Contribution of Selected Gene Mutations to Resistance in Clinical Isolates of Vancomycin-Intermediate *Staphylococcus aureus*

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Infections with vancomycin-intermediate *Staphylococcus aureus* (VISA) have been associated with vancomycin treatment failures and poor clinical outcomes. Routine identification of clinical isolates with increased vancomycin MICs remains challenging, and no molecular marker exists to aid in diagnosis of VISA strains. We tested vancomycin susceptibilities by using microscan, Etest, and population analyses in a collection of putative VISA, methicillin-resistant *S. aureus*, and methicillin-sensitive *S. aureus* (VSSA) infectious isolates from community- or hospital-associated *S. aureus* infections ($n = 77$) and identified 22 VISA and 9 heterogeneous VISA (hVISA) isolates. Sequencing of VISA candidate loci *vraS*, *vraR*, *yvqF*, *graR*, *graS*, *walR*, *walk*, and *rpoB* revealed a high diversity of nonsynonymous single-nucleotide polymorphisms (SNPs). For *vraS*, *vraR*, *yvqF*, *walk*, and *rpoB*, SNPs were more frequently present in VISA and hVISA than in VSSA isolates, whereas mutations in *graR*, *graS*, and *walR* were exclusively detected in VISA isolates. For each of the individual loci, SNPs were only detected in about half of the VISA isolates. All but one VISA isolate had at least one SNP in any of the genes sequenced, and isolates with an MIC of 6 or 8 $\mu\text{g/ml}$ harbored at least 2 SNPs. Overall, increasing vancomycin MICs were paralleled by a higher proportion of isolates with SNPs. Depending on the clonal background, SNPs appeared to preferentially accumulate in *vraS* and *vraR* for sequence type 8 (ST8) and in *walk* and *walR* for ST5 isolates. Taken together, by comparing VISA, hVISA, and VSSA controls, we observed preferential clustering of SNPs in VISA candidate genes, with an unexpectedly high diversity across these loci. Our results support a polygenetic etiology of VISA.

Vancomycin has been the mainstay of treatment for invasive methicillin-resistant *Staphylococcus aureus* infections since its introduction more than 50 years ago. Its efficacy started to diminish only about a decade ago, when high-level vancomycin-resistant *S. aureus* (VRSA) and vancomycin-intermediate *S. aureus* (VISA) isolates were first recognized (2, 8). In addition, vancomycin-susceptible *S. aureus* (VSSA) may already harbor a subset of resistant colonies, known as heterogeneous VISA (hVISA), which have also been implicated in adverse clinical outcomes (12). While *vanA* operon-mediated VRSA isolates remain rare (26), VISA and hVISA infections have emerged globally and constitute a more frequent and often underrecognized diagnostic and therapeutic challenge (22).

To date, no molecular marker is available that allows rapid and unequivocal identification of VISA infections, and the detection of reduced phenotypic vancomycin susceptibility can be technically challenging (reviewed in reference 9). Optimal testing algorithms have been controversial, as the disk diffusion method and some automated systems do not always accurately identify and may underestimate VISA and hVISA isolates. Diagnostic alternatives include the use of the Etest. The gold standard to determine hVISA is the population analysis profile (PAP) (33), but this test is time-consuming and appears unsuitable for routine clinical microbiology laboratory diagnostics.

The development of a molecular marker to aid in the diagnosis of strains with reduced vancomycin susceptibility could therefore be an important additional diagnostic tool in determining vancomycin susceptibility and in guiding optimal antibiotic treatment regimens. This approach is still hampered by an incomplete understanding of the underlying mechanisms of VISA evolution. Although it is generally accepted that the thickening of the peptidoglycan layer and abnormal autolytic activity are some of the

major phenotypic alterations in VISA (29), the genetic basis appears multifactorial. It has been suggested that VISA evolves in a stepwise process from VSSA to hVISA to VISA (4, 21). Impaired function of the accessory gene regulator (*agr*) has also been implicated as a predisposing factor for the evolution of vancomycin heteroresistance (24). Several whole-genome sequencing studies have compared sequential *S. aureus* clinical isolates that converted from vancomycin susceptibility to resistance during antibiotic therapy (10, 11, 15, 20). These studies identified accumulation of several mutations in increasingly resistant strains in different loci, including the *vraSR* operon (20), *graRS* (4, 11, 21), *rpoB* (15), and more recently *walRK* (10, 28). For several of these SNPs, complementation studies are lacking to verify their role in modulating vancomycin susceptibility (20). Nevertheless, given this variety in mutations conferring the VISA phenotype, it has been speculated that the individual clonal background of VISA strains may play a role in the acquisition of SNPs in different genes across the genome.

However, few studies have systematically studied sequence variations in all of these combined regions or carried out genotypic and phenotypic comparisons with methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) iso-

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lates. We therefore aimed to determine the prevalence of mutations in putative candidate genes for VISA in isolates with decreased vancomycin susceptibility and contrasted these with a genetically diverse group of MSSA and MRSA isolates to identify patterns that would allow for a genetic screening test for VISA in clinical isolates.

MATERIALS AND METHODS

Sample selection. Putative VISA isolates, collected between 2007 and 2008, were kindly provided by the Centers for Diseases Control (CDC), Atlanta, GA ($n = 14$) as well as the New York City Department of Health and Mental Hygiene (NYC DOHMH; $n = 24$). In addition, 39 *S. aureus* isolates (7 clinical MSSA, 22 colonizing MSSA, 1 infectious MRSA, and 9 colonizing MRSA isolates) were included in all drug susceptibility and genotyping assays for comparisons. These isolates were randomly selected by using a random number generator from a community study on *S. aureus* transmission in northern Manhattan, collected between January 2009 and February 2010 (31). Basic epidemiological data (site of infection, date of birth, hospital, and date of collection) were available for all but the CDC samples.

Antimicrobial susceptibility testing. Drug susceptibilities of all *S. aureus* isolates were determined by using the Kirby-Bauer standard disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines (23). The following antibiotics were tested: penicillin, cefoxitin, oxacillin, erythromycin, tetracycline, levofloxacin, gentamicin, clindamycin, trimethoprim-sulfamethoxazole, and rifampin.

The Etest was carried out using glycopeptide resistance detection strips (bioMérieux) with vancomycin and teicoplanin gradients of 0.5 to 32 $\mu\text{g/ml}$ as described previously (34). A single colony was inoculated in tryptic soy broth (TSB) and incubated at 37°C. At a cell density of 0.5 McFarland, bacteria were streaked evenly onto Mueller-Hinton agar–5% sheep blood plates (BD). Plates were incubated at 37°C for 24 h.

Modified PAPs were carried out as described previously (25, 33). In brief, single colonies were inoculated in TSB and grown overnight at 37°C. These cultures were standardized to an optical density of 0.5 in phosphate-buffered saline, and a 10^4 dilution was spiral plated onto brain heart infusion agar (BD) with the following concentrations of vancomycin (Sigma): 0, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 $\mu\text{g/ml}$. Colonies were counted after the plates were incubated at 35°C for 48 h. The $\log_{10}$ CFU/ml was plotted against the vancomycin concentration, and an area under the time-concentration curve (AUC) was calculated using GraphPad Prism software. The $\text{AUC}_{\text{test}}/\text{AUC}_{\text{Mu3}}$ ratio was calculated, and strains were categorized as VSSA, hVISA, and VISA according to this ratio as follows: VSSA, <0.9 ; hVISA, 0.9 to 1.3; VISA, >1.3 . Mu3 and Mu50 served as hVISA and VISA controls, respectively (8, 33).

Molecular genotyping and sequencing. All isolates were genotyped by PCR sequencing of the repeat region of the *S. aureus* protein A (*spa*) gene as previously described (7). *spa* types were automatically assigned using the Staph Type software (version 1.5; Ridom) and further clustered into *spa* clonal complexes (*spa*-CC) by using the integrated BURP (based upon repeat patterns) algorithm (16, 17) with parameters set as “cluster *spa* types into *spa*-CC if cost distances are less than or equal to 4” and “exclude *spa* types shorter than 4 repeats.” For the selected isolates with ambiguous BURP clustering or clustering as singletons, multilocus sequence typing (MLST) was carried out (5). All *S. aureus* isolates were screened for the presence of the staphylococcal chromosomal cassette (SCC) *mec* in a multiplex PCR assay (18, 19).

For all isolates, full-length forward and reverse sequences were obtained for the genes *vraR*, *vraS*, *yvaF*, SA1703, *graR*, *graS*, *walR*, *walK*, and *rpoB* from PCR-amplified fragments using the primers shown in Table S1 of the supplemental material. For a subset of VISA isolates, *rpoC*, *vraG*, SA1129, SACOL2314, and *tgt* were also sequenced (11, 20). Sequences were aligned and compared to the reference genome N315 (GenBank accession number BA000018) or according to the strain's MLST to reference genomes SACOL (CP000046), MRSA252 (BX571856), MSSA476

TABLE 1 Comparison of Etest MIC and PAP results to define VISA among strains with a vancomycin Etest MIC of ≥ 1.5 $\mu\text{g/ml}$

Etest MIC ($\mu\text{g/ml}$)	No. of strains with MIC ^a	No. of strains with PAP result of:		
		VSSA	hVISA	VISA
1.5	20	18	2	0
2	5	3	2	0
3	8	0	3	5
4	12	0	2	10
6	3	0	0	3
8	4	0	0	4

^a From among a total of 52 isolates.

(BX571857), or USA300 (CP000255) by using SeqMan software (DNASTAR).

Statistical analyses. All statistical analyses were carried out using SPSS version 18 software. Chi-square tests were used for comparisons of dichotomous variables, and Fisher's exact test was used, with an expected cell count of ≤ 5 . A *P* value of <0.05 was considered statistically significant.

RESULTS

Vancomycin susceptibilities by microscan, Etest, and PAP analysis. We first tested susceptibility to vancomycin in our collection of 38 putative VISA isolates, obtained from the CDC and the NYC DOHMH, by microscan and vancomycin-teicoplanin Etest. Microscan identified 20/38 isolates as VISA, with an MIC of ≥ 4 $\mu\text{g/ml}$. By the Etest, 26/38 isolates were VISA, with a vancomycin MIC of ≥ 3 $\mu\text{g/ml}$ (19 isolates with an MIC of ≥ 4 $\mu\text{g/ml}$) (Table 1). To further assess the presence of resistant subpopulations, we carried out modified population analysis profiles on these isolates. PAPs classified 22 isolates as VISA and 9 isolates as hVISA. The hVISA strains were detected among a vancomycin MIC range of 1.5 to 4 $\mu\text{g/ml}$ (Table 1). In contrast, none of the 39 randomly selected *S. aureus* isolates from a community study that were used for comparisons in subsequent sequence analyses (31) was VISA or hVISA by Etest or PAP analysis (data not shown).

Drug susceptibilities and *spa* and MLST genotyping of VISA and VSSA isolates. VISA and hVISA isolates differed from VSSA isolates based on an overall higher frequency of drug resistance to multiple antibiotics, including levofloxacin and trimethoprim-sulfamethoxazole (Table 2). hVISA strains were less frequently resistant to rifampin (2/9; 22%) than VISA isolates (12/22; 54%), although this was not statistically significant ($P = 0.13$). All VISA and VSSA isolates ($n = 78$) were susceptible to linezolid based on Kirby-Bauer testing. Eight VISA isolates had a decreased susceptibility to daptomycin by microscan testing (MIC, ≥ 2 $\mu\text{g/ml}$). All but three hVISA/VISA isolates were methicillin resistant. Of these, 15 isolates harbored SCC*mec* type II and 13 isolates had SCC*mec* type IV.

The genetic background of each strain was determined by combined MLST and *spa* typing. The majority of VISA strains were either Spa-CC1 (ST8; $n = 11$) or Spa-CC5 (ST5; $n = 12$) and were most frequently *spa* type t064 ($n = 9$) or t002 ($n = 8$) (Table 3; see also Table S2 in the supplemental material).

Frequency of SNPs in *vraRS*, *graSR*, *walRK*, and *rpoB* and vancomycin susceptibility. We observed multiple SNPs in the full-length sequences of the *vra* operon (20), *graRS* (11), *rpoB* (15), and *walRK* (10). To account for the high diversity in the genetic background of the strain collection, we determined SNPs

TABLE 2 Resistance to selected antistaphylococcal drugs of VSSA, hVISA, and VISA isolates^a

Drug	No. (%) of isolates, by resistance category		
	VSSA (<i>n</i> = 46)	hVISA (<i>n</i> = 9)	VISA (<i>n</i> = 22)
Penicillin	39 (85)	9 (100)	21 (96)
Tetracycline	6 (13)	2 (22)	3 (14)
Oxacillin	19 (41)	8 (89)	20 (91)
Daptomycin ^b	0 (0)	0 (0)	8 (36)
Linezolid	0 (0)	0 (0)	0 (0)
Rifampin	1 (2.2)	2 (22)	9 (41)
Levofloxacin	10 (22)	9 (100)	18 (82)
Trimethoprim-sulfamethoxazole	13 (28)	9 (100)	20 (91)
Clindamycin	6 (13)	7 (78)	12 (55)
Erythromycin	22 (48)	8 (89)	16 (73)
Gentamicin	2 (4.4)	2 (22)	7 (32)

^a All results were obtained by Kirby-Bauer susceptibility testing, except for daptomycin.

^b For daptomycin, the susceptibility was determined by microscan, with a cutoff for decreased susceptibility of ≥ 2 μ g/ml.

after alignment to the corresponding lineage-specific reference sequences.

We identified 13 distinct nonsynonymous SNPs in nearly half of the VISA (10/22; 45%) and hVISA (4/9; 44%) isolates in the *vraSR* operon (Fig. 1 and Table 3). These SNPs were distributed in *vraR* (K43E, I103T, and D194N), *vraS* (G9V, M192I, and A314T/V), *yvqF* (A59E, L85F, T125I, P174Q, and Y215S), and SA1703 (D65V and N85S). Two of the nonsynonymous SNPs (G9V and A314T/V in *vraS*) were previously reported among VISA isolates from *in vitro* selection experiments that included imipenem pressure (13). None of the other previously described mutations was identified in our isolates (Fig. 1A) (20). Three non-VISA isolates also carried nonsynonymous SNPs in the *vraSR* operon. These included F51C in *vraS* in a community-associated MRSA (CA-MRSA) infection, A152T in *yvqF* in a hospital-associated MRSA (HA-MRSA) infection, and N85S in SA1703. The latter mutation was present in both VISA (*n* = 5) and VSSA (*n* = 1) isolates, all of which were *spa* t064 isolates. There was no apparent epidemiological link between these 6 isolates, except that 2 samples were obtained from the same hospital about 15 months apart.

In *graRS*, we identified 4 nonsynonymous SNPs (E15K and S79F in *graR* and I18I and T136I in *graS*) in 5/22 (23%) of VISA isolates. Both *graR* SNPs and T136I in *graS* have previously been described (11) (Fig. 1B). hVISA and VSSA isolates all lacked mutations in *graRS*.

We detected 5 nonsynonymous SNPs at 3 positions in *walR* (T101S or T101R, V113A, and P216T or P216S) and 13 SNPs in *walK* (S9P, T217K, G223S, A243T, R282C, V383I, S437F, F483I, and H576R). SNPs in *walRK* were again predominantly clustered among VISA strains (11/23; 48%) and less frequently encountered in hVISA (2/9; 22%) (Table 4). Only three VSSA isolates, obtained from HA-MRSA infections, carried a mutation in *walK* (S221P, R374C, or E239K) but not in *walR*. None of the strains shared the same *walRK* SNPs. However, of the 14 isolates with *walRK* mutations, 10 belonged to the ST5 lineage. Upon further comparing the locations of mutations from this current collection and prior studies (10), SNPs did not cluster in particular predicted functional domains of *walRK* (Fig. 1C).

In addition to SNPs in *vraS* and *graR*, the VISA isolate Mu50 also carries an SNP in *rpoB*, which has been shown to modulate the expression of the full VISA phenotype (15, 32). In our collection, all 11 rifampin-resistant isolates harbored at least one SNP in *rpoB* (Table 4), which for the majority of isolates was H481Y (*n* = 8), but also the SNPs H481N, D471G/R483H, and A477D were found. SNPs in *rpoB* were detected in 11/22 (48%) VISA, 2/9 (22%) hVISA, and 2/46 (4.3%) VSSA isolates. Two rifampin-sensitive isolates carried an S464P *rpoB* mutation, which in *in vitro* studies has not been associated with rifampin resistance or changes in the vancomycin MIC (15).

SNP frequencies and clonal backgrounds of isolates. There appeared to be an accumulation of SNPs in either *vraSR* or *walRK*, based on the MLSTs of the isolates: 9/12 (75%) VISA/hVISA isolates of the CC1 lineage harbored SNPs in *vraSR*, as opposed to only 2/12 (17%; *P* = 0.012) SNPs in *walRK*. In contrast, VISA/hVISA isolates of the CC5 lineage more frequently contained SNPs in *walRK* (10/17; 59%) than in *vraSR* (6/17; 35%), although this was not statistically significant (*P* = 0.17).

As most of the observed nonsynonymous SNPs clustered in *walK*, we wanted to further assess if this locus is intrinsically more polymorphic than other regions of the *S. aureus* genome. We compared the frequency of SNPs per 100 bp and the frequency of nonsynonymous to synonymous SNPs of the genes sequenced here to SNPs in their corresponding MLST genes in VSSA isolates (Table 5). While most of the loci tested matched the SNP frequency of MLST genes of 2 to 5 SNPs/100 bp (Table 5), *graR* and *graS* in particular showed higher frequencies, with 7 and 14 SNPs/100 bp, respectively (Table 5).

SNP frequency and vancomycin susceptibility. When combining SNPs from the 4 sequenced loci, all but 1 VISA isolate (21/22; 91%) harbored at least 1 SNP, whereas 7/9 (78%) of hVISA and only 7/46 (15%) of VSSA strains were polymorphic at these combined sites (Table 6). All isolates (*n* = 7) with an MIC of 6 or 8 μ g/ml had at least two nonsynonymous SNPs in the 4 loci tested. For the 3 VISA/hVISA isolates lacking any SNPs, we also sequenced additional VISA candidate genes, but we failed to detect mutations in either *rpoC*, *vraG*, SA1129, SACOL2314, or *tgt* (11). However, these 3 isolates had a microscan vancomycin MIC of 2 μ g/ml. Based on microscan MICs, all isolates with an MIC of ≥ 4 μ g/ml (*n* = 20) contained at least one SNP in the 4 combined regions, compared to 25% of VSSA isolates with an MIC of ≤ 2 μ g/ml (*n* = 14/57; *P* < 0.0001). Using either the microscan or Etest to determine vancomycin MICs, we observed that the accumulation of SNPs paralleled the increasing vancomycin MICs (Table 6).

DISCUSSION

The lack of genetic markers for VISA is a major limitation in surveying for decreased vancomycin susceptibility in *S. aureus* infections and in providing information for antibiotic treatment decisions. Here we examined the prevalence of mutations in the *vraSR* operon, *graRS*, *walRK*, and *rpoB*, as these loci have recently been suggested to play a crucial role in the evolution of VISA (10, 11, 15, 20, 32). We detected multiple nonsynonymous SNPs in all of these putative target genes that were not present in a collection of VSSA isolates, suggesting an association with decreased vancomycin sensitivity. None of these genetic markers allowed us to reliably differentiate VISA from hVISA isolates. However, SNPs in

TABLE 3 Phenotypic and molecular characterizations of VISA isolates

Strain	MIC (μg/ml)			SNP(s) identified in gene or operon											
	VAN ¹⁰	TP ^a	RIF ^b	VA ^c	PAP result	spa type	vraR	vraS	yyqF	SA1703	graR	graS	rpoB	walK	walR
7374	1.5	8	S	2	1 (hVISA)	t064		A314V							
8043	1.5	4	S	1	1 (hVISA)	t002								F483I	V113A
7359	2	6	R	1	1 (hVISA)	t002	I103T	M192I		D65V			A477D		
8089	2	6	S	2	1 (hVISA)	t002									
8007	3	16	S	2	1.4 (VISA)	t008			A59E						T101S
8042	3	8	R	2	1 (hVISA)	t064									
8694	3	12	S	4	1.5 (VISA)	t098						I18L		V380I	
8712	3	4	S	2	1.3 (hVISA)	t002									
9005	3	4	S	2	1 (hVISA)	t064				N85S					
C115	3	8	S	4	2 (VISA)	t688					E15K		H481N		
C122	3	6	S	4	1.3 (hVISA)	t2487								V383I	
C407	3	32	R	4	2.1 (VISA)	t067			P174Q				H481Y		T101A
C506	3	12	S	2	1.4 (VISA)	t088									P216T
7317	4	4	S	2	1.5 (VISA)	t002			Y215S	N85S			D471G		
7321	4	12	R	4	1.5 (VISA)	t064							R483H		
7356	4	32	S	4	2.1 (VISA)	t064			T125I						
7436	4	32	S	2	1.4 (VISA)	t6863									
8393	4	32	S	4	1.4 (VISA)	t148							Insert 473-A-474		
8734	4	8	S	4	1.3 (hVISA)	t002								S9P	
C107	4	12	S	4	2.9 (VISA)	t002		A314V			E15K			A243T	
C123	4	32	R	4	2.1 (VISA)	t242							H481Y	R282C	
C126	4	32	R	4	1.4 (VISA)	t064							H481Y		
C217	4	8	S	4	2.6 (VISA)	t002						T136I			P216S
C221	4	16	S	4	1.5 (VISA)	t002	K43E								
7353	6	32	R	4	2 (VISA)	t002								T217K	
C109	6	32	S	4	1.9 (VISA)	t064	D194N		L85F	N85S			S464P		
C504	6	32	R	4	1.9 (VISA)	t002							H481Y	G223S	
7370	8	32	R	4	2.9 (VISA)	t064				N85S			D471G		
													R483H		
C119	8	32	R	8	2.8 (VISA)	t306		A314T			S79F		H481Y		
C303	8	32	S	4	2.8 (VISA)	t064								H576R	
C312	8	32	R	8	2.1 (VISA)	t004		G9V					H481Y	S437F	

^a Vancomycin (VAN) and teicoplanin (TP) MICs were determined by Etest.
^b Rifampin (RIF) MICs were determined by the Kirby-Bauer method.
^c The VAN MICs were also determined with the microscan test.

FIG 1 Schematic representation of single-nucleotide point mutations in *vraSR* (A), *graRS* (B), and *walRK* (C). SNPs identified in this study are shown above the diagrams, those previously reported in the literature (with superscript numbers to indicate reference numbers) are shown below the genes, and boxes with dashed lines indicate SNPs observed in ours and other studies.

graR and *walR* were exclusively detected among VISA isolates, although at relatively low frequencies.

One of the limitations of this study includes that it assessed associations between phenotypes and genotypes but did not directly investigate the genetic effects of newly identified SNPs on vancomycin susceptibilities. Nevertheless, it provides strong evidence for the polygenetic trait of VISA strains, as at best only about 50% of VISA isolates carried SNPs in a given candidate locus. Alternatively, this low sensitivity of an individual locus to identify VISA could also be confounded by the difficulty in phenotypically defining VISA and hVISA isolates with existing techniques. The cutoff for vancomycin susceptibility was recently lowered to ≤ 2

$\mu\text{g/ml}$ for the broth MIC, to increase the detection of heterogeneously resistant *S. aureus* (3, 30). The Etest is a diagnostic alternative, although there have been concerns that Etest MICs for vancomycin are generally higher than broth microdilution results (14). In our study, 4 isolates were vancomycin sensitive, with an MIC of 2 $\mu\text{g/ml}$ by microscan, but more consistent results for classification as VISA by Etest and PAP, and 3 isolates were hVISA by PAP. Regardless of the test used, increasing vancomycin MICs were also paralleled by a higher frequency of isolates with SNPs in the sequenced loci, which suggests that the VISA phenotype includes a spectrum of changes rather than a critical threshold. Our findings also support the hypothesis that alterations in different genetic targets can lead to VISA, and they extend findings from whole-genome sequencing projects. For example, Howden et al. identified SNPs in *graRS* and *walRK* but not in *vraR* (10, 11), whereas Mwangi detected SNPs in *vraR* but not *graRS* or *walRK* (20). In contrast, hVISA Mu3 carries the I5N SNP in *vraS*, and the subsequent VISA Mu50 strain contains an additional SNP in *graR* (N197S) (10, 21). Some of the SNPs observed in the current study have previously been shown and tested in isogenic pairs and found to confer VISA or hVISA susceptibilities (13, 32). However, the high diversity of SNPs in all of the candidate VISA loci observed here and in the literature is remarkable, and no clear clustering in specific functional regions of genes has been apparent (Fig. 1) (10, 20).

A possible explanation for the diversity of SNPs in VISA iso-

TABLE 4 Frequencies of SNPs by vancomycin susceptibility category

Gene locus	No. (%) of isolates with SNPs, by vancomycin susceptibility category, based on testing by:				
	PAP			Microscan	
	VISA (n = 22)	hVISA (n = 9)	VSSA (n = 46)	VISA (n = 20)	VSSA (n = 57)
<i>vraSR</i>	10 (45)	4 (44)	3 (6.5)	9 (45)	8 (14)
<i>graRS</i>	5 (23)	0 (0)	0 (0)	5 (25)	0 (0)
<i>walRK</i>	11 (50)	3 (33)	3 (6.5)	10 (50)	7 (12)
<i>rpoB</i>	11 (50)	2 (22)	2 (4.3)	11 (55)	4 (7)
All loci combined	21 (91)	7 (78)	7 (15)	20 (100)	14 (25)

TABLE 5 Frequencies of SNPs for MLST and VISA candidate genes^a

Gene	Genotype based on:	Length (bp)	No. of SNPs	No. of SNPs detected by type		N/S ratio	SNP/100 bp
				S	N		
<i>arcC</i>		456	13	8	5	0.63	3
<i>aroE</i>		456	23	12	11	0.92	5
<i>glpF</i>		465	10	9	1	0.11	2
<i>gmk</i>		429	9	7	2	0.29	2
<i>pta</i>		474	13	9	4	0.44	3
<i>tpi</i>		402	17	10	7	0.70	4
<i>yqiL</i>		516	15	10	5	0.50	3
<i>vraR</i>	MLST	629	12	10	2	0.2	2
	VISA isolates		15	10	5	0.5	2.4
<i>vraS</i>	MLST	1,043	32	32	0	0	3
	VISA isolates		36	32	4	0.13	3.5
SA1702	MLST	701	21	20	1	0.05	3
	VISA isolates		28	21	7	0.33	4
SA1703	MLST	386	5	2	3	1.5	1
	VISA isolates		7	2	5	2.5	1.8
<i>graR</i> *	MLST	675	42	36	6	0.17	6
	VISA isolates		47	39	8	0.21	7
<i>graS</i> *	MLST	1,041	147	109	38	0.35	14.1
	VISA isolates		149	109	40	0.37	14.3
<i>rpoB</i>	MLST	3,552	34	31	3	0.09	1
	VISA isolates		43	34	9	0.26	1.2
<i>walK</i>	MLST	1,827	27	26	1	0.04	1.5
	VISA isolates		42	28	14	0.5	2.3
<i>walR</i>	MLST	702	7	7	0	0	1
	VISA isolates		14	9	5	0.6	2

^a Abbreviations and symbols: S, synonymous; N, nonsynonymous; *, insertions and deletions were also present but were excluded from the calculation of the N/S ratio of SNPs.

lates that should be considered is the clonal background of isolates and the possibility of lineage-specific adaptation. Most of the VISA strains studied here belonged to either ST5 (USA100) or ST8 (USA300). While traditionally considered a community-associated clone, USA300 has now emerged as a major cause of health care-associated bloodstream infections in the United States (27). In our study, ST8 VISA strains were more likely to harbor SNPs in *vraSR* or *graRS*, whereas ST5 isolates mainly accumulated SNPs in *walRK*. Previously, SNPs in *vraSR* have been described in both the ST5 and ST8 lineages (6, 20). Based on the high diversity of the observed SNPs, this preferential clustering is inconsistent with an outbreak of a particular clone. Interestingly, one of the previously reported changes between paired VISA-VISA isolates identified a triplet deletion in the intergenic region downstream of *vraR* in *S. aureus* strain VM3 (20). This strain was derived from the MRSA strain COL (ST8 strain) after continuous *in vitro* exposure to vancomycin, resulting in an increase in the vancomycin MIC from 1 to 3 µg/ml. In the current study, we noted that all non-ST8 isolates lacked this triplet, whereas all ST8 strains carried this insertion. This preexisting variability may be an important factor in determining the influences of subsequent SNPs in candidate loci for development of the VISA phenotype. In addition, comparison of SNP frequencies based on MLST alone revealed that among the genes sequenced here, *graR* and *graS* in particular are highly polymorphic. The impacts of these background nonsynonymous

TABLE 6 Frequencies of SNPs by vancomycin MIC

MIC (µg/ml)	Frequency (%) of SNPs based on:	
	Microscan	Etest
≥4	20/20 (100)	18/19 (95)
3	NA ^a	7/8 (88)
2	7/16 (44)	2/5 (40)
1.5	NA	5/20 (25)
1	7/40 (17.5)	2/25 (8)

^a NA, MIC not available (could not be obtained with microscan test).

SNPs on vancomycin susceptibility or the frequency of *de novo* mutations in these two genes remain to be determined.

Overall, the VISA and hVISA isolates studied here were also more frequently resistant to other antibiotics. Therefore, some of the observed SNPs could be attributed to resistance to other drugs. For example, *rpoB* had the highest frequency of SNPs of all loci tested, although this almost exclusively correlated with the rifampin resistance of these isolates. However, it has recently been shown *in vitro* that MRSA isolates under rifampin pressure also develop decreased susceptibility to vancomycin (32), and the H481Y mutation contributes to the development of increased vancomycin resistance in Mu3 in allelic exchange experiments (15). These findings are of direct clinical relevance, as rifampin and vancomycin are frequently used in combination to treat complicated MRSA infections. In general, patients who are at risk for developing VISA infections are often exposed to multiple antibiotics. For example, the *vraSR* two-component regulatory system controls cell wall synthesis and is not only inducible by glycopeptides but also by other cell wall antimicrobials, such as β-lactams (1). Overall, we infrequently observed SNPs in *vraSR*, *graRS*, *walRK*, or *rpoB* in the sequenced non-VISA isolates. Mutations in these isolates were almost exclusively detected in hospital-associated clinical samples and may be indications of prior glycopeptide or β-lactam exposure. Alternatively, a recent study suggested that *vraS* may also serve as an on/off switch for vancomycin susceptibility (6). In a series of clinical isolates, the vancomycin MIC increase from 1.5 to 3 µg/ml was paralleled by the evolution of SNPs in *γvqF*, *vraR*, *γycH*, and *lspA*. After passage of the VISA isolate without vancomycin pressure, the subsequent revertant with an MIC of 1 µg/ml contained an additional mutation in *vraS*, a premature stop codon (6). Therefore, SNPs in *vraS* (and perhaps other loci) may also indicate the loss of vancomycin resistance.

While SNPs in *vraSR*, *graRS*, *walRK*, and *rpoB* were more frequently detected in VISA than in hVISA isolates, they did not allow reliable differentiation between the two categories. Furthermore, one VISA and three hVISA isolates did not harbor any mutation in the sequenced regions. This suggests that, in these isolates, additional yet-unidentified genes account for the change in vancomycin susceptibility. Additional candidate genes have now been proposed, such as the proteolytic regulatory gene *clp* (28).

In summary, we observed that VISA and hVISA are more resistant to antimicrobials in general and have a higher frequency of SNPs in candidate regions *vraSR*, *graRS*, *walRK*, and *rpoB*. SNPs in all of these loci contributed equally to this relationship, while only SNPs in *graR* and *walR* were unique to VISA isolates. Overall, the accumulation of SNPs was paralleled by rising vancomycin MICs. We observed that the clonal background of the isolates might play a role in the preferential accumulation of SNPs in a given locus. In

light of this high SNP diversity, a rapid PCR typing genetic test appears less promising. Perhaps targeting downstream gene expression profiles (6, 10) will provide a more feasible alternative diagnostic approach.

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