Mechanisms of vancomycin resistance in *Staphylococcus aureus*

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Vancomycin is a glycopeptide antibiotic used for the treatment of Gram-positive bacterial infections. Traditionally, it has been used as a drug of last resort; however, clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) strains with decreased susceptibility to vancomycin (vancomycin intermediate-resistant *S. aureus* [VISA]) and more recently with high-level vancomycin resistance (vancomycin-resistant *S. aureus* [VRSA]) have been described in the clinical literature. The rare VRSA strains carry transposon Tn*1546*, acquired from vancomycin-resistant *Enterococcus faecalis*, which is known to alter cell wall structure and metabolism, but the resistance mechanisms in VISA isolates are less well defined. Herein, we review selected mechanistic aspects of resistance in VISA and summarize biochemical studies on cell wall synthesis in a VRSA strain. Finally, we recapitulate a model that integrates common mechanistic features of VRSA and VISA strains and is consistent with the mode of action of vancomycin.

Introduction

Reduced vancomycin susceptibility and resistance in methicillin-resistant Staphylococcus aureus. By the end of the 1990s the relatively few multidrug-resistant and highly epidemic clones of methicillin-resistant *Staphylococcus aureus* (MRSA) had become the most frequent causative agents of *S. aureus* disease in both hospitals and communities (1). In spite of the availability of several structurally different antibacterial agents, the therapy most frequently used for treatment of MRSA infections has remained the glycopeptide antibiotics, primarily vancomycin (2). From 1980 on, there was an abrupt and continued increase in the use of vancomycin in the United States and several countries (3), which seems to parallel the increasing frequency of MRSA infections in hospitals. This illustrates the enormous selective pressure highly focused on MRSA strains worldwide.

An MRSA isolate with decreased susceptibility to vancomycin was first reported in Japan in 1997 (4). The isolate had only a modestly increased minimum inhibitory concentration (MIC) value for vancomycin, in the range of 3–8 µg/ml, and became known as vancomycin intermediate-resistant *S. aureus* (VISA). VISA isolates do not carry imported foreign genetic elements; rather, the increased vancomycin MIC values are related to mutations that appear in the invading pathogen during vancomycin therapy in vivo. VISA began to be reported with increasing frequency among MRSA isolates identified all over the world (for review, see ref. 5). In spite of their moderate increase in MIC value, vancomycin treatment of infections by VISA isolates often ended in treatment failure (6, 7). In 2002, the first vancomycin-resistant *S. aureus* (VRSA) strain (with a vancomycin MIC value greater than 100 µg/ml) was reported in the United States (8). Clearly the use of vancomycin therapy against such isolates would be impossible.

Comprehensive reviews of various microbiological and epidemiological aspects of VISA-type resistance have been published (5, 6, 9–12). Herein, we briefly discuss some of the mechanistic aspects of VISA-type antibiotic resistance, beginning with a case description that puts the emergence of a VISA mutant into an evolutionary context. Additionally, we summarize results of experiments that have begun to explore the biochemical mechanism of high-level vancomycin resistance in *S. aureus*.

VISA strains: evolution and population structure. In the overwhelming majority of cases, VISA strains have emerged in patients with MRSA infections undergoing prolonged vancomycin therapy, which often ended in treatment failure (for review, see refs. 5–7). The emergence of one of the first VISA derivatives in the United States was documented in detail in the clinical and microbiological history of a 79-year-old patient with end-stage renal disease who received repeated courses of vancomycin therapy against an MRSA blood stream infection (13). The first MRSA isolate, named PC1 and recovered December 13, 1997, had a vancomycin MIC of 2.0 µg/ml. However, examination of the PC1 culture by population analysis showed that it was heterogeneous; it contained subpopulations of bacteria with a frequency of 5×10^{-4} that could grow on 2 µg/ml vancomycin. In addition, bacteria that could grow on 4 µg/ml vancomycin were also present, with a frequency of 6×10^{-6}. Upon readmission of the patient to the hospital on January 10, 1998, an MRSA isolate named PC2 was recovered from the bloodstream. The vancomycin MIC of PC2 was still 2 µg/ml, and the culture was still heterogeneous, but the size of the subpopulation capable of growing in the presence of 2 µg/ml had increased to 10^{-3}. Approximately 2 months later, the same patient was readmitted to the hospital with a fatal MRSA blood stream infection. The vancomycin MIC of the strain named PC3 had now increased to 8 µg/ml and had become homogeneous: its MIC was shared by the entire bacterial population. Strains PC1 through PC3 shared an identical pulsed-field gel electrophoresis (PFGE) profile, indicating that these isolates were genetically identical. Like that of all other VISA isolates described in the clinical literature, the evolutionary history of PC1 through PC3 begins and ends in a particular patient.

Increased vancomycin MIC and problems in chemotherapy. At first sight, the high frequency of failed vancomycin therapy (14–17) is surprising given the rather modest increase in the MIC value of the isolates and the fact that VISA strains showed decreased virulence potential when tested in a variety of animal models (18–21). Thus, a major contributor to the frequent lack of success of prolonged chemotherapy may be the poor health status of the patients, who

are often elderly and have serious underlying health conditions (2, 5, 18). Nevertheless, some data in the literature raise the possibility that factors in the invading bacterium may also contribute. Examination of the vancomycin MIC value of MRSA strains that were recovered from patients with failed therapy showed that therapeutic failure correlated significantly with the vancomycin MIC value of the strains, even when this antibiotic was not used for therapy (15, 16, 22–24). These data suggest that the vancomycin susceptibility profile of MRSA strains correlates with some as-yet-unidentified aspects of the invasive potential of the bacteria.

Population structure of VISA isolates. A characterization of VISA isolates using the method of population analysis first developed for the characterization of MRSA strains with heterogeneous resistance to β-lactam antibiotics (25) has led to the identification of "hetero-VISA" strains (9), which represent the great majority of VISA isolates worldwide (5) and were proposed to be precursors of the less frequent homogeneously resistant VISA strains (9, 26). In cultures of hetero-VISA strains, the great majority of cells have low vancomycin MIC values, close to those of susceptible strains. However, the same cultures also contain low-frequency subpopulations of bacteria with increased vancomycin MIC value. Analysis of a collection of MRSA isolates recovered from hospitals in New York City identified numerous heterogeneously vancomycin-resistant strains. All of these isolates, including PC1 through PC3, belonged to the New York/Japan clone of MRSA (ST5, SCC*mec* II), which was widely spread in hospitals of New York, New Jersey, and Pennsylvania in the late 1990s (27).

Abnormal phenotypes of VISA isolates. All VISA isolates described above share some abnormal properties, including an excess of cell wall material (best visualized in electron microscopic thin sections), an aberrant separation of daughter cells at the end of cell division, and altered (most often decreased) rates of autolysis (5, 9–12, 28, 29).

Genetic/biochemical basis of VISA-type resistance. Elucidation of the genetic basis of VISA-type resistance requires the availability of both the resistant and the isogenic susceptible (parental) isolates recovered prior to the beginning of chemotherapy — ideally from the same patient. Comparison of such susceptible and resistant isolates can then be used to identify the altered genetic determinant in the VISA strain. This approach has identified a variable number and kinds of mutations in different VISA isolates (18, 28, 30–40).

The most frequently mutated determinants identified in 22 clinical VISA isolates from the collection of the CDC and the New York City Department of Health and Mental Hygiene were the *walkR, vraSR,* and *rpoB* genes (31). In another study, the predominant genetic change was in the *yvqF/vraSR* system (41), while Watanabe et al. showed that most of the 38 VISA strains recovered from 10 different countries carried a mutated *rpoB* (42). In most, but not all, cases, the relevant mutated genes appear to be directly or indirectly involved with the biosynthesis/metabolism of the staphylococcal cell wall, specifically, two-component sensory regulatory systems controlling the transcription of genes in cell wall synthesis (32, 33, 43). However, mutations in the RNA polymerase gene *rpoB* were also detected in VISA isolates (42).

At least some of the extensive transcriptional changes observed in VISA strains could also be induced by treating the corresponding parental strain with vancomycin (44).

Identification of genetic stages in the development of VISA-type resistance by whole genome sequencing. In an attempt to better understand the evolution of VISA mutants, the emergence of VISA-type antibiotic resistance was followed in a patient undergoing extensive chemotherapy (36). Data available on this case suggest that the primary infection site was most likely the heart valve. Isolates were recovered from the blood stream of the patient at different times during chemotherapy and were tested for the MIC values of vancomycin. The MRSA strain causing the infection was a single locus variant of the multidrug-resistant New York/Japan clone (ST105), a frequent cause of hospital-acquired infections. The initial isolate (JH1) had a vancomycin MIC value of 1.0 μg/ml. In the second isolate, JH2, the vancomycin MIC value increased to 4.0 μg/ml. In the sixth isolate, JH6, the vancomycin MIC value reached 8 μg/ml. Full genome sequencing was done on the last isolate, JH9 (recovered just before the patient expired), and on the initial fully susceptible isolate JH1, and the rate of appearance of the total of 33 mutated genes identified in JH9 was determined in the early and intermediate isolates by PCR sequencing (36). Consecutive isolates showed a gradual increase in vancomycin MIC value accompanied by a stepwise appearance and accumulation of 16 of the 33 mutations identified in strain JH9 (36). Determining which of these mutations actually contribute to the stepwise increase in vancomycin resistance will require complementation experiments. Also, the nature of the "excess" mutations in JH9, i.e., the 17 genetic alterations that appear only in isolate JH9, remains to be determined. These 17 mutations do not appear in isolate JH14, which had the same vancomycin MIC value as JH9 but was recovered directly from the infected heart valve of the patient during a heart valve replacement surgery performed late in the clinical history of this infection. The excess genetic alterations may represent mutations that contributed to the survival of the VISA strain JH9 in the hostile host environment.

In two recent studies similar in design to that used by Mwangi et al. (36), whole genome sequencing was done on each of the MRSA isolates recovered at various times from the blood stream of patients undergoing chemotherapy with various antimicrobial agents including vancomycin. In one of these studies, full genome sequencing was done on each of six consecutive isolates of the Brazilian clone of MRSA (ST239), which were recovered from the patient over a period of 77 days (45). In the second study, isolates were recovered and sequenced from a patient with a bloodstream infection by the Taiwan clone of MRSA (ST59) (30). These studies allowed identification of the emergence and shifts in the composition of entire subpopulations of bacteria in response to antibiotic treatment, including conversion of vancomycin-susceptible cells to hetero-VISA, followed by the eventual emergence of VISA mutants and the acquisition of multiple mutations in genes that control cell wall synthesis and autolysis (30, 45).

Another such genetic comparison of parental and corresponding VISA isolates was recently described in an MRSA strain belonging to the widely spread USA300 clone (ST8) (28). In this case, the parental, vancomycin-susceptible strain SG-S (recovered from the patient before the beginning of therapy), the VISA isolate SG-R, and a spontaneous revertant of the VISA strain (which had recovered the normal parental level of vancomycin susceptibility) were available for comparison. The SG-S and SG-R isolates showed differences in five genetic determinants, of which one, the mutated *yvqF* (a regulator of the *vraSR* genetic system involved in the control of cell wall synthesis), was confirmed by complementation experiments to be responsible for the increased vancomycin MIC value. This was also consistent with the single genetic change identified in the revertant strain, which carried a complementary mutation in *vraSR* (28). The role of the four other mutations identified in SG-R is not known.

Multiple mutations, in addition to the mutated gene responsible for the VISA phenotype, have been identified in several genetically characterized VISA isolates (18, 28, 33, 36). The contribution of these mutated genes to the VISA phenotype is not clear. In many cases, complementation experiments with the determinant responsible for the VISA phenotype have led to a complete recovery of the wild-type (parental) phenotype of the bacterium, but the extra mutations were still retained by the cells (18, 28, 33, 40, 46). These extra mutations may represent alterations in genes that contribute to the survival and/or progress of the invading bacterium en route to anatomical sites of infection in the antibiotic treated host.

VISA mutants in epidemic MRSA clones. VISA-type mutants have now been identified in every one of the major epidemic clones of MRSA (4, 28, 30, 36, 47–53). Cross-infection with VISA isolates has been described only in two reports, one from a French and another from an Italian hospital in which "epidemic" VISA strains appeared and spread in the hospital environment (54, 55). Clearly, acquisition of traits that would make VISA strains epidemic would be of concern.

The mutated genes identified in the VISA mutants of various MRSA clones varied considerably in nature. Therefore, it is conceivable that the particular mutated genes responsible for the VISA phenotype may be unique to the particular MRSA lineage, i.e., each MRSA clone may have a preferred genetic pathway to VISA-type resistance; however, an experimental test disproved this hypothesis. The MRSA strain JH1 (belonging to the New York/Japan clonal lineage) was used to test whether vancomycin selection in the laboratory and in an experimental system mimicking in vivo exposure of bacteria to vancomycin would produce VISA mutants with genetic change in the same determinants that were identified in VISA-type mutants of strain JH1 that emerged in vivo in a patient during chemotherapy by vancomycin (36). While VISA mutants were recovered from strain JH1 in both the in vitro and semi–in vitro systems, the genetic alterations involved were different from the mutations identified in the in vivo VISA derivatives of strain JH1 (56). Similar conclusions were reached in several additional studies (33, 46).

VISA isolates were also identified in methicillin-susceptible *S. aureus* (MSSA) (57).

Decreased virulence and VISA-type resistance. Given the frequent therapeutic failure of VISA infections, the demonstration of decreased virulence in all VISA isolates (18–21) tested so far came as a surprise. In the VISA strain SG-R, belonging to the MRSA clone USA300, there was massive downregulation in the transcription of virulence determinants, including seven of the major virulence genes of *S. aureus*. Most importantly, normal or even above normal transcription of the same genes was obtained when the resistant isolate SG-R was "converted" to vancomycin susceptibility — by complementation experiments in the laboratory. These experiments demonstrate that decreased virulence was associated with the same mutated gene that was also responsible for the increased vancomycin MIC value. It was proposed that downregulation of virulence may represent a "stealth" strategy of the bacteria to evade surveillance by the host immune system (28).

Arrival of VRSA. The first *S. aureus* isolates exhibiting high-level vancomyin resistance (MIC ≥16 μg/ml) were described in 2002 in Michigan and Pennsylvania and in 2004 in New York (8). The strains carried plasmid-borne copies of the transposon Tn*1546,* which was acquired from vancomycin-resistant *Enterococcus faecalis.* Most strains appeared in a unique epidemiological scenario in diabetic wounds of patients that were infected by both vancomycin-

resistant enterococci (VRE) and VRSA. As of the end of 2013 the number of VRSA isolates in the United States was 13 (58). Twelve of the thirteen Tn*1546*-containing *S. aureus* strains belong to the clonal complex CC5, an MRSA lineage that has been repeatedly isolated from clinical specimens (59). The most recently identified VRSA, which was recovered in Delaware, belongs to the lineage USA1100 (CC30) (58). Full genome sequencing of VRSA isolates has been described (59, 60). A VRSA isolate was also recently identified in Portugal, also in the diabetic wound of a patient (61). Additional reports from other parts of the world have not yet been confirmed.

The biochemical mechanism of action of vancomycin is based on the high affinity of this antibiotic for the D-alanyl-D-alanine (D-ala-D-ala) residue, a ubiquitous component of the bacterial cell wall precursor Lipid II. The mechanism of resistance identified in the Tn*1546*-based antibiotic resistance was shown to involve alteration of this dipeptide residue from D-ala-D-ala to D-alanyl-D-lactate (D-ala-D-lac), a dipeptide with substantially lower affinity for the antibiotic (62, 63).

Biochemical studies with the MRSA strain COL carrying plasmid-borne copies of Tn*1546* demonstrated that this strain produced the same altered cell wall already described in VRE strains. The terminal D-ala-D-ala residues in the resistant bacterium were replaced by D-ala-D-lac, a structure to which the antibiotic has a greatly decreased affinity (64). This particular strain also carried the *mecA* determinant. Interestingly, the gene product of the *mecA* determinant penicillin-binding protein 2A (PBP2A), a key component of the β-lactam resistance mechanism in MRSA, was unable to utilize the structurally altered cell wall precursor. Cell wall synthesis utilizing the altered D-ala-D-lac cell wall precursors was catalyzed in this strain by the native PBP2 of *S. aureus* (64). These findings indicate some degree of antagonism between the *mecA*-dependent and Tn*1546*-based resistance mechanisms.

A model for common features of vancomycin resistance in VISA and VRSA. Since genetic and other evidence indicates that the VISA-type resistance also involves the staphylococcal cell wall, it was tempting to speculate about a biochemical mechanism in VISA-type resistance in terms of some modification of cell wall structure. In order to allow cell wall synthesis to continue in VISA strains in the presence of vancomycin, the VISA mutants must somehow protect Lipid II, which carries the building blocks of cell wall peptidoglycan terminating with the D-ala-D-ala residues. One way of achieving this could be to introduce into the mature cell wall of VISA isolates an excess of D-ala-D-ala–terminating cell wall chains, which could then act as false targets for the antibiotic and trap vancomycin at sites that are distant from the sites of bacterial cell wall biosynthesis. Mutations that decrease crosslinking of the staphylococcal cell wall could potentially produce excess D-ala-D-ala residues. In fact, chemical analysis of the cell wall of strain JH9, one of the VISA mutants recovered from the blood stream of a patient who underwent extensive chemotherapy, was shown to contain peptidoglycan with decreased crosslinking and with greatly decreased activity of PBP4, a transpeptidase with an important role in the crosslinking of staphylococcal peptidoglycan (29). A gradual decrease in the crosslinking of peptidoglycan, i.e., the production of free D-ala-D-ala residues, was also demonstrated in laboratory mutants of the MRSA strain COL that were selected for increasing levels of vancomycin resistance (65). However, analysis of the cell wall composition of strain SG-R, another genetically characterized VISA isolate, did not show similar extensive alterations in the cell wall peptidoglycan (28). A selective enrichment of peptidoglycan

in D-ala-D-ala residues in the proximity of cell wall synthetic sites may not be resolvable by currently used analytical techniques and is likely to require more complex experimental design (9).

In an experimental test of the "false target" hypothesis described above, fluorescence-labeled vancomycin was used to determine the amount of antibiotic bound by resistant and susceptible *S. aureus* isolates. Both laboratory mutants and VISA isolates bound more vancomycin; however, the amounts bound were not proportional to the MIC value of the bacteria (66). In a more sophisticated design of the vancomycin binding experiment, it was possible to demonstrate that in VISA strains, the rate of arrival of vancomycin molecules to sites of staphylococcal cell wall synthesis at the bacterial septum was delayed in resistant bacteria due to the presence of excess D-ala-D-ala residues, which could capture and slow down the progress of the antibiotic to the site of cell wall biosynthesis. In this model, the false binding sites (D-ala-D-ala residues) that actually contribute to the increased MIC value are located close to the cell wall synthetic sites at the bacterial septum. With this modification of the model taken into account, the vancomycin binding test produced data that were consistent with the vancomycin MIC value of the bacteria (66).

Summary

The worldwide spread of multidrug-resistant MRSA clones during the past several decades has led to the frequent use of vancomycin to treat MRSA infections. This greatly increased selective pressure resulted in the emergence of MRSA isolates with reduced susceptibility to vancomycin — the so-called VISA strains — and, most recently, to the appearance of VRSA strains with high-level resistance to the antibiotic. VISA strains represent bacterial mutants with modestly increased vancomycin MIC (3–8 μg/ml), slightly greater than the susceptibility breakpoint. VISA strains emerge in vivo during the chemotherapy of patients with vancomycin. We describe the clinical history of a vancomycin-treated MRSA infection, which illustrates the stepwise appearance of mutants initially with a heterogeneous and subsequently with a more homogeneous VISA-type resistance. VISA isolates have been identified in each one of the major multidrug-resistant MRSA clones, and genetic analysis identified mutations in determinants that control the biosynthesis of bacterial cell wall and/or mutations in the ribosomal gene *rpoB* as the most frequent genetic alterations associated with the VISA phenotype. The most recently identified VRSA isolates with a vancomycin MIC ≥100 μg/ml were identified in diabetic wounds coinfected by resistant *E. faecalis* and MRSA. In spite of their basic differences, the biochemical mechanisms of VISA and VRSA strains appear to share several common features.

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