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Genomic Analysis Reveals a Point Mutation in the Two-Component Sensor Gene *graS* That Leads to Intermediate Vancomycin Resistance in Clinical *Staphylococcus aureus*[▽]

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Methicillin-resistant *Staphylococcus aureus* (MRSA), once restricted to hospitals, is spreading rapidly through the wider community. Resistance to vancomycin, the principal drug used to treat MRSA infections, has only recently emerged, is mainly low level, and characteristically appears during vancomycin therapy (vancomycin-intermediate *S. aureus* [VISA] and hetero-resistant VISA). This phenomenon suggests the adaptation of MRSA through mutation, although defining the mutations leading to resistance in clinical isolates has been difficult. We studied a vancomycin-susceptible clinical MRSA isolate (MIC of 1 μg/ml) and compared it with an isogenic blood culture isolate from the same patient, despite 42 days of vancomycin treatment (MIC of 4 μg/ml). A whole-genome sequencing approach allowed the nearly complete assembly of the genome sequences of the two isolates and revealed only six nucleotide substitutions in the VISA strain compared with the parent strain. One mutation occurred in *graS*, encoding a putative two-component regulatory sensor, leading to a change from a polar to a nonpolar amino acid (T136I) in the conserved histidine region of the predicted protein. Replacing the *graS* allele of the vancomycin-susceptible parent strain with the *graS* allele from the VISA derivative resulted in increased vancomycin resistance at a level between those of the vancomycin-susceptible *S. aureus* and VISA clinical isolates, confirming a role for *graRS* in VISA. Our study suggests that MRSA is able to develop clinically significant vancomycin resistance via a single point mutation, and the two-component regulatory system *graRS* is a key mediator of this resistance. However, additional mutations are likely required to express the full VISA phenotype.

Methicillin-resistant *Staphylococcus aureus* (MRSA) emerged as a major nosocomial pathogen in the early 1970s, necessitating the increased use of vancomycin. Fully vancomycin-resistant MRSA resulting from the acquisition of the enterococcal *van* genes has been described (34); however, such strains are rare. In contrast, vancomycin treatment failure due to vancomycin-intermediate *S. aureus* (VISA) or hetero-resistant VISA (hVISA) is being increasingly recognized (4, 15, 30), but the underlying molecular mechanisms of this clinically important low-level resistance remain to be fully elucidated.

VISA and hVISA were first reported in 1997 (11, 12) and are being increasingly reported globally (30). In many cases, hVISA (defined by vancomycin population analysis profile [PAP] testing [35]) or VISA (defined as having a vancomycin broth MIC of 4 to 8 μg/ml [5]) have been detected after a patient, initially infected with a vancomycin-susceptible strain, remained unwell despite vancomycin treatment. Subsequent cultures revealed MRSA which had evolved to hVISA or VISA (13).

Phenotypically, hVISA/VISA strains have thickened cell walls (7, 8, 13), reduced autolytic activity (13, 26, 29), increased production of abnormal muropeptides, increased numbers of D-Ala-D-Ala residues, and reduced peptidoglycan cross-linking (9, 29). Although the phenotypic changes have been well characterized, the genetic changes leading to the hVISA/VISA phenotype are poorly understood. Microarray data suggest that global regulators are involved in the expression of the hVISA/VISA phenotype, often leading to cell wall thickening (18, 19, 22, 28). Attempts to define the mutations causing resistance by sequencing loci such as *vraSR*, *saeSR*, and *agr*, known to be involved in global regulation, have not been successful (14).

Recently, a number of mutations were detected in the VISA strain JH9 compared to the vancomycin-susceptible strain JH1. These mutations had been acquired in vivo during persistent infection (24). A potentially important mutation was detected in the *vraSR* operon which has been linked to the activity of the "cell wall stimulon." Although mutations in this operon were found in a small number of additional VISA isolates by Mwangi et al. (24), we found no mutations in the *vraSR* operon among our pairs of vancomycin-susceptible and -resistant clinical isolates (14), suggesting that other mutations are responsible for resistance in our strains. In addition, confirmation of the impact of the mutation found by Mwangi et al. by the introduction of the mutation into a sensitive strain was not performed. Neoh et al. recently sequenced hVISA (Mu3) and VISA (Mu50) strains isolated from different patients and found 16 nucleotide differences (25). A mutation in the response regulator *graR* was linked to a change in vancomycin resistance from hVISA to VISA status; however, introduction of the mutation into a vancomycin-susceptible strain did not alter susceptibility (25), suggesting that the mutation found in

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Property(ies)	Source or reference
Strains		
Isolate pairs		
JKD6009	VSSA strain used for genome sequencing	13
JKD6008	VISA strain used for genome sequencing	13
JKD6000	Isolate pair 2, VSSA	13
JKD6001	Isolate pair 2, VISA	13
JKD6021	Isolate pair 3, VSSA	13
JKD6023	Isolate pair 3, VISA	13
JKD6052	Isolate pair 4, VSSA	13
JKD6051	Isolate pair 4, hVISA	13
JKD6004	Isolate pair 5, VSSA	13
JKD6005	Isolate pair 5, hVISA	13
Others		
RN4220	*S. aureus* strain capable of stably maintaining shuttle plasmids	17
JKD6112	In vitro-derived VISA strain (vancomycin MIC of 4 μg/ml) from JKD6009 by incremental broth culturing	This study
JKD6118	In vitro-derived VISA strain (vancomycin MIC of 4 μg/ml) from JKD6009 on vancomycin gradient plates	This study
JKD6196	JKD6009 Δ*graRS*	This study
JKD6205	JKD6196 with pCU1	This study
JKD6206	JKD6196 with pJKD6151	This study
JKD6207	JKD6196 with pJKD6148	This study
JKD6208	JKD6009 with point mutation in the *graS* product, resulting in T136I mutation	This study
Escherichia coli DH5α	F^- *endA1 hsdR17*(r_K^- m_K^-) *thi-1* λ^- *recA1 gyrA96 relA1 rhoA supE44 deoR* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*	NEB
Plasmids		
pCU1	Ap^r Cm^r, *E. coli*/*S. aureus* shuttle-cloning vector	2
pJKD6148	pCU1 HindIII Ω (3-kb PCR product from JKD6008 containing the region upstream of SACOL0715 to the region downstream of SACOL0717, generated using primers insertF and insertR); contains the T136I point mutation	Recombination
pJKD6151	pCU1 HindIII Ω (3-kb PCR product from JKD6009 containing the region upstream of SACOL0715 to the region downstream of SACOL0717, generated using primers insertF and insertR); does not contain a point mutation	Recombination
pKOR1	*E. coli*/*S. aureus* shuttle vector for the construction of allelic-exchange mutants	3
pJKD6191	pKOR1 with the *graRS* knockout construct	This study
pJKD3138	pKOR1 with the *graRS* loci from JKD6008, generated with DAP1367_attB1 and DAP1365_attB2	This study

graR could not fully explain the resistant phenotype, and additional mutations are required for resistance in strain Mu50. The recent development of high-throughput genome sequencing technologies has significantly reduced the time and cost of complete bacterial genome sequencing. In this study, we have performed whole-genome comparisons of a vancomycin-sensitive and -resistant pair of clinical MRSA isolates from the same patient who experienced vancomycin treatment failure. We then used allelic replacement to test the contribution of a single nucleotide substitution found in a putative two-component regulatory locus for increased vancomycin resistance.

MATERIALS AND METHODS

Bacterial strains for whole-genome sequencing. The *S. aureus* strains JKD6009 and JKD6008 were isolated from a hospitalized patient who developed a postsurgical wound infection and subsequent MRSA bacteremia and native valve endocarditis, despite treatment with vancomycin. JKD6009 was a surgical wound isolate obtained before the exposure of the patient to glycopeptide antibiotics. JKD6008 was a blood culture isolate obtained after 42 days of vancomycin therapy. Both isolates were multilocus sequence type ST239, *spa* type 3, identical by pulsed-field gel electrophoresis (PFGE), and multiresistant—specifically, resistant to ciprofloxacin, tetracycline, co-trimoxazole, and gentamicin (13). JKD6009 is vancomycin susceptible (vancomycin broth MIC of 1 μg/ml; PAP area under the curve ratio of <0.9) (35). JKD6008 is a VISA isolate with a vancomycin MIC of 4 μg/ml (5) (Table 1).

454 Genome sequencing and mutation detection. Genomic DNA was extracted from JKD6008 and JKD6009 using the GenElute bacterial genomic DNA kit (Sigma) according to the manufacturer's instructions and then subjected to single-read 454 pyrosequencing using the GS-20 sequencing system as described previously (21). Sequences were assembled using Newbler software with reference to the MRSACOL genome sequence (GenBank accession number CP000046). All large contigs (≥500 bp) were joined by 17 N′s to generate pseudomolecules for JKD6008 and JKD6009. For mutation detection, reciprocal BLASTN analysis between each strain was performed for all large contigs of JKD6008 versus the JKD6009 pseudomolecule and vice versa. PCR amplification and Sanger sequencing confirmed potential regions of difference. The oligonucleotides used in this study are listed in Table 2. All mutations were mapped to a location on the MRSACOL genome sequence.

Screening of additional isolate pairs for mutations. The same regions were also PCR amplified and sequenced from four other clinical vancomycin-susceptible *S. aureus* (VSSA) and hVISA/VISA pairs (13) to determine whether mu-

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)	Note(s)
0714F	CAAAGCCAACGAGTTGTTCA	3-kb product, SACOL0714 to SACOL0717
0717R	CTTATTATAACGTTGAAGCTGC	
1694F	TCGACTTGATAAAGCACCTCAA	2.2-kb product, SACOL1694
1694R	AGTCCAAAGGACATCGCATT	
2314F	TCAAAGCTTAACATAGTCCG	1-kb product, SACOL2314
2314R	GTAACCTTACTGGTCTTATGG	
2600F	AGGTTCATTAGGTATGGGCG	1.3-kb product, SACOL2600
2600R	ATTTCTCAAGGATAGGTC	
IGF	CGGTCATGTAGCATTTGTTGA	582-bp product, intergenic region
IGR	TGGAGCTGTATTGAGCACGGC	
insertF	GCCAAGCTTCAAAGCCAACGAGTTGTTCA	To generate an insert for cloning into pCU1
insertR	GCCAAGCTTCTTATTATAACGTTGAAGCTGC	To generate an insert for cloning into pCU1
DAP1367_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTGAAATTTT AAAACATTTGC	To generate an insert for cloning into pKOR1
DAP1365_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTATAA CGTTGAAGCTGC	To generate an insert for cloning into pKOR1
DAP852	CAAAGCCAACGAGTTGTTCA	To confirm the generation of the *graRS* knockout of JKD6196 and the allelic exchange in JKD6208
DAP1436	CGACTTGTGAGCCTTCCTTTA	To confirm the generation of the *graRS* knockout of JKD6196 and the allelic exchange in JKD6208

tations found in JKD6008 were also detected in the same loci in other hVISA/VISA strains of the same genetic background (Table 1, isolate pairs 2 to 5).

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Staphylococcal strains were stored in glycerol broth at −80°C and normally subcultured twice onto Columbia blood agar (Oxoid) for 48 h before being used in an experiment. Unless otherwise indicated, all *S. aureus* isolates were grown in brain heart infusion broth (BHIB; Oxoid), and *Escherichia coli* was grown in LB broth (Oxoid). When required, media were supplemented with the following antibiotics at the indicated concentrations: for *E. coli*, 100 μg/ml ampicillin; for *S. aureus* RN4220, 10 μg/ml chloramphenicol; and for *S. aureus* clinical isolates, 25 μg/ml chloramphenicol.

Susceptibility tests. Vancomycin MICs were determined by broth microdilution in Mueller-Hinton broth and read at 24 h according to CLSI criteria (5). Using the new criteria, *S. aureus* strains with a vancomycin MIC of 4 to 8 μg/ml were defined as VISA. The vancomycin PAP was determined by serial dilution of an overnight BHIB culture and by inoculation of BHI agar (BHIA) containing 0 to 8 μg/ml of vancomycin. Colonies were counted after incubation for 48 h in air at 37°C and plotted as numbers of CFU/ml versus the vancomycin concentration. A macromethod Etest to test susceptibility to vancomycin and teicoplanin was performed with a 200-μl inoculum onto BHIA, and results were read after 48 h of incubation at 37°C as previously described (33).

DNA methods and molecular techniques. Standard procedures were used for DNA manipulations and molecular techniques (27). *E. coli* plasmid DNA was isolated by the alkaline lysis method according to the manufacturer's instructions (High Pure plasmid isolation kit; Roche). *S. aureus* plasmid DNA was also isolated using alkaline lysis after an initial 2 h of incubation in TES [*N*-tris(hydroxymethyl)methyl-2- aminoethanesulfonic acid] buffer (50 mM Tris HCl, 5 mM EDTA, 50 mM NaCl; pH 8.0) with lysostaphin and lysozyme at 37°C. PCR amplification of DNA was carried out using *Taq*, a DNA polymerase (Roche Molecular Biochemicals). DNA sequencing was performed using the BigDye Terminator version 3.1 cycle sequencing kits (Applied Biosystems), and the reaction mixtures were analyzed with a model 3730 DNA analyzer (Applied Biosystems). Sequencing results were analyzed using Sequencher version 3.0 (Gene Codes Corporation) and Artemis version 6 (Sanger Institute). Nucleotide and amino acid sequence comparisons were performed using the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST), resources at the Sanger Institute (http://pfam.sanger.ac.uk), and the Victorian Bioinformatics Consortium (http://vbc.med.monash.edu.au). PFGE and *spa* typing were carried out as described previously (13).

In vitro generation of VISA from JKD6009. VISA strains were generated from the clinical VSSA isolate JKD6009 by two methods. In the first approach, JKD6009 was sequentially subcultured for 48 h in BHIB with increasing concentrations of vancomycin to generate the VISA strain JKD6112, which was isolated after growth in 7 μg/ml vancomycin. In the second method, JKD6009 was repeatedly subcultured onto a BHIA-vancomycin gradient plate. After incubation, the colonies growing at the highest vancomycin concentrations were transferred to a new gradient plate, resulting in the VISA strain JKD6118. Molecular typing by PFGE and *spa* typing was performed on JKD6112 and JKD6118 to allow comparisons with the parent strain, JKD6009.

Construction of mutants. The cloning and transformation of *E. coli* were carried out using standard techniques. Electroporation of *S. aureus* was performed as previously described (20). Restriction endonuclease and T4 DNA ligase (Promega) reactions were carried out according to the manufacturers' instructions. To test the impact on vancomycin susceptibility of the single-base mutation in *graS* leading to the T136I mutation, two approaches were employed. The *E. coli*/*S. aureus* shuttle vector pKOR1 was used to conduct allelic replacement and allele deletion experiments as previously described (3). The plasmid pCU1 was used for the complementation of deletion mutants (2).

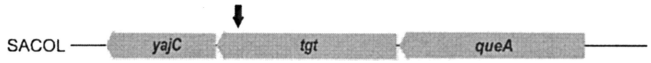
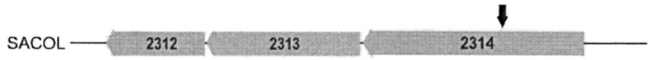
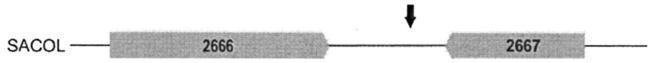
Nucleotide sequence accession numbers. The whole-genome shotgun sequences have been deposited at DDBJ/EMBL/GenBank under accession numbers ABRZ00000000 (JKD6008) and ABSA00000000 (JKD6009).

RESULTS

Whole-genome sequencing of VSSA and VISA and mutation detection. For both the vancomycin-susceptible and -intermediate *S. aureus* strains JKD6009 and JKD6008, respectively, sequencing yielded approximately 2.82 Mb of mappable data and resulted in the assembly of 130 large contigs (>500 bp), with an average 20-fold coverage. After generation of pseudomolecules and reciprocal BLASTN analysis, only six nucleotide substitutions were found between JKD6009 and JKD6008, suggesting that one or more of these mutations in JKD6008 was responsible for the resistant phenotype that had arisen after 42 days of exposure to vancomycin therapy. PCR and Sanger sequencing confirmed the six mutations in JKD6008. The location of each mutation was mapped to positions on the *S. aureus* COL genome (Table 3).

Although six mutations were found in JKD6008 compared to JKD6009, one potentially important mutation led to a predicted

TABLE 3. Summary of mutations

Mutation	Locus (SACOL)	Locus map (SACOL gene identification no.)[a]	Function of locus	Effect of mutation
C to T	SACOL0717		Sensor kinase, homologue of *graS*	T136I
G to A	SACOL0971		*rexA*, exonuclease	Synonymous
T to A	SACOL1694		*tgt*, queuine tRNA-ribosyltransferase	F365Y
C to T	SACOL2314		Sodium/bile acid symporter family protein	P128S; returns to SACOL sequence
G to A	SACOL2600		Hypothetical protein	G268D
C to T	Intergenic		NA[b]	NA

[a] SACOL0715, hypothetical protein; SACOL0716, response regulator homologue of *graR*; SACOL0717, sensor kinase homologue of *graS*; SACOL0718, ABC transporter, homologue of *vraF*; SACOL0720, ABC transporter, homologue of *vraG*; SACOL0970, *rexB* exonuclease; SACOL0971, *rexA* exonuclease; SACOL2312, hypothetical protein; SACOL2313, hydrolase; SACOL2314, sodium/bile acid symporter family protein; SACOL2599, hypothetical protein; SACOL2600, hypothetical protein; SACOL2666, *N*-acetylmuramoyl-l-alanine amidase domain protein; SACOL2667, isochorismatase family protein; downward-pointing arrow, location of mutation.

[b] NA, not applicable.

polar (threonine)- to nonpolar (isoleucine)-amino-acid substitution that was 7 amino acids downstream of the conserved histidine in the SACOL0717 kinase (Fig. 1) at amino acid position 136 (T136I). The mutation in SACOL0971 (*rexA*) was synonymous, while the mutation in SACOL2314 returned the nucleotide sequence to the SACOL reference sequence. The mutation in the intergenic region was downstream of two loci and was considered less likely to affect function. The mutation in SACOL1694 (queuine tRNA-ribosyltransferase) led to an amino acid change at position 365 (F365Y). SACOL1694 is involved in translation and exchanges the guanine residue with 7-aminomethyl-7-deazaguanine in tRNAs with GU(N) anticodons. The mutation in the hypothetical protein SACOL2600 led to a G268D amino acid change. This hypothetical protein has conserved domains with sequence similarity to thioredoxin reductase, an enzyme that maintains thioredoxin in a reduced state and plays a role in the protection of cells against toxic oxygen species (32).

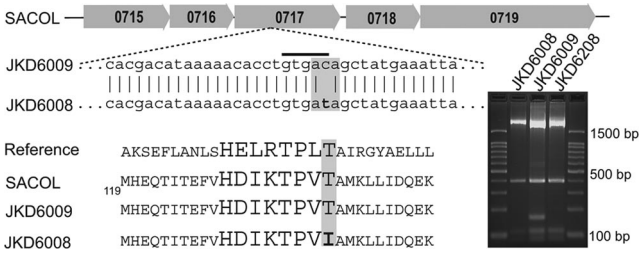


FIG. 1. Schematic diagram of the *graRS* loci. The point mutation in the nucleotide sequence of the sensor region of SACOL0717 is shown (the Tsp45I site is underlined) and is close to the conserved histidine in the sensor histidine kinase. SACOL0715, hypothetical protein; SACOL0716, response regulator (*graR*); SACOL0717, sensor kinase (*graS*); SACOL0718, ABC transporter (*vraF*); SACOL0720, ABC transporter (*vraG*). The reference sequence is the Sanger Pfam sensor histidine kinase reference sequence (http://pfam.sanger.ac.uk). Also shown is an agarose gel displaying the Tsp45I restriction digest pattern of a PCR product generated with oligonucleotides DAP1367_attB1 and DAP1365_attB2, which confirm that the *graS* allele from JKD6008 has been inserted into JKD6009. Lanes 1 and 5, 100-bp ladder (Qiagen); lane 2, JKD6008; lane 3, JKD6009; lane 4, JKD6208.

Screening for mutations in other VSSA and hVISA/VISA isolate pairs. The primers in Table 2 were used for DNA amplification and sequencing of the loci where the mutations in JKD6008 were found, except for the synonymous mutation in SACOL0971. This included sequencing the whole *graRS* loci from all isolates. DNA sequencing of the regions that spanned each of the five mutations in our four other pairs of VSSA and hVISA/VISA clinical strains did not reveal any changes in these regions. To determine whether the same mutations occur in an in vitro-derived VISA strain generated from JKD6009, two VISA strains were generated (JKD6112 and JKD6118; both had a vancomycin broth MIC of 4 μg/ml). By PFGE, isolates JKD6009, JKD6112, and JKD6118 had different banding patterns. JKD6009 and JKD6112 demonstrated a three-band difference, while JKD6009 and JKD6118 had only a single-band difference. The *spa* sequences were identical for the in vitro-derived VISA strains and JKD6009, indicating that the VISA strains were derived from JKD6009. Sequencing of the six loci where mutations were found in JKD6008 did not demonstrate any mutations in VISA strains JKD6112 and JKD6118.

Introduction of the SACOL0717 (*graS*) T136I mutation into JKD6009 increases vancomycin resistance. To test the impact of the T136I mutation on vancomycin resistance, an allelic-replacement experiment was performed by taking *graS* from the VISA strain JKD6008 and using it to replace *graS* in the vancomycin-susceptible parent, JKD6009. The *graRS* loci from JKD6008 were amplified by PCR using primers DAP1367_attB1 and DAP1365_attB2 (Table 2), and the resulting amplicon was cloned into pKOR1 to create pJKD3138. This construct was

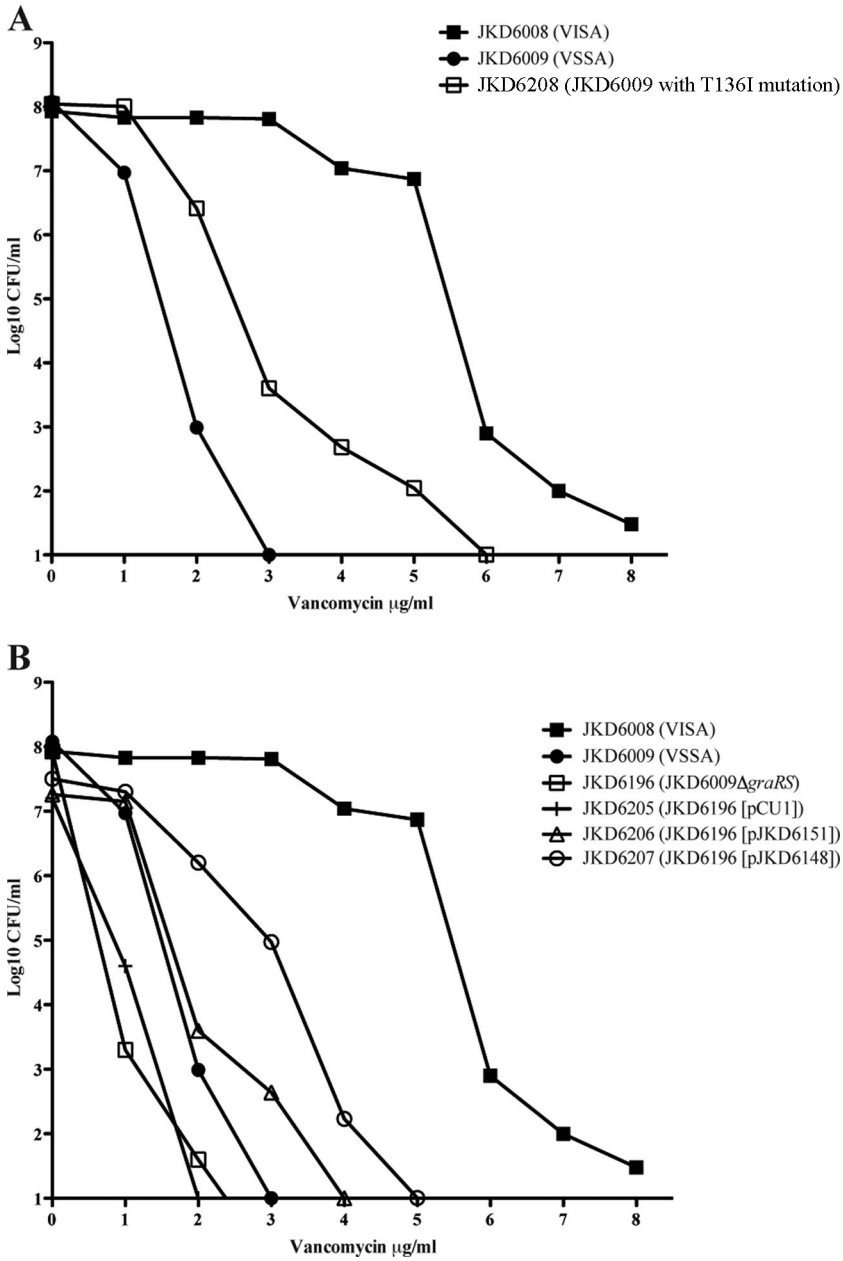


FIG. 2. Vancomycin PAPs for clinical isolate JKD6009 (VSSA), JKD6008 (VISA), and mutant strains. (A) *graS* allelic-exchange strain JKD6208 (JKD6009 with a single-base mutation in *graS* leading to a T136I change), demonstrating increased resistance to vancomycin compared to that of JKD6009. (B) *graRS* knockout strain JKD6196 and JKD6196 complemented with pCU1 (JKD6205), demonstrating increased vancomycin susceptibility compared to that of JKD6009. JKD6196 harboring pJKD6148 (pCU1 with the *graS* sequence from the VISA strain JKD6008), demonstrating increased resistance compared with that of JKD6009.

transformed sequentially into *E. coli* DH5α and then into *S. aureus* RN4220 and finally JKD6009. Several potential double-crossover mutants were analyzed by PCR and restriction fragment length polymorphism analysis, as the T136I mutation disrupts a Tsp45I restriction site. An isolate (JKD6208) with a *graRS* restriction profile matching that of JKD6008 (Fig. 1) was further analyzed by sequencing of the entire locus, including flanking sequences, to ensure that no additional changes had been introduced during homologous recombination.

Vancomycin susceptibility testing of JKD6208 revealed a distinct increase in resistance. The MICs to vancomycin and teicoplanin as determined by the macromethod Etest increased (JKD6009, vancomycin MIC of 2 μg/ml, teicoplanin MIC of 3 μg/ml; JKD6208, vancomycin MIC of 6 μg/ml, teicoplanin MIC of 12 μg/ml), and a change was also observed in the vancomycin PAP curve (Fig. 2 and Table 4). The macromethod Etest was used, as it is more sensitive than standard MIC testing in detecting changes in vancomycin resistance

TABLE 4. Macromethod Etest results for strains[a]

Strain	Vancomycin 2McF Etest MIC (μg/ml)	Teicoplanin 2McF Etest MIC (μg/ml)
JKD6009	2	3
JKD6008	8	8
JKD6208 (JKD6009 with T136I mutation)	6	12
JKD6196 (JKD6009 Δ*graRS*)	1.5	2
JKD6205 [JKD6196(pCu1)]	1.5	2
JKD6206 [JKD6196(pJKD6151)]	3	3
JKD6207 [JKD6196(pJKD6148)]	4	6

[a] 2McF, McFarland standard inoculum of 2.

(33). These observations confirm that the single nucleotide change observed in *graS* was a major contributor to the emergence of this VISA isolate.

In a second approach to explore the contribution of *graRS* to VISA, the locus was disrupted in the susceptible parent JKD6009 by homologous-recombination-mediated deletion, using pKOR1 as previously described (3, 25), to generate the strain JKD6196. To complement the mutation in JKD6196, the *graRS* locus was PCR amplified on a 3-kb fragment from JKD6008 that included a 300-bp region upstream of SACOL0715 and the 3′ end of SACOL0717 using the primers insertF and insertR (Table 2) and cloned into the HindIII site of pCU1. This construct (pJKD6148) was transformed sequentially into *E. coli* DH5α, *S. aureus* RN4220, and then JKD6196 to generate JKD6207. In addition, a control mutant was generated by the amplification and cloning of the identical 3-kb region from JKD6009 (without the mutation in the sensor region) and electroporated into JKD6196 to generate JKD6206. Additionally, pCU1 was electroporated into JKD6196 as an empty vector control (JKD6205). The plasmids extracted from *E. coli* were sequenced to confirm the correct insert sequence before electroporation into RN4220. Testing of the *graRS* knockout, JKD6196, showed a decrease in vancomycin resistance (from 2 μg/ml to 1.5 μg/ml) and teicoplanin resistance (from 3 μg/ml to 2 μg/ml) compared to that of JKD6009, as measured by the macromethod Etest and PAP analysis (Table 4; Fig. 2B). Complementation of JKD6196 with the entire *graRS* locus from JKD6009 (susceptible parent) resulted in a restoration of the original JKD6009 VSSA MIC profile, and complementation of JKD6009 with an empty vector had no effect on the MIC profile (Fig. 2B; Table 4). Complementation of JKD6196 with the *graRS* locus from the VISA strain JKD6008 (strain JKD6207) led to increased vancomycin resistance compared with that of JKD6009 but did not produce an MIC as high as that for JKD6008. Unfortunately, JKD6008 was not electrocompetent, and hence, no genetic experiments could be performed directly on this strain.

DISCUSSION

We have studied an isogenic pair of clinical MRSA isolates obtained from a patient who initially had an MRSA surgical wound infection but who subsequently developed MRSA endocarditis and persistent bacteremia despite 42 days of vancomycin therapy. Although the change in vancomycin MIC in the clinical context was small (from 1 μg/ml to 4 μg/ml), it was linked to an unequivocal failure of antibiotic therapy. By taking a combined comparative genomic and genetic approach, we have shown for the first time that a single base substitution increases vancomycin resistance in an initially vancomycin-susceptible isolate. By sequencing the isogenic pair of clinical isolates obtained from this patient before and after vancomycin treatment failure, we identified a mutation that affects a putative sensor histidine kinase, encoded by *graS*. We then demonstrated by allelic replacement that a single amino acid substitution within the histidine kinase domain was a major factor in the emergence of the hVISA/VISA phenotype from a vancomycin-susceptible strain.

The two-component regulator *graRS* is one of many regulatory systems that are found in *S. aureus*. The functions of *graRS* are only just being uncovered, with recent comparative transcriptomics suggesting that the locus might control at least 248 genes (10). It has been shown to control the expression of the ABC transporters *vraF* and *vraG* (23), two genes that are upregulated in VISA (19). Overexpression of *graRS* has also been linked to the VISA phenotype (6), and recently, a *graR* knockout mutant of the VISA strain Mu50 demonstrated increased susceptibility to vancomycin (23). We have confirmed these findings but have also demonstrated increased susceptibility to glycopeptides in the *graRS* knockout strain generated from the already vancomycin-susceptible strain JKD6009. The recent demonstration that a mutation in the response regulator *graR* is linked to an increase in vancomycin resistance from hVISA to VISA status confirms the importance of the *graRS* loci in the expression of hVISA/VISA (25). Neoh et al. suggested that a mutation in *graRS* could not generate hVISA/VISA from a vancomycin-susceptible strain but could only increase resistance in a strain that already demonstrated the hVISA phenotype, as found in their study (25). However, our study clearly demonstrates that a single nucleotide change in the *graRS* loci is able to generate increased vancomycin resistance in a fully vancomycin-susceptible *S. aureus* isolate (Fig. 2A). Mwangi et al. (24) linked an increase in vancomycin MIC from 1 to 4 μg/ml to seven different point mutations at other sites. These and other data (including our own) suggest that the emergence of the hVISA/VISA phenotype arises from a variety of mutations in different genetic loci.

Although swapping *graS* from the VSSA parent with *graS* from the VISA progenitor (Fig. 2A) caused an increase in vancomycin resistance, it did not generate the expression of the full-resistance phenotype of the clinical isolate JKD6008. Similarly, full resistance was not restored by complementation of the VSSA *graRS* knockout with the VISA *graRS* loci. These data suggest that one or more of the five other mutations may play a role in resistance in JKD6008. Based on predicted function and the location of the mutations, it is not apparent which mutation is most likely to contribute. In particular, three of the mutations (SACOL0971, SACOL2314, and the intergenic region) might not be expected to alter function, unless they encode undiscovered regulatory RNAs. The mutations in SACOL1694 and SACOL2600 are also unlikely to produce the significant phenotypic alterations and global transcriptional changes that are associated with reduced vancomycin susceptibility in JKD6008 (13, 14). It appears that the mutation in *graS* is the major factor leading to reduced vancomycin susceptibility in JKD6008; however, a complete understanding of the impact of each of the additional mutations will require significant work to individually generate the mutations and

assess their impact on resistance. To better understand the pathways to intermediate vancomycin resistance, it will also be important to develop systems for the delivery of DNA to VISA strain JKD6008, as it is refractory to transformation by electroporation, presumably attributable to the cell wall changes noted in this strain (13). Alternative methods, such as conjugation, are currently being explored to address this issue.

Previous studies have focused on the role of the cell wall stimulon and mutations in the *vraSR* operon as important in the expression of the VISA phenotype (18, 22, 24, 28). Of interest, upregulation of *vraS* and related genes was detected in JKD6008, compared to JKD6009, in our previous microarray analysis (14). Because we found no mutations in the *vraSR* operon in JKD6008, we can conclude that mutations in other regions of the genome (including in the *graRS* operon) can lead to the upregulation of the cell wall stimulon.

We generated laboratory-induced VISA strains from JKD6009 to determine whether consistent mutations would lead to VISA in isolates of the same genetic background. The laboratory-derived VISA strains JKD6112 and JKD6118 had different PFGE banding patterns from JKD6009, and mutations were not found in the same loci in these laboratory-derived strains, providing further evidence that mutations in different chromosomal regions are linked to the VISA phenotype. This included sequencing the whole *graRS* locus. This supports the results of our previous microarrays where divergent transcriptional patterns were found, even in closely related strains, suggesting that different transcriptional pathways (and presumably different mutations) are linked to resistance (14). Importantly, there were three or fewer bands of difference in the PFGE profiles, and the *spa* sequences were identical between strains JKD6009, JKD6112, and JKD6118, indicating that JKD6112 and JKD6118 were indeed derived from JKD6009.

The rapid rise of community-onset MRSA (1, 16), and the persistently high prevalence of hospital MRSA (31, 36), will continue to promote increasing vancomycin use. We have demonstrated that a single base substitution can be associated with the evolution of hVISA/VISA from VSSA during persistent infection associated with vancomycin treatment failure. Given the ease with which this evolution occurred, which results in levels of resistance that have been clearly linked to vancomycin treatment failure, it is likely that VISA will remain a major antimicrobial resistance problem. Further comparative and functional genomics will be essential if we are to develop a more complete understanding of the genetics of the VISA phenotype and use this knowledge to improve our strategies for treating infection with *S. aureus*.

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