Alternative Mutational Pathways to Intermediate Resistance to Vancomycin in Methicillin-Resistant Staphylococcus aureus

Celine Vidaillac,1,a Susana Gardete,4,8 Ryan Tewhey,5 George Sakoulas,6 Glenn W. Kaatz,1,2,3 Warren E. Rose,7 Alexander Tomasz,4 and Michael J. Rybak1,2

1Anti-Infective Research Laboratory, Eugene Applebaum College of Pharmacy and Health Sciences, and 6School of Medicine, Wayne State University, and 7John D. Dingell V A Medical Center, Detroit, Michigan; 8The Rockefeller University, New York, New York; 4Scripps Translational Science Institute, The Scripps Research Institute, and 6University of California–San Diego School of Medicine, La Jolla, California; 6University of Wisconsin, Madison; and 3ITQB, Oeiras, Portugal

(See the editorial commentary by Deresinski on pages 7–9.)

Background. We used 2 in vitro experimental systems to compare phenotypic and genotypic changes that accompany selection of mutants of methicillin-resistant Staphylococcus aureus (MRSA) strain JH1 with low-level vancomycin resistance similar to the type found in vancomycin-intermediate S. aureus (VISA).

Methods. The previously described MRSA strain JH1 and its vancomycin-intermediate mutant derivative JH2, both of which were recovered from a patient undergoing vancomycin chemotherapy, were used in this study. Mutants of JH1 were selected in vitro by means of a pharmacokinetic/pharmacodynamic (PK/PD) model of simulated endocardial vegetations (SEVs) and by exposure to vancomycin in laboratory growth medium. Phenotypic abnormalities of JH1 mutants generated by each in vitro experimental system were compared to those of JH2, and whole genomes of 2 in vitro JH1 mutants were sequenced to identify mutations that may be associated with an increased vancomycin minimum inhibitory concentration.

Results. JH1R1 was selected from the PK/PD model, and JH1R2 was selected in laboratory growth medium. Both mutants displayed reduced vancomycin and daptomycin susceptibility and phenotypic alterations (eg, thicker cell walls and abnormal autolysis) that are typical of in vivo VISA mutants. Genome sequencing of JH1R1 identified point mutations in 4 genes, all of which were different from the mutations described in JH2, including 1 mutation in yycG, a component of the WalKR sensory regulatory system. Sequencing of the JH1R2 genome identified mutations in 7 genes, including 2 in rpoB.

Conclusion. Our findings indicate that JH1 is able to develop VISA-type resistance through several alternative genetic pathways.

Keywords. PK/PD model; JH1; VISA; genetic alterations; phenotypic alteration; genome sequencing; cell wall thickness; pharmacokinetics/pharmacodynamics; vancomycin resistance.

Staphylococcus aureus has remained one of the most important human pathogens in our era, causing a wide variety of infections, both in hospitals and in the community. The success of S. aureus as a pathogen is based on its possession of an impressive number of virulence factors and its extraordinary capacity to adapt and survive under different hostile conditions [1]. Since the introduction of antimicrobial therapy, this species has shown a remarkable propensity to develop resistance to antibiotics rapidly, including antistaphylococcal penicillins, resulting in the emergence of methicillin-resistant S. aureus (MRSA) strains, which commonly
are also resistant to several additional antibiotics, making them truly multidrug resistant. The number of therapeutic options against MRSA infections has significantly decreased over time, with the glycopeptide vancomycin currently the primary treatment. The increasing use of vancomycin has predictably led to the emergence of resistance and to the consequent reduction in its efficacy against MRSA in the clinical environment. The first S. aureus strain with intermediate resistance to vancomycin (VISA) was reported in Japan, in 1997 [2], and other strains were subsequently observed in many other countries [3]. VISA-type resistance is associated with a minimum inhibitory concentration (MIC) of 3–8 µg/mL, and the mechanism appears to involve an adaptive process accompanied by mutations in several genetic determinants. These mutations differ from one VISA isolate to another and may, to some degree, reflect the different genetic backgrounds of the particular MRSA lineage [3–7].

Thus, mutation in the graS gene was identified in VISA-type mutants in MRSA strains belonging to sequence type 239 (ST239) and ST5 [8, 9]. Other VISA isolates, belonging to ST239, ST247, and ST5, carried a mutated yycF, part of the 2-component sensory regulatory system WalKR [5, 6, 10]. Mutation in the β subunit of rpoB was associated with resistance in strains belonging to ST105 and ST239 [11]. The VISA-type mutants studied in this communication emerged in strain JH1, which belongs to the subtype of the US/Japan clone ST105 [12]. The first VISA mutant, JH2 (vancomycin MIC, 3 µg/mL), was recovered from the bloodstream of a patient undergoing vancomycin therapy and carried a mutation in yycF [4], a gene that appears to be a negative regulator of the 2-component sensory regulatory system vraSR [7, 13].

Despite this variation in primary genetic determinants, all VISA isolates described so far were shown to exhibit common abnormalities in a variety of morphological and physiological properties, such as thickness of cell wall, abnormal cell separation at the end of cell division, and abnormal rates of autolysis [3, 7, 12].

The primary purpose of the study described here was to test whether, under selective pressure, the same JH1 parental strain would develop VISA-type resistance linked to the same genetic determinant yycF that was identified in vivo in the VISA mutant JH2, which would indicate that the genetic background of an S. aureus strain may restrict the mechanism of VISA-type resistance to mutations in a unique set of preferred genetic determinants.

To test this hypothesis, the parental strain JH1 was exposed to vancomycin selective pressure in 2 in vitro experimental systems. The first system was the pharmacokinetic/pharmacodynamic (PK/PD) model with simulated endocardial vegetations (SEVs) [14], which was developed specifically to mimic human PK antimicrobial exposures. Strain JH1 was inoculated at a high cell concentration (10⁶ colony-forming units [CFU]) into a large number of SEVs, which were submerged in growth medium and exposed to a simulated regimen involving 500 mg of vancomycin every 12 hours for 30 days in order to reproduce the PK/PD of vancomycin therapy in humans [15]. In another experimental system, strain JH1 was exposed to static but increasing concentrations of vancomycin in laboratory growth medium.

The second purpose of our study was to test whether the mutants recovered from the 2 in vitro experimental systems also exhibited phenotypic abnormalities typical of in vivo VISA isolates [3, 7, 12]. In 2 of the mutants, mutant JH1R1, recovered from the PK/PD model, and mutant JH1R2, isolated under laboratory growth conditions, the nature of genetic alteration was also determined by whole-genome sequencing.

### MATERIAL AND METHODS

#### Strains and Growth Conditions

S. aureus strains used in this study are listed in the Table 1. Tryptic soy agar (TSA; Difco Laboratories, Detroit, MI), brain heart infusion (BHI; Difco) agar, Mueller-Hinton (MH; Difco) agar, and Luria-Bertani (LB) broth were used for routine growth of bacterial strains, selection of resistant populations, and vancomycin population analysis profiles (PAPs). MH broth (Difco) supplemented with 25 mg/L calcium and 12.5 mg/L magnesium was used for antibiotic simulations in the in vitro PK/PD model and for susceptibility testing with vancomycin. For oxacillin or daptomycin MIC determinations, MH broth was supplemented with 4% NaCl or 50 mg/L calcium, as recommended by Clinical and Laboratory Standards Institute guidelines [16, 17]. Vancomycin and oxacillin were obtained from Sigma Aldrich (St. Louis, MO). Antibiotic stock solutions were freshly prepared following the recommendations of the manufacturer.

#### In Vitro PK/PD Model

SEVs consisted of human cryoprecipitate and platelets (American Red Cross, Detroit, MI), bovine thrombin (Sigma Aldrich), and a bacterial inoculum (approximately 10⁹ CFU/g of SEV). SEVs containing approximately 3–3.5 g/dL of albumin content and 6.8–7.4 g/dL of protein [18] were attached to a sterile monofilament line and suspended through ports in a 250-mL 2-compartment glass model maintained at 37°C. A magnetic stir bar ensured adequate mixing of medium, and peristaltic pumps (Masterflex; Cole-Parmer Instrument, Chicago, IL) were set to simulate the vancomycin half-life (6 hours). The simulated antibiotic regimens used were vancomycin 500 mg every 12 hours (maximum concentration, 20 µg/mL) for 30 days. SEVs were removed every 24 hours prior to the next dose interval and homogenized with trypsin. Serial dilutions of the bacterial samples were performed in physiologic saline, and viable counts (log₁₀ CFU/g) were determined by spreading 20 µL of the appropriate dilutions on TSA plates. Bactericidal activity was defined as a >3 log₁₀ decrease in the initial inoculum [19].
Susceptibility Testing and Vancomycin PAP

To assess the development of resistance, 250-µL samples of SEVs were plated daily onto BHI and MH agar plates containing vancomycin or daptomycin at 3 times the initial MIC. Plates were incubated at 35°C, and isolates growing after 24–48 hours of incubation were further evaluated. Vancomycin and daptomycin MICs were determined in duplicate for 5 consecutive days for each of the selected resistant isolates in order to test their stability. Vancomycin, daptomycin, and oxacillin MICs of the overall population recovered from the SEVs were also assessed on a daily basis, using both the Etest and the broth microdilution method [16].

S. aureus ATCC 25 213 was used as a quality control strain. Finally, vancomycin PAPs were performed as previously described [20].

In Vitro Selection of a JH1 Derivative With Decreased Susceptibility to Vancomycin

JH1R2 is a laboratory-derived mutant of JH1 obtained by step selection with increasing concentrations of vancomycin. Briefly, an overnight culture of JH1 was plated at various dilutions (10⁻¹, 10⁻², 10⁻³, and 10⁻⁴) on TSA plates containing increasing concentrations of vancomycin. The plates were incubated at 37°C for 48 hours. A colony was selected from a plate containing 1 µg/mL of vancomycin and was grown for 2 consecutive days in tryptic soy broth (TSB) supplemented with 1 µg/mL of vancomycin for stabilization. On the third day, an overnight culture grown with 1 µg/mL of vancomycin was diluted (1:500) in TSB supplemented with 2 µg/mL of vancomycin. The culture was grown for 2 consecutive days in TSB supplemented with 2 µg/mL of vancomycin and used for further phenotypic and genotypic characterization.

Pulsed-Field Gel Electrophoresis (PFGE)

To confirm the clonal identity of the selected mutants, PFGE was performed on Smal-digested DNA. Gels were run at 6 V/cm at a temperature of 14°C and an included angle of 120° on a 1.2% agarose gel with pulse times of 5–35 seconds for 21 hours. The clonal identity of the isolates were determined by visual inspection of the gel [21].

Triton X-100–Stimulated Autolysis

Cells were grown to mid-exponential phase, chilled in an ice-ethanol bath, harvested, and washed with ice-cold water. Cells were then suspended to an OD₆₀₀ of 1.0 in lysis buffer (50 mM glycine buffer, pH 8.0, containing 0.01% Triton X-100) as previously described [22]. The rate of autolysis was measured at 37°C every 15 minutes for 3 hours as a decrease in OD₆₀₀.

Biofilm Quantiﬁcation

Biofilm production was assessed for the mutants, using the crystal violet method, as described elsewhere [23]. Strains ATCC 35 556 (methicillin-susceptible S. aureus), NARSA 101 (methicillin-resistant Staphylococcus epidermidis [MRSE]), and ATCC 12 228 (MRSE) were used as controls, and cut offs for high-, medium-, and low-biofilm–producing strains were determined as previously described [24].

Susceptibility to Human Cathelicidin LL-37 and Platelet Microbicidal Protein (tPMP)

Human LL-37, a cationic cathelicidin antimicrobial peptide prevalent in skin and neutrophils, was purchased from AnaSpec (Fremont, CA). The tPMPs were prepared from freshly collected rabbit platelets and the bioactive equivalency determined as previously described [25, 26]. Bacteria were

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Selection Method</th>
<th>MIC, µg/mL</th>
<th>Biofilm Production&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mutations Associated With VAN Resistance</th>
<th>Cell Wall Thickness</th>
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<tr>
<td></td>
<td></td>
<td>VAN DAP OXA RIF</td>
<td></td>
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<tr>
<td>JH1 In vivo (patient isolate)</td>
<td>1 0.25 0.75 0.01</td>
<td>1</td>
<td>NA</td>
<td>yvgF (H164R), rpoB (D471Y, A473S, A477S, E478D), rpoC (E854A), SaurJH1-0530 (frameshift), saurJH1-0745 (I29L), saurJH1-1650 (S14I)</td>
<td>31 ± 6.6</td>
</tr>
<tr>
<td>JH2 In vivo (patient isolate) (28 d of VAN therapy)</td>
<td>4 1 25 16</td>
<td>0.373</td>
<td>yycG (G223D), SaurJH1-0530 (frameshift), saurJH1-0745 (I29L), saurJH1-1650 (S14I)</td>
<td>29.6 ± 4.8</td>
<td>14</td>
</tr>
<tr>
<td>JH1R₁ SEV model (25 d of VAN 500 mg every 12 h)</td>
<td>3 1 0.75 0.01</td>
<td>1.366</td>
<td>yycG (G223D), SaurJH1-0530 (frameshift), saurJH1-0745 (I29L), saurJH1-1650 (S14I)</td>
<td>35.8 ± 7.2</td>
<td>38</td>
</tr>
<tr>
<td>JH1R₂ In vitro VAN plate (4 µg/mL)</td>
<td>3 1 16 24</td>
<td>0.344</td>
<td>rpoB (R484C, N641K)</td>
<td>35.8 ± 7.2</td>
<td>38</td>
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Abbreviations: DAP, daptomycin; MIC, minimum inhibitory concentration; NA, not applicable; OXA, oxacillin; RIF, rifampin; VAN, vancomycin.

<sup>a</sup> OD₆₀₀ ratio of the value for the mutant to the value for JH1.

<sup>b</sup> Data are in nanometers and were obtained from 65 to 278 measurements, depending on the strain.

<sup>c</sup> Data are percentage increase in the cell wall thickness, compared with JH1.
grown to stationary phase (16–20 hours) in LB broth, pelleted, and washed in assay buffer (Roswell Park Memorial Institute medium + 5% LB broth for LL-37; phosphate-buffered saline for tPMP). Initial bacterial inocula of $10^8$ CFU/mL were mixed with peptide concentrations of 288 mg/L (64 μM) for LL-37 and 2.5 mg/L for tPMP in a final volume of 100 μL. The mixture was incubated for 2 hours at 37°C and then plated on blood agar plates to determine the number of surviving bacteria. Results represent 3 separate experiments each performed in duplicate.

**Analysis of Staphyloxanthin Pigment Production**

Bacteria were grown in BHI broth for 48 hours at 37°C with shaking at 200 rpm. Aliquots (1 mL) were centrifuged for 5 minutes at 11 000 rpm. The supernatant was discarded, pellet washed twice in phosphate-buffered saline, resuspended in 150 μL of methanol, and incubated at 42°C for 30 minutes. After removal of the bacteria by centrifugation, the OD$_{450}$ of the supernatant containing the extracted staphyloxanthin was measured. ATCC 29 213 and ATCC 33 591 were used as control strains. The experimental assay was performed in duplicate.

**Cytochrome C Binding as a Determinant of Relative Surface Charge**

Bacteria were grown overnight to stationary phase in BHI broth. Cells (5-mL aliquots) were recovered by centrifugation, washed twice in 5 mL of MOPS buffer (20 mM, pH 7.0), and resuspended in 1 mL of MOPS buffer. The OD$_{600}$ of a 1:10 dilution was measured and found to be approximately, 0.9–1.0. The volume of bacteria needed to give OD$_{600}$ of 4.0 in 1 mL was added to a clean Eppendorf tube, the volume was adjusted to 0.9 mL with MOPS, and 0.1 mL cytochrome C (5 mg/mL in MOPS) was added. The mixture was vortexed for 10 seconds and incubated at room temperature for 30 minutes. The suspension was centrifuged at 13 000 rpm for 5 minutes, and cytochrome C in the supernatant was quantitated using a spectrophotometer, as previously described [27].

**Hemolysin Assay**

Hemolysin activity was determined on sheep blood agar plates as previously described [28].

**Transmission Electron Microscopy**

Bacterial samples were centrifuged and washed twice with cold sodium phosphate buffer. The pellet was fixed in electron microscopy buffer, postfixed in 1% osmium tetroxide in electron microscopy buffer, and dehydrated before being embedded in Spurr’s epoxy resin. Ultrathin sections of the samples were examined using a Philips CM120 electron microscope and ImageJ 1.39t software. The cell wall thickness and septum were measured (100–150 measurements) from each side of the cell for a total of 4 measurements per cell.

**Whole-Genome Sequencing**

Whole-genome sequencing was performed on a multiplexed lane of an Illumina HiSeq at Scripps Translational Science Institute, using standard multiplexed protocols [29, 30]. More than 3 million 100-bp paired end reads were acquired for each sample using Illumina’s v3 sequencing chemistry. Samples were processed using a hybrid assembly pipeline consisting of both assembly and mapping, allowing us to confidently call single-nucleotide events, insertion/deletions, as well as large structural changes. Reads were first de novo assembled using the overlap consensus assembler EDENA v3 with an overlap of 55 and an end trimming of 10 reads [31]. Final contigs accounted for an average of 2.88 Mb of combined sequence per isolate, spanning across an average of 31 contigs with an n50 of 208 kb. Assembled contigs were mapped back to the JH1 reference genome by using Blat, and the alignments were processed using custom perl scripts. Variation between the reference and de novo contigs were tabulated, and read mapping using BWA was performed against both alleles [32]. The most likely genotype was chosen on the basis of mapping depth and quality statistics. Nonreference alleles were incorporated into the JH1 reference, and a final round of mapping was performed with the Stampy aligner, using BWA premapping [33]. High-quality single-nucleotide variants and small insertion/deletions (indels) were called with the SAMtools/bcftools suite and custom Perl scripts.
RESULTS

Population-Level Changes in Strain JH1 Exposed to Vancomycin in the PK/PD Model

The PK/PD model allowed us to examine alterations in a bacterial population exposed for prolonged times to high concentrations of vancomycin but prior to the appearance of actual VISA-type mutant derivatives. Some of these alterations may represent changes in the phenotype of an invading S. aureus strain exposed to high concentration of vancomycin at sites of infection.

Prior to the appearance of the first mutant, the entire population of bacteria in the SEVs underwent extensive (3-log10) reduction in viable bacterial titer, from the initial value of approximately 10^9 CFU/g to 10^6 CFU/g after 7–9 days of exposure. During the rest of the extended treatment period, the viable titer per SEV fluctuated around 10^6 CFU/g (Figure 1). The stabilization in the titer of viable bacteria that followed the initial and extensive loss of viability did not seem to involve selection of bacterial mutants that were tolerant of or persistently resistant to the bactericidal activity of vancomycin [34]: the surviving bacteria recovered after 13 days of vancomycin treatment showed the same degree of loss in viability when exposed to comparable concentrations of vancomycin in the laboratory (data not shown).

Additional changes that occurred in the entire bacterial population included a gradual decrease in the average colony size and reduced hemolytic activity. Slow growth rate and decrease in hemolysis are typical features of VISA strains emerging in the clinical environment. Also, colony pigmentation gradually changed from beige to white (Figure 1). The alterations in colony size, hemolytic activity, and pigment production appeared to be irreversible in the sense that these properties were retained when bacteria from the SEVs were passaged on TSA in the laboratory (data not shown).

Emergence and Properties of Mutants With Increased Vancomycin MICs

Daily testing of the SEVs for the emergence of resistance led to the identification of bacterial populations with increased vancomycin and daptomycin MICs. Properties of the in vitro-selected mutants JH1R1 and JH1R2 summarized in Table 1 include vancomycin, daptomycin, oxacillin, and rifampin MICs and physiological properties, such as increased thickness of cell wall and capacity to produce biofilms.

Genomes of JH1R1 and JH1R2 were also sequenced to identify mutations that may be associated with the increased vancomycin MICs. Mutated genes are listed in Table 1, together with the mutations identified earlier in the in vivo mutant JH2 [4]. The PFGE profiles of JH1R1 and JH1R2 were indistinguishable from that of the susceptible parental strain JH1 (Figure 2A). Figure 2 also illustrates the altered antibiotic resistance profiles and altered autolytic properties of JH2, JH1R1, and JH1R2. Enrichment of each of the mutant cultures for bacteria with an increased resistance level is documented in the PAPs (Figure 2B). Each of the 3 resistant mutants had abnormal autolytic properties: JH1R1 and JH2 demonstrated slow autolysis, while JH1R2 demonstrated faster autolysis than JH1.

Figure 3 illustrates some additional altered properties associated with VISA-type resistance in JH2, JH1R1, and JH1R2. Compared with JH1, the 3 mutants had thicker cell walls, as determined by electron microscopy of thin sections (Figure 3A). Each mutant had decreased production of staphyloxanthin, which parallels the already noted shift in the color of colonies from beige to white (Figure 3B). The resistant bacteria also had less capacity to bind cytochrome C (Figure 3C) and differed from the parental cells in their susceptibility to killing by the human cathelicidin LL-37 (Figure 3D) and TMP (Figure 3E). These altered properties are similar to the aberrant phenotypes of VISA mutants recovered from clinical specimen in vivo.

DISCUSSION

The development of VISA-type vancomycin resistance in the MRSA strain JH1 was originally observed in vivo, in blood samples of a patient undergoing prolonged vancomycin therapy [4]. Because a collection of blood isolates only represents samples recovered at loci distant from the primary site of infection, in vivo studies may not be optimal to identify all of the changes occurring in the cell populations during vancomycin therapy at the site of infection, such as a damaged heart valve.

Use of the PK/PD model allowed observation of changes in vancomycin-treated bacteria before the appearance of mutants with decreased susceptibility to this antibiotic. In this model, bacteria were entrapped at high bacterial concentrations (10^9 CFU/g) in SEVs, a model mimicking a damaged heart valve, which is the site of the primary lesion in staphylococcal endocarditis [18]. Freshly grown cultures of strain JH1 were entrapped in a large number of SEVs, which were exposed to concentrations of vancomycin comparable to serum concentrations used during patient therapy. SEVs were removed daily from the system and were tested to determine the number of bacteria that survived antibiotic treatment, possible alterations in the morphology and physiological properties of the cells, and the emergence of resistant mutants.
Figure 2. Properties of parental strain JH1 and vancomycin-intermediate *Staphylococcus aureus*–type resistant isolates JH2, JH1R1, and JH1R2. A, Pulsed-field gel electrophoresis banding patterns of SmaI-digested chromosomal DNA. Lanes 1 and 6, λ marker; lane 2, JH1; lane 3, JH2; lane 4, JH1R1; and lane 5, JH1R2. B, Vancomycin population analysis profiles of strains JH1, JH2, JH1R1, and JH1R2. C, Triton X-100–stimulated autolysis was determined as described in Methods.

The findings described in this communication indicate that VISA-type mutants, which were recovered from the SEV model or by means of the completely in vitro technique, shared properties with the VISA-type mutants that appeared in the clinical environment from the same parental strain JH1. In addition to the increased resistance to vancomycin, the resistant phenotype in mutants JH1R1 and JH1R2 included the same complex morphological and physiological changes that are characteristic of VISA mutants recovered during chemotherapy. These included resistance to daptomycin, abnormal rate of autolysis, decreased hemolytic activity, decreased staphyloxanthin production, and decreased capacity to bind cytochrome C, suggesting a more positively charged cell surface.

The most interesting aspect of these findings is that, starting with a common susceptible bacterial strain (ie, JH1), the bacteria acquired comparable levels of vancomycin resistance through at least 3 distinct genetic pathways. In the case of the in vivo isolate JH2, the mutations associated with resistance were completely different from the 4 mutations identified in JH1R1, the mutant derivative of JH1 recovered from the SEV system. The derivative of strain JH1 that was entirely generated in vitro, JH1R2, acquired a comparable level of vancomycin resistance, presumably via the acquisition of mutations in yet another genetic determinant, rpoB. Mutations in rpoB have been identified in clinical and laboratory mutants with VISA-type resistance [4–6, 11]. Thus, the identical genetic background of the parental strain JH1 has allowed evolution of VISA-type resistance through at least 3 different genetic pathways. Our findings are also consistent with the pleiotropic pathways to VISA-type resistance described recently in several clinical VISA isolates [35].

Complementation experiments indicate that the genetic alteration critical for the increased vancomycin MIC of the in vivo isolate JH2 was a mutation in yqfE [4, 7]. One of the mutated genes identified in JH1R1 was yycG, the histidine kinase–encoding gene of the WalKR 2-component sensory regulatory system that had already been identified in the VISA-type resistance of some clinical isolates [5, 6, 10].

The similar, abnormal phenotype—intermediate resistance to vancomycin—among VISA mutants is consistent with the findings that mutations responsible for the increased vancomycin MIC are most often in genetic determinants (such as vraSR, graSR, and walkR) that control the transcription of a large number of genetic determinants that are involved with cell wall synthesis and cell division of the bacteria.

The observations described here document the additional insights that the use of the SEV model can provide for understanding the mechanism of emergence of VISA-type resistance, by allowing documentation of physiological/morphological alterations in the bacteria that precede the appearance of mutations associated with the increased vancomycin MIC. Because of the similarity of the SEV system to the conditions invading bacteria may encounter when growing on a damaged heart valve, further studies of this model may provide additional mechanistic insights into the emergence of vancomycin resistance and the pathology of staphylococcal endocarditis.

Notes

Financial support. This work was supported by the US Public Health Service (grant 2 R01 AI457838-13 to A. T.); Fundação para a Ciência e Tecnologia, Portugal (grant SFRH/BPD/25403/2005 to S. G.); the Palm Beach Infectious Disease Institute, Florida (fellowship to S. G.); and the National Center for Research Resources (grant UL1 RR025774 to R. T.) and the National Institutes of Allergy and Infectious Diseases (grant R21AI0925055 to M. J. R.), National Institutes of Health.

Potential conflicts of interest. G. S. has served on speakers bureaus of or received grant support from Cubist, Forest, and Pfizer. W. R. has served as a consultant for, received grant support from, and served on speakers bureaus of The Medicines Company, Cubist, Astellas, and Visante. M. J. R. has served on speakers bureau of, consulted for, and received grant support from Cubist, Forest, Cepheid, Cereca, Novartis, and Theravance. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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