clinical isolates. However, which genetic elements respond to these external factors in staphylococci and how they are connected to the methicillin resistance are still unknown.[36]

Signal transduction may also play an important role in methicillin resistance. Substances that are able to interact with the cell membrane, such as surfactant glycerol monolaurate, were shown to inhibit β-lactamase induction, probably by interfering with signal transduction. The detergent Triton-X100, which stimulated both the release of acylated lipoteichoic acids and bacterial autolysis, was shown to reduce methicillin resistance when added in subinhibitory concentrations. The effect is similar to that of polidocanol, a nonionic detergent, which has been shown to increase the susceptibility of MRSA to β-lactam antibiotics. [37] These substances presumably act at the cell membrane level, disturbing signal transduction and enhancing β-lactam action by interfering with cell wall metabolism. Mutants resistant to these effects might help identify additional factors involved in β-lactam action and resistance.

Tn*551*-mediated insertional mutagenesis of the staphylococcal genome has been used to generate susceptible mutants from a methicillin resistant strain. The genes identified were termed factors essential for methicillin reistance (*fem*) or auxillary (*aux*) factors. These Tn*551*-mediated inserts were then mapped and found to be numerous and scattered around the staphylococcal genome. According to the effect of their inactivation on methicillin resistance, they can be separated into two classes. Inserts in *femA* and *femB* abolish methicillin

resistance completely, including even the formation of a highly resistant subpopulation. Other factors, such as *femC* and *femD*, reduce the basal resistance level but still allow formation of highly resistant revertants. All of these factors belong to the normal set of genes of the staphylococcal genome of both susceptible and resistant strains and are involved directly or indirectly in specific steps of cell wall biosynthesis. Inactivation of these factors in a susceptible strain results in hypersusceptibility to β-lactams.[38]

The *femAB* operon was mapped downstream or *trpA* in the *Sma*I-A fragment in *S. aureus* 8325. It is involved in the peptidoglycan pentaglycine interpeptide bridge formation. Mutants that lack FemB are able to attach only three glycines to the cross-bridge. No *femA* zero mutant is available, but peptidoglycan composition of a polar mutant allowing less than 10 percent of *femAB* transcription suggests that FemA directs incorporation of glycines two and three. The first glycine appears to be added independently of FemA and FemB.[39] The precise role of these two cytoplasmic proteins, which are very similar to one another and have an approximate size of 49kDa, is unknown. The glycines are added to the peptidoglycan stem-pentapeptide by three glycyl-tRNA's that are specific for cell wall synthesis and different from the glycyl-tRNA used in protein biosynthesis. In *S. aureus*, glycine is loaded on each of these tRNA's by a single glycyl t-RNA synthetase. However, FemA and FemB lack homology to any published protein sequences, including glycyl t-RNA synthetase. Therefore, it is not possible to predict their precise functions.[40]

The long flexible pentaglycine side chain is responsible for the high cross-linking values characteristic of the staphylococcal peptidoglycan. The shortened glycine side chain in *femAB* mutants leads to reduced peptidoglycan cross-linking and to higher resistance to lysostaphin, a glycyl endopeptidase. Additional defects in *femAB* mutants include aberrant septum formation, thickened cross walls, and a retarded cell separation. Neither PBP2' synthesis nor that of the normal PBP's is affected in *femAB* mutants. PBP's are produced in normal amounts, but their function is impaired – the cells become hypersusceptible to β-lactams. This is clinically significant because inhibition of FemA of FemB to any antibacterial agent should therefore restore the efficacy of β-lactams against MRSA.

The *femC* and *femD* loci were also found to be located on the chromosomal *Sma*I-A fragment of the *S. aureus* 8325 chromosome. Inactivation of *femC* reduces only the basal resistance level in MRSA but still allows formation of a highly resistant subpopulation. The *femC* mutation produces a metabolic block in glutamine production. Inactivation of the glutamine synthetase repressor (*glnR*) has a polar effect on transcription of the 3' glutamine synthetase gene, *glnA*, in the *glnRA operon*, leading to reduces production of glutamine synthetase and consequently to a lack of glutamine. This has an indirect effect on peptidoglycan composition, reducing the degree of cross-linking in the peptidoglycan. Addition of glutamine to the growth medium can restore methicillin resistance. The mutation *femD* interferes with peptidoglycan precursor production. It has been postulated to be a defect in a gene controlling

the rate of biosynthesis of the unsubstituted disaccharide pentapeptide precursor. [41]

Little information is available on *femE*, although it has been identified as a Tn*551* insertion in the *Sma*I-A fragment, in a site distinct from *femAB* and *femC*. No significant changes in peptidoglycan composition were detected in this mutant.[42] *femF* mutants are impaired in an early step of precursor muropeptide synthesis and the gene has been localized to the *Sma*I-B fragment.[43]

In addition to the fem (factors associated with methicillin resistance), a gene, *llm*, coding for a 38-kDa lipophilic protein, has been identified that increases autolysis rates and reduces methicillin resistance upon inactivation, as do the *fem* factors. The *llm* gene has been identified in all *S. aureus* strains, but has not been found in other staphylococcal species. *llm* shows strong similarities to *mraY*, the first enzyme involved in the lipid cycle of peptidoglycan biosynthesis.[44] From the growing list and functions of *fem* factors, it seems that any disturbances in the well-balanced biosynthesis of peptidoglycan and possibly alterations in membrane composition as well, reduce the optimal function of PBP2' when resident PBP's are saturated by methicillin. As previously stated, the *fem* factors seem to belong to the normal housekeeping genes of staphylococci and apparently function normally in clinical methicillin-resistant isolates. Although their experimental disruption by transposon insertion leads to a reduction in methicillin resistance, it is not clear that any of the *fem* genes or loci are directly involved in the expression of methicillin resistance in clinical isolates or that they are altered in their expression or function by the presence of

the *mec* determinant. Future studies could possible use techniques other than gene inactivation to find these resistance-associated factors.

Unlike the *femAB* mutants, the *femC*, *femD*, and *llm* mutants produce a small number of highly methicillin-resistant revertants when plated on high concentrations of methicillin. Reversion takes place in a site distant from the corresponding *fem* or *llm* mutation. In *femC* mutants, this reversion affects an unidentified gene(s), termed *hrmC* (for high methicillin resistance reversion in *femC*), and does not restore the glutamine synthetase activity. The corresponding *hmrD* revertants of *femD* and the highly resistant revertants of *llm* likewise occur at sites involving neither *femD* nor *llm*. These revertants are highly and homogeneously resistant, similar to the highly resistant subpopulation formed by a heterogeneous MRSA in the presence of high concentrations of methicillin. However, the chromosomal *chr** mutation involved in high-level resistance of wild-type MRSA strains could not substitute for the *hmrC* mutation. This means that in MRSA, multiple paths may lead to high-level resistance.[45]

Standard susceptibility tests sometimes fail to distinguish between an MRSA with a very low level basal resistance and the clinically less relevant borderline resistant strains mentioned earlier that carry no *mec* determinant. Two types of borderline resistant strains have been encountered: BORSA and MODSA. The first type, termed BORSA, are hyperproducers of β-lactamase or strains specifically producing a methicillinase. This resistance mechanism is not intrinsic because it involves partial hydrolysis of penicillinase-resistant penicillins. However, it appears that their hyperproduction of β-lactamase is not sufficient,

and that specific, yet-to-be defined host background factors are also needed to establish borderline resistance.[46]

The second type of borderline resistant isolates, termed MODSA, are strains with modifications in their own PBP's. Such strains can also be obtained in vitro from a susceptible *S. aureus* and selected for growth on increasing concentrations of β-lactams. Multiple factors that have yet been identified are involved in this process, and changes in the amount and/or affinity of the existing PBP's of the cells have been described. Increase in resistance by this mechanism is usually impaired with decreasing growth rates. In contrast to *mecA*-dependent methicillin resistance, no heteroresistance is observed in MODSA strains – all descendents of the culture are uniformly resistant.[47] Clinically, neither the BORSA nor the MODSA have become as relevant as MRSA but, because of their phenotypic overlap with low-level resistant MRSA, identification methods have been used that can distinguish between the presence or absence of the *mecA* gene.

In April 2001, Kuroda et al publishes the genome sequence (about 2.8 million base pairs) for two strains of MRSA, Mu50 and N315.[48] Many believe this is the breakthrough needed to finally begin to "win" the struggle against MRSA.

III. RESISTANCE TO ANTIMICROBIAL AGENTS OTHER THAN β-LACTAMS

Resistance to a wide range of antimicrobial agents, including antibiotics, antiseptics/disinfectants, and inorganic or heavy metal ions, has been reported in *S. aureus* and other staphylococci.[49] In many instances, the resistance elements

are either plasmid-encoded or associated with other mobile genetic elements. These determinants mediate resistance through a number of different biochemical mechanisms that can be classified into several common categories: inactivation mechanisms, bypass mechanisms, target site alteration, efflux mechanisms, and sequestration.

In the first example, the antimicrobial agent is inactivated by chemical modification by either intracellular or extracellular detoxifying enzymes (e.g. the inactivation of chloramphenicol by chloramphenical acetyltransferase). Secondly, staphylococci can bypass the effect of the antimicrobial agent by substitution of an alternate pathway or enzyme, as in resistance to trimethoprim due to the synthesis of an alternative trimethoprim-insensitive dihydrofolate reductase enzyme. Thirdly, target site alteration leads to a reduced affinity of the target site for binding with the antimicrobial agent, as is observed with in resistance to macrolides, lincosmaides, and streptogramin (MLS) type B antibiotics, where there target site is structurally modified (methylation of the 23S rRNA adenine residues). Fourthly, the antimicrobial agent can be actively pumped out of the bacterial cell (e.g. the active export of toxic organic cations). Lastly, the antimicrobial agent can be specifically and stoichiometrically bound by a cellular protein such that its antimicrobial action is blocked, an example of which can be seen in the binding and sequestration of bleomycin. Some of these examples and others will be discussed in detail in the next sections.[50] For each antibiotic, examples of each will be given along with their mechanism of action and prevalence of use for staphylococcal infections. Also discussed will be

cloning and sequence analysis of proposed resistance determinants, location (where they are located chromosomally, types of plasmids, etc), and homology to other known determinants, as well as other factors relevant to describing the genetic and molecular bases of resistance.

AMINOGLYCOSIDES

Aminoglycoside antibiotics, including gentamicin, kanamycin, streptomycin, and neomycin, inhibit protein synthesis by binding to the 30S ribosomal subunit. Aminoglycosides have been widely used to treat staphylococcal infections and are still used in combination with other antistaphylococcal agents. The major mechanism of aminoglycoside resistance observed in the staphylococci is drug inactivation by cellular enzymes, such as aminoglycoside acetyltransferases (AAC), adenyltransferases (AAD), or phosphotransferases (APH). Aminoglycosides modified at the amino groups (by AAC's) or hydroxyl group (by AAD's or APH's) lose their ability to bind ribosomes, and hence, do not inhibit protein synthesis.[51]

Resistance to gentamicin and concomitant resistance to tobramycin and kanamycin in the staphylococci is mediated by a bifunctional enzyme displaying both AAC And APH activity, which has been purified and characterized biochemically. [52] The *aacA-apdD* gene encoding this enzyme has been cloned from the plasmid pSK1 and sequenced. Amino acid sequence analysis suggests that it contains two separate domains, an N-terminal domain with homology to AAC enzymes and a C-terminal domain with homology to APH enzymes. Mutagenesis of the *aacA-apdD* gene supports the notion that two separate

domains in this enzyme are responsible for the two aminoglycoside-modifying activities. The *aacA-apdD* gene has been shown to be encoded on the composite transposon Tn*4001*. Tn*4001* and Tn*4001*-like elements have been found to be widely distributed in both *S. aureus* and CoNS, being found on pSK1 family plasmids, conjunctive plasmids, occasionally on β-lactamase/heavy metal resistance plasmids, and also in various chromosomal locations.[53]

Resistance to neomycin, kanamycin, tobramycin, and several other aminoglycosides in the staphylococci is mediated by an AAD encoded by the *aadD* gene. The *aadD* gene is often carried on small plasmids, such as pUB110. The complete nucleotide sequence of pUB110 has also been determined, and the AAD product of *aadD* has been purified and biochemically characterized. Copies of the *aadD*-encoding pUB110-like plasmids have also been found integrated into large conjunctive plasmids and into the methicillin resistance *mec* region of the chromosome of some *S. aureus* isolates. Additionally, a variety of other plasmids encoding AAD have been detected and probably also carry an *aadD* gene.[54]

Staphylococcal streptomycin-resistance genes have also been identified. Sequencing of the streptomycin resistance gene *str*, identified on small plasmids such as pS194, has demonstrated that its product is homologous with AAD enzymes from other bacteria. High-level streptomycin resistance has been associated with chromosomal mutations (*strA*) that affect the ribosome. Low-level cross-resistance to most aminoglycosides appears to be due to a chromosomal mutation that affects the membrane's permeability to

aminoglycosides. A streptomycin resistance APH encoded by an *aphC* gene has also been found in *S. aureus*, but not well characterized. Additionally, a plasmid-borne *aadA* gene encoding an AAD that confers resistance to streptomycin and spectinomycin has been detected.[55]

CHLORAMPHENICOL

Chloramphenicol is a bacteriostatic antibiotic that binds to the 50S ribosomal subunit and inhibits the transpeptidation step in protein synthesis. It has not been widely used as an antistaphylococcal agent, however resistance to chloramphenicol in the staphylococci has been reported and is due to the inactivation of the antibiotic by a chloramphenicol acetyltransferase enzyme (CAT), which acetylates the antibiotic.[56] *cat* genes have been located on a wide variety of staphylococcal plasmids, and three main families of cat-encoding plasmids have been identified, typified by the plasmids pC194, pC221, and pC223. The nucleotide sequences of the *cat* genes on *S. aureus* plasmids such as these have been determined and their products are homologous both with each other and with *cat* enzymes from other bacteria. Expression of the *cat* gene is typically inducible and has been studied in detail in the case of the pUB112 *cat* gene. Expression seems to be regulated by translational attenuation in a similar manner to *ermC* expression, which will be discussed in the next section. The *cat* gene is preceded by a 9-amino acid leader peptide, and the leader mRNA can form a stable stem-loop structure, which masks the ribosome binding site of the *cat* gene. Chloramphenicol appears to cause the ribosome to stall on the leader sequence, opening the stem-loop structure, thereby exposing the *cat*

ribosomal binding site, allowing *cat* expression, and therefore resistance to chloramphenicol.[57]

MACROLIDES, LINCOSAMIDES, STREPTOGRAMINS (MLS)

Macrolides, such as erythromycin and oleandomycin; lincosamides, such as lincomycin and clindamycin; and streptogramin antibiotics have a bacteriostatic effect similar to that of chloramphenicol - by binding the 50S ribosomal subunit and arresting protein synthesis. Erythromycin, clindamycin, and lincomycin have been used extensively in the treatment of both minor and major staphylococcal infections. Currently, resistance to these agents is highly prevalent among the staphylococci. Streptogramins, such as pristinamycin, have been used in Europe as antistaphylococcal agents, but the prevalence of resistant strains remains low.[58] A number of determinants conferring resistance to this group of antibiotics via a variety of mechanisms have been identified in the staphylococci.

Three homologous staphylococcal determinants, *ermA*, *ermB*, and *ermC* have been identified that confer resistance to the MLS antibiotics by target-site alteration of the ribosome. The best example of these genes, *ermC*, has been demonstrated to encode a methylase, which catalyzes the dimethylation of an adenine residue in the 23S ribosomal subunit, thereby reducing the affinity between the ribosome and the MLS antibiotic. The *ermC* gene is typically carried on small staphylococcal plasmids, such as pE194, and its expression is inducible and is regulated at the level of mRNA secondary structure, as has been thought for *cat* regulation. The *ermC* mRNA contains a leader sequence coding for a 19-

amino acid leader peptide, and this leader mRNA is capable of forming a stable stem-loop structure that sequesters the *ermC* binding site. Erythromycin-induced stalling of the ribosome during translation of the leader sequence opens up the stem-loop, permitting ribosomal access to the *ermC* ribosomal binding site and subsequent translation of *ermC*. Both genetic and physical evidence strongly support this model of translational attenuation and have suggested that codons 5 to 9 of the leader sequence are essential for induction while codons 9 and 10 are in physical contact with the stalled ribosome. Although *ermC*-encoded MLS resistance is characteristically inducible, both naturally occurring and in vitro mutants of *ermC* have been isolated that express constitutive MLS resistance. [59]

The *ermA* gene also confers inducible resistance to MLS and has been shown to be encoded on the transposon Tn*554*. Tn*554* also carries the streptomycin resistance *spc* gene encoding an adenyl transferase, similar to AAD of aminoglycoside-resistant strains. The complete sequence of Tn*554* has been determined and the *ermA* gene demonstrated to encode a product homologous to the RNA methylase encoded by *ermC*. Tn*554* has been shown to insert at a specific site in the *S. aureus* chromosome but has also been found at a number of chromosomal sites, including within the *mec* region and may also be plasmid located. The *ermA* gene is preceded by a leader sequence coding for a 19 amino-acid peptide homologous with the *ermC* leader peptide. This suggests that *ermC* is also regulated by translational attenuation; supporting this, erythromycin-induced stalling of the ribosome on the *ermA* leader has been observed. Erythromycin may also play a role in stabilizing the *ermA* mRNA.

The *ermB* gene confers constitutive resistance to MLS and has been detected on some β-lactamase/heavy metal plasmids where it is encoded on the transposon Tn*551*. Transposition of Tn*551* has been demonstrated in vivo and the transposon has been partially sequenced at this time. [60]

Distinct from the *erm* genes, *msrA* gene confers resistance only to 14-and 15-member macrolides, such as erythromycin, and to type B streptogramins, possibly by an efflux mechanism. *msrA* is frequently plasmid encoded and is widely disseminated in clinical *S. epidermidis* strains, as well as being found in *S. aureus* strains. *msrA* has been cloned and sequenced from the *S. epidermidis* plasmid pUL5050 and found to encode a hydrophobic protein with sequence similarity to the ATP-binding components of the ABC superfamily ATP-dependent transport systems. The region upstream of *msrA* resmbles the control regions of *ermA/C* genes regulated by translational attenuation. [61] Epidemiologic studies of erythromycin resistance have indicated that *ermC* and *msrA* are the most frequent causes of erythromycin resistance and that *ermA* and *ermB* are only present in a minority of isolates.[62]

TETRACYCLINES

Tetracycline and related antibiotics are bacteriostatic agents that bind to the 30S ribosomal subunit, preventing stable association with the aminoacyl-tRNA, resulting in the inhibition of protein synthesis. Tetracycline is a very commonly used antibiotic, but has not been widely used for treatment of staphylococcal infections, however a semi-synthetic tetracycline, minocycline, is used in some countries for treatment of multi-resistant staphylococcal strains.[63]

Two different resistance mechanisms to tetracycline have been identified in the staphylococci. First, the chromosomally encoded *tetA*(M) gene confers resistance to tetracycline and to semisynthetic analogues, including minocycline, by a mechanism known as ribosomal protection. The *tetA*(M) gene from a clinical *S. aureus* isolate has been cloned and sequenced and found to share a high level of sequence similarity at the nucleotide and amino acid level with class M *tet* determinants from other gram-positive and-negative organisms. Southern hybridization analysis has suggested that there is no indication that *tetA*(M) in *S. aureus* is located on a transposable element. Expression of *tetA*(M) gene has been shown to be induced by the tetracycline. The TetM protein shares sequence similarity with elongation factor G, which translocates the peptidyl-tRNA during protein synthesis, and other GTP-binding proteins. tRNA modification activity has been suggested to be necessary for TetM-mediated tetracycline resistance, but to date, the biochemical basis remains unclear. One possibility is that TetM stabilizes the ribosome-tRNA interaction in the presence of tetracycline.[64,65]

The second tetracycline resistance mechanism identified in staphylococci is active efflux that can be encoded by either of to determinant *tetA*(K) or *tetA*(L). Both confer inducible resistance to tetracycline, but not its semisynthetic analogues. *tetA*(K) is found on small plasmids, and also has been found chromosomally due to IS*257*-mediated integration of a plasmid. *tetA*(K) has been cloned an sequenced from staphylococcal plasmids. *tetA*(L) is also typically found on small plasmids in *Bacillus sp.*, but it also has been seen in

staphylococci. Both *tetA*(K) and *tetA*(L) are probably regulated by translational attenuation in a similar manner to that of *cat*, *ermA*, and *ermC* expression. The products of *tetA*(K) and *tetA*(L) are homologous to each other, as well as to a range of drug efflux proteins in the major facilitator superfamily, some of which are discussed later. TetK and TetL are both thought to be membrane proteins with 14 transmembrane-spanning segments. Like other members of the major facilitator superfamily, TetK and TetL mediate drug efflux driven by the proton motive force (pmf) of the transmembrane electrochemical proton gradient, rather than by ATP hydrolysis. Transport assays have indicated that *tetK* confers tetracycline resistance – assays of tetracycline uptake in everted membrane vesicles have indicated that TetK is functionally similar to the well-characterized TetB tetracycline efflux pump encoded in enterobacteria. In a similar manner to TetB, TetK-mediated tetracycline efflux was demonstrated to be dependent on the presence of divalent cations, such as Co^{2+}, which are co-transported with tetracycline – transport of such a metal ion/tetracycline complex is coupled with proton transport.[66]

TRIMETHOPRIM AND SULFONAMIDES

Trimethoprim and the sulfonamides are synthetic agents that affect the biosynthesis of hydrofolic acid, an essential derivative used in amino acid and nucleotide synthesis. Sulfonamides are analogues of *p*-aminobenzoic acid that competitively inhibit the enzyme dihydropteroate synthase (DHPS), which catalyzes the condensation of dihydropteridine with *p*-aminobenzoic acid, an early step in tetrahydrofolic acid synthesis. Trimethoprim is an analogue of

dihydrofolic acid that competitively inhibits the enzyme dihydrofolate reductase (DHFR). DHFR catalyzes the reduction of dihydrofolic acid to tetrahydrofolic acid, the final step of tetrahydrofolic acid synthesis. Cotrimoxazole, a combination of trimethoprim with a sulfonamide, has been used extensively to combat staphylococcal infections.[67]

Both high- (MIC greater than 1,000 μg/ml) and low- (MIC less than 100 μg/ml) level trimethoprim resistance has been reported in both *S. aureus* and CoNS. In some cases, chromosomally encoded low-level trimethoprim resistance may be due to overproduction of host DHFR. More commonly, however, resistance is encoded by the *dfrA* gene carried on the transposon-like element Tn*4003*. *dfrA* codes for a trimethoprim-resistant type S1 dihydrofolate reductase that has been both biochemically purified and characterized. Tn*4003* has also been sequenced and it or similar *drfA* encoding elements have been found on pSK1 family plasmids, large conjunctive plasmids, and on various other plasmids in both *S. aureus* and CoNS. Transposition of Tn*4003* has not been experimentally demonstrated; rather, its apparent motility may be due to IS*257*-mediated recombination events. The *drfA* gene appears to be responsible for both high- and low-level resistance to trimethoprim in both *S. aureus* and CoNS. The differences in resistance correlate with differences in transcription caused by deletions adjacent to a copy of IS*257* in Tn*4003* affecting the promoter used by *drfA*.[68]

The chromosomal trimethoprim-sensitive DHFR gene from *S. aureus* has been cloned and sequenced and found to be distinct from *drfA* (80 percent

identical at the amino acid level) and not located within an operon, suggesting that *drfA* did not originate from the *S. aureus* chromosomal DHFR gene. In contrast, the *S. epidermidis* trimethoprim-sensitive chromosomal DHFR gene differs from *drfA* by only 4 bp and is found in an operon-like structure, strongly suggesting that *drfA* originate from the *S. epidermidis* chromosomal gene. Site-directed mutagenesis of *drfA* and kinetic analyses of purified DHFR's has indicated that a single alteration is largely responsible for the trimethoprim resistance.[69]

FLUOROQUINOLONES

Fluoroquniolones such as norfloxacin, ciprofloxacin, and pefloxacin are synthetic broad-spectrum antimicrobial agents that interfere with the function of DNA gyrase, an essential enzyme involved in DNA replication and repair. Fluoroquinolone resistance has arisen rapidly in the staphylococci following the use of these agents in recent years to treat both gram-positive and gram-negative bacterial infections. Three distinct loci have been identified in S. aureus that are involved in conferring resistance to fluoroquinolones.[70]

First, target alterations in DNA gyrase can decrease the fluoroquinolone sensitivity of this enzyme. DNA gyrase is a tetrameric enzyme with two subunits (A and B) encoded by the *gyrA* and *gyrB* genes, which are homologous to their counterparts from other bacteria such as *E. coli* and *B. subtilis*. Sequencing of mutant alleles of *gyrA* from a very large number of independent fluoroquinolone-resistant isolates has indicated that mutations conferring fluoroquinolone resistance are located within a single region near codon 84. Additionally, *gyrB*

mutants have been identified that confer lower levels of resistance than *gyrA* mutants. These mutants were found to have substitutions at codon Asp-437 or Asp-458. Transformation of these *gyrA* or *gyrB* fluoroquinolone-resistant mutants with wild type *gyrA* or *gyrB* plasmids reconferred fluoroquinolone susceptibility in these strains, confirming that mutations in *gyrA* and *gyrB* are in fact responsible for the fluoroquinolone resistance. In addition, purified DNA gyrase from fluoroquinolone-resistant *gyrA* mutants has been shown in vitro to be resistant to approximately 20-fold higher concentrations of the agent than purified wild-type enzyme from fluoroquinolone-sensitive strains.[71]

Secondly, low-level fluoroquinolone resistance appears to be associated with mutations in the chromosomal *grlA* gene of *S. aureus*. The *S. aureus grlA* and *grlB* genes encode for homologues of GyrA and GyrB, which have been demonstrated to catalyze ATP-dependent DNA relaxation. Analysis of fluoroquinolone-resistant *S. aureus* mutants has indicated that mutants resistant to low levels of fluoroquinolone contained substitutions at Ser-80 of GrlA. Work with stepwise-selected mutants has indicated that mutations in *grlA* are required for expression of resistance due to *gyrA* mutations. Thus, low-level resistance is conferred by *grlA* mutations, but high-level resistance is a result of mutations in both *grlA* and *gyrA*. Thirdly, a chromosomally encoded active fluoroquinolone efflux system encoded by the *norA* gene has been described in *S. aureus*. The *norA* gene has been cloned and sequenced and found to encode a membrane protein. Sequence analyses have indicated that NorA is a member of the major facilitator superfamily of transporter proteins and is closely related to other drug

efflux proteins, some of which were mentioned earlier. Drug transport studies in whole cells and everted membrane vesicles have indicated that NorA mediates the active efflux of fluoroquinolone driven by the pmf. *norA* has also been shown to confer resistance to a range of compounds structurally unrelated to the fluoroquinolones and the *norA* gene has also been found in fluoroquinolone-sensitive staphylococcal strains. An initial report suggested that fluoroquinolone resistance was due to a single base pair change in *norA*, resulting in a single amino acid change in its product. However, sequencing of the complete *norA* gene from both sensitive and resistant *S. aureus* strains failed to detect any differences within the gene.[72]

MUPIROCIN

The antibiotic mupirocin (pseudomonic acid A) is an isoleucine analogue that competitively inhibits isoleucyl-tRNA synthetase (IRS), preventing protein synthesis. Mupirocin has been used as a topical agent in the treatment of gram-positive bacterial infections since its introduction in 1985. Interestingly, *mupA* has been detected in staphylococcal strains dating from 1965, well before the introduction of mupirocin as a topical agent. High- (MIC greater than 1,000 μg/ml) and low- (MIC 8 to 256 μg/ml) resistance to mupirocin has been detected in both *S. aureus* and CoNS. Low-level resistance is probably due, in most cases, to mutation in the host IRS.[73] Strains resistant to high levels of mupirocin have been shown to contain two biochemically distinct IRS enzymes: the naïve mupirocin-sensitive IRS enzyme and an additional IRS enzyme that is less sensitive to inhibition by mupirocin. The *mupA* gene that codes for a mupirocin-

resistant shares significant sequence similarity with a number of different bacterial IRS enzymes and contains characteristic IRS-specific motifs involved in ATP hydrolysis and amino acid activation. The *mupA* gene is, however, distinct from the chromosomal IRS gene, as they share only 30 percent similarity at the amino acid level. Thus, high-level mupirocin resistance in *S. aureus* appears to be due to the acquisition of an IRS gene from another organism, possibly in an analogous manner to the acquisition of trimethoprim resistance, as discussed previously. The *mupA* gene has demonstrated to be plasmid encoded in a rage of epidemiologically independent *S. aureus* and CoNS strains. Analysis of the *mupA* plasmids of pGO400 and pI3358 has indicated that the gene is flanked by directly repeated copies of the insertion sequence IS257. Analysis of other staphylococcal plasmids has suggested that IS257 has played a role in horizontal transmission of the resistance gene, as was suggested for previous examples.[74]

VANCOMYCIN AND TEICOPLANIN

Glycopeptide antibiotics, such as vancomycin and teicoplanin, form complexes with the peptidyl-D-Ala-D-Ala terminus of the peptidoglycan precursors at the outer surface of the cytoplasmic membrane, leading to the inhibition of transglycosylation and transpeptidation steps of cell wall synthesis. Since the development of multiresistance staphylococcal strains, vancomycin has frequently been the only drug available to the clinician for the treatment of serious infections caused by MRSA. Staphylococcal strains with reduced susceptibility to both vancomycin and teicoplanin have been isolated. In clinical strains of CoNS, resistance to both has also been noted, although more

commonly, strains are resistant to teicoplanin (MIC less than 128 μg/ml) but sensitive to vancomycin (MIC less than 4 μg/ml). In particular, *S. haemolyticus* displays a propensity for developing resistance to glycopeptides in a single step upon exposure. Clinical isolates of *S. aureus* have been detected which have low-level resistance to both vancomycin and teicoplanin, while mutants of *S.aureus* have been selected in vitro with decreased susceptibility to both as well.[75] A number of studies of glycopeptide-resistant staphylococcal strains have indicated that resistance is typically constitutively expressed, chromosomally encoded, and is stable in the absence of selective pressure. Clinical isolates of teicoplanin resistant staphylococci have been found to express a 39-kDa protein associated with the membrane to cell wall of the organisms. Kaatz et al detected a teicoplanin-resistant isolate from a single patient following unsuccessful therapy. Tn*551* insertion mutants obtained of this strain regained teicoplanin susceptibility. These insertions were mapped to a single Sma1 chromosomal fragment. Analysis of the resistant strain indicated that expressed a 35-kDa membrane protein and had increased expression of both PBP2 peptides relative to the Tn551 insertion mutants. Moreover, its cell wall physiology was altered in some aspects.[76] In a similar study, analysis of clinical *S. aureus* isolates with decreased susceptibility to vancomycin from 12 patients indicated that the majority expressed a 35-kDa membrane protein with increased expression of both PBP2 peptides as well.[77]

Vancomycin- and teicoplanin-resistant *S. aureus* mutants have been obtained in vitro by serial incubation in the presence of a low concentration of

either compound. Daum et al isolated mutants exhibiting low-level vancomycin resistance that showed alterations in cell wall organization and constitutively produced a 35-kDa protein. The *ddh* gene encoding this protein has been cloned and sequenced and codes for a cytoplasmic protein with sequence similarity to D-lactate dehydrogenase (D-LDH) and to the enterococcal VanH protein (discussed later). *ddh* was shown to be expressed in wild-type staphylococci, and expression was found to be increased in a glycopeptide-resistant mutant, which was associated with increased D-LDH activity. This possibly suggests that overexpression of D-LDH in *S. aureus* may contribute to low-level glycopeptide resistance. As mentioned earlier, decreased susceptibility to glycopeptides in the staphylococci also appears to be associated with expression of a 35- or 39-kDa membrane protein, often accompanied by increased expression of PBP2 peptides and apparent alterations in the cell wall physiology of the organism. Despite the similarity in size, these 35- or 39-kDa proteins probably do not correspond to Ddh, as Ddh is a cytoplasmic protein. Therefore, the mechanism(s) of glycopeptide resistance in these strain(s) still remain to be clarified.[78]

In the better-studied enterococci, transferable high-level resistance to vancomycin (MIC greater than 512 μg/ml) with concomitant resistance is commonly seen and is referred to as VanA type resistance. This phenotype has been shown to be encoded by the *vanA* gene cluster on the 10.8 kb transposon Tn*1546*, which is often carried by conjunctive plasmids with a broad host range in gram-positive bacteria. Tn*1546* includes three genes essential for glycopeptide

resistance: *vanA*, which encodes an alternative D-Ala-D-Ala ligase that produces altered peptidoglycan side chains, containing an terminal dipeptide (e.g. D-Ala-D-lactate) with greatly reduced affinity for glycopeptide antibiotics; *vanH*, which encoded a dehydrogenase that reduces pyruvate to D-lactate, a substrate for the *vanA* product; and *vanX*, which encodes a D, D-dipeptidase that hydrolyzes D-Ala-D-Ala produced by the host ligase. Tn*1546* also includes two accessory glycopeptide–resistance genes: *vanY*, encoding a D, D-carboxypeptidase that cleaves the C-terminal D-Ala of peptidoglycan precursors, and *vanZ*, which encodes a teicoplanin-resistance determinant; two genes, *vanR* and *vanS*, encoding a two-component system that regulates expression of glycopeptide resistance; and two transposition genes. Two other types of glycopeptide resistance, VanB and VanC, have also been identified in the enterococci. Like VanA resistance, these rely on the production of D-Ala-D-Ala ligases. The *vanB* gene cluster, which encodes a vancomycin-resistant, teicoplanin-sensitive phenotype, is encoded on the 64-kb composite transposon Tn*1547*. Significantly, Tn*1547* is bounded on one side by the staphylococcal insertion sequence IS*256* and on the other by IS*16*, which is highly similar to IS*256*.[79]

Unlike the enterococci, reduced susceptibility to vancomycin and teicoplanin in the staphylococcal appears not to be due to ligases with altered substrate specificity, since resistant staphylococci produce peptidoglycan precursors terminating in D-Ala-D-Ala. High-level vancomycin resistance as seen in the enterococci has not been observed in clinical staphylococcal strains. However, the conjunctive co-transfer both in vitro and in vivo of vancomycin

resistance, along with other resistances, from *E. faecalis* to *S. aureus* has been demonstrated. The *S. aureus* transconjugants were subsequently resistant to high levels of vancomycin, although they were unable to transfer resistance to other *S. aureus* strains and resistance was lost in the absence of selective pressure.[80] Thus, it seems likely that the emergence of staphylococcal strains resistant to high levels of vancomycin is only a matter of time. This scenario would have great ramifications for the treatment of serious S. aureus infections. However, it is hoped that with the growing knowledge of the mechanistic basis of resistance and its evolution at the molecular and genetic level, new strategies can be devised to reduce the impact of this devastating public health problem.

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Figure 1: Kinetic parameters of the four variants of β-lactamase of *Staphylococcus aureus*

	Type A			Type B			Type C			Type D		
β-lactam	Kcat	Km	REH	Kcat	Km	REH	Kcat	Km	REH	Kcat	Km	REH
Cephaloridine	1.95	10.9	100	6.65	19.4	191	5.61	11.5	273	1.25	7.9	88
Cefazolin	1.01	18.4	31	4.68	254	10	3.94	267	9	1.91	49.9	21
Nitrocefin	23.2	6.4	2,034	16.5	15.7	587	13.7	20.4	293	25.6	5.9	2,424
Benzylpenicillin	171	51.1	1,869	156	38.6	2,258	210	55.9	2,098	46	112.6	228
Ampicillin	308	255	675	412	160	1,438	355	122	1,685	133	129	571

Abbreviations: Kcat molecules of substrate hydrolyzed per second; Km, in μm; REH, relative efficiency of hydrolysis (Kcat/Km) relative to cephaloridine set at 100
(Data from Rowland et al)