Vancomycin Resistance in *Staphylococcus aureus*

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The evolution of *Staphylococcus aureus* during the modern antibiotic era has been delineated by distinct strain emergence events, many of which include acquisition of antibiotic resistance. The relative high burden of methicillin-resistant *S. aureus* (MRSA†) in healthcare and community settings is a major concern worldwide. Vancomycin, a glycopeptide antibiotic that inhibits cell wall biosynthesis, remains a drug of choice for treatment of severe MRSA infections. *S. aureus* strains exhibiting increased resistance to vancomycin, known as vancomycin intermediate-resistant *S. aureus* (VISA) (MIC = 4-8 µg/mL), were discovered in the 1990s. The molecular basis of resistance in VISA is polygenic and involves stepwise mutations in genes encoding molecules predominantly involved in cell envelope biosynthesis. *S. aureus* isolates with complete resistance to vancomycin (MIC ≥ 16 µg/mL) are termed vancomycin-resistant *S. aureus* (VRSA)—they were first reported in the U.S. in 2002. Resistance in VRSA is conferred by the *vanA* gene and operon, which is present on a plasmid. Although treatment of VRSA infections is challenging, the total number of human VRSA infections to date is limited (14 in the U.S.). By comparison, the burden of VISA is relatively high and the molecular mechanisms of resistance are less well-defined. VISA are associated with persistent infections, vancomycin treatment failure, and poor clinical outcomes. Here, we review in brief progress made toward understanding the acquisition of antibiotic resistance in *S. aureus*, with an emphasis on the molecular mechanisms underlying vancomycin resistance.

**INTRODUCTION**

*Staphylococcus aureus* is an important human pathogen and was first recognized as the etiological agent of suppurative abscesses more than 130 years ago [1]. *S. aureus* infections range from mild skin and soft-tissue infections to life-threatening endocarditis, chronic osteomyelitis, pneumonia, or bacteremia, which are associated with significant morbidity and mortality [2-6]. The advent and use of antibiotics such as penicillin and methicillin in the mid-20th century initially proved effective against *S. aureus*. However, *S. aureus* rapidly acquired resistance to these antibiotics and infections with penicillin-resistant *S. aureus* (PRSA), and in turn methicillin-resistant *S. aureus* (MRSA), were difficult to treat. Although progress has been made, MRSA remains a significant threat to human health globally. For example, *S. aureus* isolates represent 29 percent of all reported bacterial isolates in Europe, and an estimated 72,444 cases of invasive MRSA infections occurred in the United States in 2014 [7-9]. Importantly,

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the glycopeptide antibiotic vancomycin has proven effective in treating severe MRSA infections [10]. However, S. aureus clinical isolates with reduced susceptibility to vancomycin, and less commonly, with complete resistance to vancomycin have emerged within the past 20 years [11-13]. This review highlights features of vancomycin intermediate-resistant S. aureus (VISA, MIC = 4-8 µg/mL) and vancomycin-resistant S. aureus (VRSA, MIC ≥ 16 µg/mL) in the context of epidemiology, mechanisms of resistance, and human infections.

ANTIMICROBIAL RESISTANCE

The modern antibiotic era began with the discovery of penicillin by Sir Alexander Fleming. Many infectious diseases became treatable with antibiotics, including those caused by S. aureus [14]. However, the rapid acquisition of antibiotic resistance by S. aureus is a significant problem for treatment of human infections caused by this organism. A timeline illustrating emergence of antibiotic-resistant S. aureus following the introduction of key antibiotics is provided in Figure 1.

Mobile genetic elements (MGEs) play an integral part in the ability of S. aureus to adapt to environmental stresses, which include exposure to antibiotics. MGEs are a primary means by which genetic information is exchanged between bacteria via horizontal gene transfer. S. aureus strains in general contain a relatively large variety of MGEs, including plasmids, transposons, bacteriophages, pathogenicity islands, and staphylococcal cassette chromosomes. Plasmids and staphylococcal cassette chromosomes in particular have played a central role in conferring resistance to β-lactam antibiotics and vancomycin [15-18].

Penicillin & Methicillin Resistance

PRSA was reported in the early 1940s, a few years after the first use of penicillin for treatment of human infections [19,20]. Infections caused by PRSA were widespread among hospitals and in community settings throughout the 1950s and early 1960s [20,21]. A large number of clinical isolates at this time were categorized by phage-type as 80/81 (the pandemic S. aureus phage-type 80/81 strain), which later were predominantly classified as multilocus sequence type (MLST or ST) 30 and clonal complex 30 (CC30) [22,23]. S. aureus resistance to penicillin is primarily conferred by the blaZ gene, which encodes a β-lactamase. β-lactamase inactivates penicillin by hydrolyzing the β-lactam ring of penicillin [24]. Methicillin, a semi-synthetic beta-lactam antibiotic, was introduced in the late 1950s as a therapy for PRSA infections. Despite efficacy of methicillin for treatment of PRSA infections, the first methicillin-resistant S. aureus (MRSA) strains were reported within two years of clinical use [25]. The burden of MRSA worldwide has increased over many decades [26]. Currently, MRSA accounts for a large proportion of hospital-associated S. aureus infections and is associated with significant morbidity and mortality [7,27-29]. The recent emergence of community-associated MRSA (CA-MRSA) further underscored S. aureus as a serious infectious disease threat globally. Notably, CA-MRSA causes infections in otherwise healthy individuals outside of healthcare settings—thus anyone is at risk for infection [30-32]. The mecA gene, which encodes a low-affinity penicillin-binding protein (PBP2a or PBP2’), confers resistance to methicillin. mecA was discovered more than twenty years after the first reported cases of MRSA [25,33]. It is encoded on a mobile genetic element called staphylococcal cassette chromosome (SCC) [34]. Prototype hospital-associated and community-associated MRSA strains harbor distinct SCCmec variants, highlighting the central role that MGEs play in facilitating the evolution of S. aureus antibiotic resistance [33,35]. Inasmuch as there is a high burden of CA-MRSA in the U.S., selected CA-MRSA strains and most notably the epidemic USA300 strain, have moved into the healthcare setting [36-38]. Interestingly, the most prominent healthcare-associated MRSA strains have largely failed
to move into the community setting. An explanation for this phenomenon is multifactorial, but involves limited transmission among healthy individuals, fitness burden imparted by the SCCmec element, and/or limited strain virulence capacity [39].

**Vancomycin-resistant S. aureus (VRSA)**

Despite being approved for use in humans in 1958, vancomycin became an antibiotic of choice for treatment of MRSA infections in hospital settings in the late 1980s [10,40,41]. Resistance to vancomycin was discovered in enterococci in the 1980s, and this finding elicited significant concern with regard to the future use of vancomycin as an effective treatment for MRSA [42]. Shortly thereafter, *S. aureus* isolates with reduced susceptibility to teicoplanin—a structural relative of vancomycin—emerged in Europe [43,44]. The first VRSA isolate in the United States was reported in 2002 [45,46]. Since that time, there have been a total of 14 isolates reported in the United States [47].

Complete vancomycin resistance in *S. aureus* (MIC ≥ 16 µg/ml) is conferred by the vanA operon encoded on transposon Tn1546, originally a part of a vancomycin-resistant enterococci (VRE) conjugative plasmid [48]. *S. aureus* can acquire enterococcal plasmids during discrete conjugation events. Vancomycin resistance in *S. aureus* is maintained by retaining an original enterococcal plasmid or by a transposition of Tn1546 from the VRE plasmid into a staphylococcal resident plasmid (Figure 2) [49,50]. To better understand the molecular mechanism by which the vanA operon confers resistance, it is necessary to understand primary components of the *S. aureus* cell wall and the mechanism of action of vancomycin. The *S. aureus* cell wall lies just beneath the outermost polysaccharide capsule layer. The cell wall is essential for preserving cell integrity as well as facilitating host-pathogen interactions [51]. The principle component of the cell wall is heavily cross-linked peptidoglycan, which is itself made up of glycan chains NAG (N-acetylglicosamine) and NAM (N-acetylmuramic acid) cross-linked to one another by glycine bridges and stem pentapeptides (UDP-MurNAc-L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala). As a new cell wall is formed, each precursor component is synthesized in the cytoplasm and transported to the division septum of the growing cell wall for further assembly [52].

In Gram-positive bacteria, vancomycin interferes with late-stage peptidoglycan synthesis by forming non-covalent hydrogen bonds with the penultimate D-Ala-
D-Ala residues of newly synthesized UDP-MurNAc-pentapeptides, thereby disrupting downstream peptidoglycan assembly. Ultimately, cell wall synthesis is inhibited and bound vancomycin-pentapeptide complexes accumulate within the cell [53,54]. Two key events are necessary for vanA operon-mediated vancomycin resistance: 1) hydrolysis of dipeptide D-Ala-D-Ala peptidoglycan precursors, which bind vancomycin, and 2) synthesis of D-Ala-D-lactate peptidoglycan precursors, which cannot bind vancomycin [55]. A schematic diagram that depicts acquisition and molecular mechanism of vanA-type vancomycin resistance is provided in Figure 2. The vanA operon is comprised of vanA, vanH, vanX, vanS, vanR, vanY, and vanZ genes. It is controlled via a two-component sensor-regulator system encoded by vanS and vanR that sense vancomycin and activate transcription of the operon respectively [56]. VanA, VanH, and VanX together are essential for the vancomycin resistance phenotype. VanA and VanH are responsible for synthesizing the depsipeptide D-Ala-D-Lac. VanA is a ligase that catalyzes the ester-bond formation of the D-Ala-D-Lac depsipeptide and VanH is a dehydrogenase that forms D-Lac by reducing pyruvate [55]. VanX is a D,D-dipeptidase that hydrolyzes the D-Ala-D-Ala ester bond, ensuring the newly formed D-Ala-D-Lac depsipeptide has little competition to bind the UDP-linked tripeptide peptidoglycan precursor [57]. VanY is a D-carboxypeptidase that performs a similar—but not essential—function by facilitating the cleavage of D-Ala-D-Ala dipeptides already attached to the C-terminal end of stem pentapeptide structures [58]. The role of VanZ is not well understood, but it may confer S. aureus resistance to teicoplanin. Incorporation of altered D-Ala-D-Lac into peptidoglycan yields a cell wall that is no longer susceptible to vancomycin.

**Intermediate Resistance to Vancomycin**

In 1997, a S. aureus clinical isolate from a patient in Japan was found to have reduced susceptibility to vancomycin [59]. This was the first reported VISA isolate. However, retrospective studies suggest that S. aureus susceptibility to vancomycin dates back at least to 1987 in the United States [60]. VISA is typically associated with hospitalization, persistent infection, prolonged vancomycin treatment and/or treatment failure. The VISA phenotype is frequently preceded by an intermediate phenotype known in the clinical laboratory as heterogenous VISA (hVISA) [61-63]. An hVISA phenotype refers to a mixed cell population—derived originally from a single colony of S. aureus—in which the majority of cells have little or no resistance to vancomycin (MIC ≤ 2 µg/ml) and a sub-population of cells is resistant to the antibiotic at the level of VISA (MIC ≥ 4 µg/ml) [64]. The molecular mechanisms that underlie development of hVISA are incompletely defined, although progress has been made. For example, Roch and colleagues demonstrated that an hVISA phenotype can be triggered by exposure of S. aureus to non-glycopeptide antibiotics such as β-lactams [65]. Consistent with these findings, Haaber et al. reported recently that exposure of strain USA300 to colistin caused enhanced resistance to vancomycin, a phenomenon that is regulated at the level of gene expression and thus reversible [66]. Collectively, these studies provide support to the notion that development of hVISA is an epigenetic process rather than one based on gene mutation [65,66]. A current accepted hypothesis is that VISA, which has homogeneous resistance to vancomycin, develops from hVISA in individuals treated with glycopeptide antibiotics over extended time periods [64,65].

Fundamental characteristics of the VISA phenotype include increased cell wall thickness, caused by differentially regulated cell wall biosynthesis and stimulatory pathways [67-70], reduced cross-linking of peptidoglycan, decreased autolytic activity of the enzymes responsible to cell-wall turnover [61,71-76], altered surface protein profile, dysfunction of the agr system and changes to growth characteristics [73,77-81]. Multiple approaches have been used to investigate the molecular genetic basis of the VISA phenotype. Such studies have employed transcriptome, proteome, and comparative genomics profile analyses to compare VISA and vancomycin susceptible S. aureus (VSSA) isolates, including comparisons between closely related VSSA and VISA, sequentially isolated strains from patients undergoing vancomycin therapy, and analysis of laboratory-derived VISA strains [62,67-79,75,80]. These studies identified several genes and/or mutations in genes that contribute to the vancomycin intermediate-resistance phenotype (Table 1). VSSA strains most likely develop vancomycin intermediate-resistance in a step-wise manner, acquiring mutations that each play a role in reducing susceptibility to vancomycin (reviewed in [64]). As proof of this concept, Katayama et al. generated a laboratory-derived VISA strain by introducing sequential mutations in six different genes in the VSSA strain, N315 [82].

Although our understanding of the molecular basis of the VISA phenotype is incomplete, several genes / mutations are known to contribute to the development of VISA. Of particular significance are mutations within genes encoding two-component regulatory systems, such as graRS and walKR, which have been linked to glycopeptide resistance [83,84]. A gene encoding the DNA-dependent RNA polymerase β-subunit (rpoB) is also commonly associated with increased resistance to vancomycin, prolonged propagation time, and increased cell wall thickness [62,85,86]. GraRS differentially regulates transcription of cell wall biosynthesis genes and has been associated with a broad array of genes and regulators that play a role in the intermediate resistance
Table 1. Genes associated with the VISA phenotype.

| Phenotype                        | Associated Genes                                                                 | Role in VISA Phenotype                                                                 | Reference         |
|----------------------------------|----------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|-------------------|
| Cell Wall Thickening and Reduced Autolytic Activity | **graSR** Up-regulates vraSR, dtl operon, capsule operon, mprF/fmtC, mgrA and rot. Down-regulates walKR and agr. Associated with nucleotide metabolism. |                                                                                       | [83, 87, 88]     |
|                                  | **walkKR (yycFG)** Limited walKR activity lowers rates of autolysis and increases cell wall thickness. |                                                                                       | [84, 124, 126]    |
|                                  | **yycH** Lowered expression of genes associated with autolysis. |                                                                                       | [62]              |
|                                  | **pbp4** Reduced rates of peptidoglycan cross-linking and transpeptidation.      |                                                                                       | [137]             |
|                                  | **sarA** Reduced production of autolysins responsible for cell wall recycling. |                                                                                       | [138]             |
|                                  | **mgrA** Reduced production of autolysins responsible for cell wall recycling.  |                                                                                       | [138]             |
|                                  | **clpP** Cell wall thickening, slow growth, and reduced autolysis.              |                                                                                       | [85]              |
|                                  | **stp1** Cell wall thickening, slow growth, and reduced autolysis.              |                                                                                       | [139]             |
|                                  | **Up-regulated Cell Wall Stimulon** **vraSR** Up-regulation of VraSR, reduced susceptibility to vancomycin. |                                                                                       | [88, 95]          |
|                                  | **vraFG** Associated with reduced susceptibility to vancomycin.                 |                                                                                       | [83]              |
|                                  | **mprF/fmtC** Increased net negative charge of cell wall and reduced peptidoglycan cross-linking. |                                                                                       | [89, 91]          |
|                                  | **spoVG** Increased capsule production.                                         |                                                                                       | [140]             |
|                                  | **capA-capP** Increased capsule production.                                      |                                                                                       | [88, 95]          |
|                                  | **isdE** Associated with reduced susceptibility to vancomycin.                  |                                                                                       | [62]              |
|                                  | **prsA** Associated with reduced susceptibility to vancomycin.                  |                                                                                       | [62]              |
|                                  | **Down-regulated Global Regulators** **agr** Attenuation of virulence and reduced susceptibility to vancomycin. |                                                                                       | [62, 81]          |
|                                  | **rot** Attenuation of virulence and reduced susceptibility to vancomycin.       |                                                                                       | [87]              |
|                                  | **rpoB** Associated with reduced susceptibility to vancomycin.                  |                                                                                       | [85, 86]          |
|                                  | **rsbU** Associated with reduced susceptibility to vancomycin.                  |                                                                                       | [64, 140]         |
|                                  | **yjbH** Associated with reduced susceptibility to vancomycin.                  |                                                                                       | [139]             |
clientes de tratamiento de la clínica. En general, se considera que el tratamiento con vancomicina es eficaz en la infección por Staphylococcus aureus, pero puede ser menos eficaz en ciertos casos, como las infecciones por cepas de S. aureus con resistencia a la vancomicina (VRSA).

**VISA phenotype** [87]. Específicamente, GraRS up-regulates genes in the capsule biosynthesis operon, leading to increased capsule production [87]. Se han realizado dos estudios separados que encontraron que mutaciones dentro de las genes **graRS** reduce la susceptibilidad a la vancomicina [83,88]. Además, GraRS up-regulates the **dlt** operon and the **mprF/fntC** genes, which are linked to teichoic acid alanylation and alteration of cell wall charge [89-91].

**Agr** (accessory gene regulator) [92]. Altered expression of global gene regulators has a tremendous downstream effect, and thus could play a role in a VISA phenotype. VISA isolates have been shown to have non-silent mutations in **vraSR**. Such mutations could lead to downstream up-regulation of over 40 cell wall synthesis genes, including genes required for producing cell wall derivatives such as D-Ala-D-Ala [92-95]. WalKR is another two-component gene regulatory system associated with the VISA phenotype. Down-regulation of the **walKR** operon by acquired mutations or insertion of **ls256** leads to increased capsule synthesis, cell wall thickness increases, and reduced autolysis [62,84,95,96].

**VISA phenotype** [98]. The increasing prevalence of hVISA/VISA poses a significant threat, as these organisms often cause infections for which vancomycin treatment fails [104-106]. There are many factors that contribute to the challenges associated with assessing the clinical impact of VISA and hVISA. One important factor is the lack of prospective comparative studies that definitively relate low-level vancomycin resistance in S. aureus to vancomycin treatment failure and poor clinical outcomes. This issue is compounded by the use of multiple testing methods (e.g., eS, trip, broth microdilution, etc.).

**Clinical Implications of Intermediate Resistance to Vancomycin**

The increasing prevalence of hVISA/VISA poses a significant threat, as these organisms often cause infections for which vancomycin treatment fails [104-106].
agree that for infections with VISA having an MIC greater than 8 µg/ml, treatment with glycopeptide antibiotics is not optimal [110,111]. In addition, surgical intervention can be considered for treatment of hVISA infections related to deep abscesses, osteomyelitis, and endocarditis for which there are high numbers of bacteria [112,113]. Interestingly, reduced susceptibility to glycopeptide antibiotics, including vancomycin, has been associated with increased susceptibility to beta-lactams [114,115]. Studies of this phenomenon, termed the “see-saw effect,” have produced conflicting clinical reports [116-118] and more work is needed in this area. The treatment guidelines of the Infectious Diseases Society of America (IDSA) stipulate that an alternative to vancomycin should be utilized for the management of persistent MRSA bacteremia and vancomycin treatment failures with an observed reduction in vancomycin susceptibility (MIC > 2 µg/ml). Visible alternatives to vancomycin include a combination of high-dose daptomycin with another antibiotic such as gentamicin, rifampin, linezolid, trimethoprim-sulfamethoxazole (TMP-SMX), or a β-lactam. Similarly, if reduced susceptibility to daptomycin is observed alongside reduced vancomycin susceptibility, then a combination or single use of the following is recommended; quinupristin-dalfopristin, TMP-SMX, linezolid, or telavancin [119].

CONCLUSIONS AND OUTLOOK

*S. aureus* is notorious for its ability to acquire and/or develop resistance to antibiotics. This attribute, coupled with the high burden of *S. aureus* infections is a problem for treatment. Inasmuch as vancomycin is important for treatment of severe MRSA infections, the ability of *S. aureus* to become completely resistant to vancomycin is disconcerting. Fortunately, strains that have complete resistance to vancomycin (VRSA) are rare, despite wide use of vancomycin for treatment of severe MRSA infections. The paucity of VRSA may be attributed to a fitness cost associated with acquisition of vanA-mediated vancomycin resistance and the infrequency of horizontal gene transfer from enterococci, robust *S. aureus* restriction modification systems that prevent foreign DNA uptake, and strain-lineage specificity that enable certain strains of *S. aureus* to more readily take up enterococcal plasmids [49,120]. Also, it is not known why the majority of VRSA strains isolated in the U.S. have similar genetic background—i.e., categorized as belonging to clonal complex (CC) 5 by multilocus sequence typing and USA100 by pulsed field gel electrophoresis [121-123]. Association of VRSA with one *S. aureus* genetic background might be explained at least in part by the high prevalence CC5 in healthcare settings. The prevalence of hVISA/VISA is much greater than that of VRSA, but the propensity for spread of these strains appears limited at present [105,115,123]. The failure of these strains to spread is perhaps linked to the transient nature of the hVISA phenotype, as the organism can revert rapidly to VSSA in the absence of selective pressure imparted by glycopeptide antibiotics. hVISA/VISA strains are largely hospital-adapted MRSA strains that belong to CC5 and CC8 [124]. These findings are consistent with the notion that selective pressures driving the step-wise evolution of hVISA/VISA strains are greater in the hospital setting than in the community. Unlike VRSA, hVISA/VISA has been associated with many *S. aureus* genetic backgrounds, including CC5, CC8, CC30, and CC45 [125,126]. From a practical standpoint, and considering these characteristics collectively, it can be argued that VISA is a much greater problem for the clinic than is VRSA. *S. aureus* with reduced susceptibility to vancomycin is not restricted to humans. Recently, VRSA and/or VISA have been isolated from pigs, goats, and cattle [127-129]. Although resistance in the livestock-associated VRSA isolates reported by Bhattacharyya et al. was not conferred by presence of the vanA gene and operon, the MIC for vancomycin was ≥ 16 µg/ml for two of the isolates [127]. Inasmuch as vancomycin is not used in livestock in that region of the world, Bhattacharyya et al. suggested that isolates either originated from humans or resistance developed as a result of the continuous exposure of animals to other antibiotics [127]. Consistent with the later hypothesis, these authors reported that the livestock VRSA and VISA isolates were resistant to multiple antibiotics, including β-lactams. The recovery of vancomycin non-susceptible isolates from livestock highlights a potential issue with antibiotic use in livestock and use of antibiotics as a feed supplement [127-129]. Whether an increased burden of such resistance in livestock translates directly to a problem for treatment of human infections remains incompletely determined.

More work is needed to advance identification of hVISA and VISA isolates, which in turn can be used to better assess prevalence of vancomycin intermediate resistance, as well as to facilitate development of optimal treatments. Next-generation sequencing technologies and comparative genomics are tools that can be deployed routinely to aid clinicians in identifying VISA as a cause of infection [130-133]. There a number of hurdles that must be overcome before these approaches can be used for diagnostics on a routine basis, including cost, time, automation, bioinformatics analyses, and standardization (quality control) [130,134]. On a positive note, these tools are available and in use for such purposes at select institutions [135,136].

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