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Novel single-nucleotide variations associated with vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*

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Abstract: Prolonged vancomycin usage may cause methicillin-resistant *Staphylococcus aureus* to become vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA). Mechanisms of vancomycin resistance of VISA and hVISA are still unclear. In this study, analyses of nucleotide sequence variations in 30 vancomycin-sensitive *S. aureus* (VSSA), 41 hVISA and 16 VISA isolates revealed 29 single-nucleotide variations in 12 genes (*fmtC*, *graR*, *graS*, *htrA*, *mecA*, *pbp2*, *pbp4*, *srtA*, *tcaA*, *upps*, *vicK* and *vraR*) that are related to cell wall synthesis or the two-component system. Six of these 29 single-nucleotide variations were novel and resulted in the following amino acid changes: Q692E in FmtC; T278I, P306L and I311T in HtrA; and I63V and K101E in Upps. Since P306L and I311T in HtrA and I63V in Upps were present in the majority (76.7%–86.7%) of VSSA isolates, these three amino acid variations may not be associated with vancomycin resistance. The other three amino acid variations (T278I in HtrA, K101E in Upps and Q692E in FmtC) were present in the majority (87.5%–93.8%) of hVISA and VISA isolates, but only in a small number (22.9%–25.7%) of VSSA isolates, suggesting that they are associated with vancomycin resistance.

Keywords: MRSA, VSSA, hVISA, VISA

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of nosocomial infection.[1] Vancomycin is usually used to treat MRSA infections, but high-level vancomycin-resistant *S. aureus* (VRSA) has emerged mainly due to the acquisition of the *vanA* operon from Enterococci.[2] There are also VRSA isolates with lower levels of vancomycin resistance, including vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA).[3] The causes for the different levels of vancomycin resistance in MRSA are not completely clear.[4,5]

Identification of VISA and hVISA is conventionally done by determination of population analysis profile and area under the curve ratio (PAP/AUC). However, PAP/AUC is very labor-intensive and not practical for clinical diagnosis. Transcriptome and proteome analyses of MRSA isolates have revealed a link between nucleotide sequence variations of several genes and vancomycin resistance.[6,7] These genes include those of the two-component systems, such as *vraRS*, *graRS* or *walRK*,[6,8,9] and those involved in cell wall synthesis, such as *upps*, *fmtC* and *srtA*.[9–11] Single-nucleotide variations (SNVs) of genes related to antibiotic resistance have also been described.[8,9,12,13] These SNVs may allow differentiation between vancomycin-sensitive *S. aureus* (VSSA) and VISA.

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Previous studies on hVISA and VISA strains have revealed 60 genes that may be associated with vancomycin resistance.[4,9,10,14–23] We hypothesized that some nucleotide sequence variations in these genes correlate with the difference in vancomycin susceptibility of VSSA and VISA isolates. To test this hypothesis, we investigated the prevalence of SNVs of these 60 genes and found 29 SNVs (in 12 genes) that were more common in hVISA and VISA than in VSSA isolates. Among them, three novel amino acid sequence variations including Q692E in FmtC, T287I in HtrA and K101E in Upps proteins were found to be significantly associated with vancomycin resistance.

Materials and methods
Bacteria strains and growth conditions
In total, 87 MRSA isolates were used in this study, including 72 from Chang Gung Memorial Hospital, 3 from National Taiwan University Hospital, 8 from Tri-Service General hospital (TSGH) and 2 each from Chi Mei Medical Center and China Medical University Hospital. These isolates were collected from 2009 to 2014. Detailed information of these isolates is presented in Table 1. All isolates were stored at −80°C and grown on tryptic soy agar plates at 37°C for 16 hours for the studies.

Whole-genome sequencing and polymerase chain reaction (PCR)
The whole genome of two VISA isolates (TSGH205 and TSGH243) was sequenced. Genomic DNA of each isolate was isolated and sonicated to generate fragments of 300–500 bp for construction of a DNA library, which was then subjected to Illumina next-generation sequencing. Raw sequence data generated were filtered and assembled for analysis using the CLC Genomics Workbench (QIAGEN, Venlo, the Netherlands). Genes selected for investigation

of vancomycin resistance and PCR primers used to amplify these genes are shown in Table 2. PCRs were performed in a buffer containing 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$ and 50 mM KCl under the following conditions: 5 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C for denaturation, 30 seconds at 55°C for primer annealing and 1 minute at 72°C for extension, and 5 minutes at 72°C for final extension. PCR products were verified by sequencing.

Minimum inhibition concentration (MIC) E-tests
E-test was performed to determine the vancomycin MIC of VSSA, hVISA and VISA isolates. Cells of an overnight culture of each isolate were suspended in 0.9% NaCl solution, adjusted to 0.5 McFarland unit and spread evenly on a Mueller–Hinton agar plate. An E-test strip (bioMérieux, Durham, NC, USA) was placed on the agar plate, which was then incubated at 35°C overnight to determine MIC values.

Population analysis profile/area under the curve
PAP/AUC was performed to identify hVISA and VISA isolates as previously described.[24,25] Briefly, serial dilutions of the overnight culture of an isolate were plated on brain heart infusion agar plates containing different concentrations of vancomycin (0, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 µg/mL). After incubation at 35°C for 48 hours, colony-forming units (CFUs) of the isolate were determined and graphed as $\log_{10}$CFU/mL value versus vancomycin concentration to calculate the AUC. Reference strains Mu3 and Mu50 were analyzed in an identical manner to serve as hVISA and VISA controls, respectively. To distinguish VSSA, hVISA and VISA, the ratio (PAP/AUC value) of the AUC of an isolate to the AUC of Mu3 was calculated. The following ratios were used for

Table 1 MRSA strains used in this study

Vancomycin phenotype (number)	Source[a] (number)	Strain IDs	Reference
VSSA (30)	CGMH (30)	43, 46, 49, 64, 827–851	This study
hVISA (41)	CGMH (34)	2, 6, 9, 10, 22, 23, 33, 37, 42, 50, 55, 57, 60, 62, 72, 75, 82, 85, 103, 104, 108, 199, 208, 215, 216, 222, 224, 232, 236, 238, 241, 246, 247, 250	This study
	TSGH (2)	5046, 5047	1, 2
	NTUH (3)	9140, 9148, 9095	
	CMMC (2)	2012, 2086	
VISA (16)	CGMH (8)	36, 80, 852–857	This study
	TSGH (6)	188, 204, 205[b], 218, 243[b], 257	1, 3, 4
	CMUH (2)	2021, 4022	2

Notes: [a]All strains were collected from blood cultures at different times. [b]Whole-genome sequenced strains.
Abbreviations: CGMH, Chang Gung Memorial Hospital; CMMC, Chi Mei Medical Center; CMUH, China Medical University Hospital; hVISA, heterogeneous vancomycin-intermediate *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*; NTUH, National Taiwan University Hospital; TSGH, Tri-Service General hospital; VISA, vancomycin-intermediate *Staphylococcus aureus*; VSSA, vancomycin-sensitive *Staphylococcus aureus*.

Table 2 Primers used in this study

Primer	Sequence (5′→3′)
fmtC-F1	GGAGATCCGTTAGGTGATGAAA
fmtC-R1	TGGTAAATCTAACTCTGGCAACC
graRS-F	TATTTTGGCCGATTTATTACTTTA
graSR-R	CCTTTAGGCTTTGGCACTTGT
htrA-F	GATTCAGACAGCTCGATGCAG
htrA-R	GGCCAATACATCATCCAAAAC
mecA-F	GTGGAGACGAGCACTAATAACC
mecA-R	GAAGTTGTAGCAGGAACACAAATG
pbp2-F	GAACTTGACTGGTGGATTTGG
pbp2-R	GCCCATCCACACTGACATAG
pbp4-40R	TACAGAAGGCATTTCGACG
pbp4-F1	AGTATGGACAATCGCAGACC
srtA-F	GCAGCATATTTGTTTGCTAAACC
srtA-R	CAATGACACGTCGTCATTGG
tcaA-R1	TCTTGCGAGCCTTGTTCAAG
tcaA-R-new	GCACCTACCAAGCAACCAAT
upps-F	TTATGGATGGTAATGGGCGA
upps-R	GCGTCTTTGACGTGACTGAT
vicK-F	GAGTATGCCAACCGTCAAGA
vicK-R	ACGATACGAATACGTCCACG
vraSR-F1	TCAGGTACACGTATCGAGGT
vraR-R1	TTGTCGGTGCTGAAATCAAT

identification of VSSA, hVISA and VISA populations: VSSA, <0.9; hVISA, 0.9–1.3; VISA, >1.3.

Results
Nucleotide sequence variations in genes related to vancomycin resistance

The 87 MRSA isolates used in this study included 30 VSSA, 41 hVISA and 16 VISA isolates (Table 1). To test the hypothesis that some SNVs in the previously identified 60 genes (Table S1) correlate with the difference in vancomycin susceptibility of VSSA and VISA isolates, the nucleotide sequences of these 60 genes in these MRSA isolates were examined. To simplify the bioinformatics work, this examination was performed in a stepwise manner to gradually narrow down the scope of analysis. Genes with SNVs were first identified, followed by determination of the prevalence of the SNVs in the MRSA isolates, identification of novel SNVs and then correlation of these novel SNVs with vancomycin resistance.

For the first step of analysis, the sequences of the 60 genes in two each of VSSA (N315 and JH1) and VISA (TSGH205 and TSGH243) isolates were aligned and compared. These four strains were selected because their entire genome had been sequenced. The two VSSA strains (N315 and JH1) were found to differ in nucleotide sequences in 13 genes, whereas the two VISA strains (TSGH205 and TSGH243) differed in

nucleotide sequences in 25 genes, indicating that genes in the VISA isolates are more variable. Most of these sequence variations are SNVs. After excluding SNVs that were common in the two VSSA and two VISA strains, 29 SNVs in the following 12 genes were found in both VISA strains (TSGH205 and TSGH243): *fmtC, graR, graS, htrA, mecA, pbp2, pbp4, srtA, tcaA, upps, vraR* and *vicK* (Table 3). These genes have been shown to be related to the two-component system (*graR, graS, vicK* and *vraR*), cell membrane synthesis (*tcaA*), cell wall synthesis (*mecA, pbp2, pbp4* and *srtA*) or vancomycin resistance (*htrA, fmtC* and *upps*).[10,11]

Prevalence of the 29 SNVs in VSSA, hVISA and VISA isolates

In the second step of analysis, the association of these 29 SNVs with vancomycin resistance was determined. To achieve the goal, the nucleotide sequences of the aforementioned 12 genes of 16 additional clinical isolates were compared, including five VSSA, nine hVIS and two VISA strains. Results showed that two VSSA, five hVISA and all VISA isolates had the same 29 SNVs. Six of these SNVs were novel and resulted in the following amino acid changes: Q692E in FmtC; T278I, P306L and I311T in HtrA; and I63V and K101E in Upps (Table 4).

Amino acid sequence variations in HtrA, FmtC and Upps associated with vancomycin resistance

In the third step of analysis, the role of these six novel SNVs in vancomycin resistance was then investigated by detecting their presence in 69 additional MRSA isolates. These isolates included 25 VSSA, 32 hVISA and 12 VISA isolates. Among the six amino acid variations, P306L and I311T in HtrA and I63V in Upps were present in the majority of VSSA isolates (P306L, 86.7%; I311T, 86.7%; I63V, 76.7%), as shown in Table 5, suggesting that these three amino acid variations are not associated with vancomycin resistance. The other three amino acid variations (T278I in HtrA, K101E in Upps and Q692E in FmtC) were found to be present in the majority of hVISA and VISA isolates, but only in a small number of VSSA isolates. The percentages of isolates with these sequence variations were the following: HtrA T278I: 87.8% hVISA, 93.8% VISA and 25.7% VSSA isolates; Upps K101E: 87.8% hVISA, 87.5% VISA and 22.9% VSSA isolates; and FmtC Q692E: 87.8% hVISA, 87.5% VISA and 25.7% VSSA isolates (Tables 5–7).

Table 3 Sequence variations in VSSA and VISA strains

Gene ID	Gene name	Amino acid variation (nucleotide variation)	VSSA N315	VSSA JH1	VISA TSGH243 (MIC=4)	VISA TSGH205 (MIC=8)
SA1193	*fmtC*	Q692E (C2074G)	Q	Q	E	E
SA0614	*graR*	**D148Q** (G442C, T444G)	D	D	Q	Q
SA0615	*graS*	**L26F** (G78C)	L	L	F	F
		I59L (A175T)	I	I	L	L
		T224I (C671T)	T	T	I	I
SA0879	*htrA*	T278I (C833T)	T	T	I	I
		P306L (T917C)	L	L	P	P
		I311T (T932C)	I	I	T	T
SA0038	*mecA*	N146K (T438A)	N	N	K	K
		N204K (T612G)	N	N	K	K
		G246E (G738A)	G	G	E	E
SA1283	*pbp2*	C197Y (G591A)	C	Y	Y	Y
		A420V (C1259T)	A	A	V	V
		A557T (G1669A)	A	A	T	T
SA0598	*pbp4*	S189T (T565A)	S	S	T	T
		S395C (A1183T)	S	S	C	C
		A409T (G1225A)	A	A	T	T
SA2316	*srtA*	N57K (T171A)	N	N	K	K
		E167G (A500G)	E	E	G	G
SA246	*tcaA*	**L218P** (T653C)	L	P	P	P
		Y237H (T709C)	Y	Y	H	H
		T262S (A784T)	T	T	S	S
		R283H (A848G)	R	R	R	H
		G312D (G935A)	G	G	G	D
SA1103	*upps*	I63V (A187G)	I	I	V	V
		K101E (A301G)	K	K	E	E
SA1700	*vraR*	E59D (A177T)	E	E	E	D
SA0018	*vicK*	R222K (G665A)	R	R	K	K

Note: Sequences in bold typeface have been reported in previous studies.
Abbreviations: TSGH, Tri-Service General hospital; VISA, vancomycin-intermediate *Staphylococcus aureus*; VSSA, vancomycin-sensitive *S. aureus*.

Discussion

Previous studies have revealed 60 genes that may be related to vancomycin resistance,[6,9] suggesting that vancomycin resistance is a complex phenomenon and is mediated not by a single gene but by a group of genes. Among these 60 genes, 41 were found to be associated with vancomycin resistance by expression analysis;[10,14,16,20–23] 8 were found by overexpression, deletion or insertion mutations;[10,15,18,19] 11 were found by analysis of SNVs.[4,9,11,17,20] In this study, we found 29 SNVs in the following 12 genes: *fmtC*, *graR*, *graS*, *htrA*, *mecA*, *pbp2*, *pbp4*, *srtA*, *tcaA*, *upps*, *vraR* and *vicK* (Table 3). Six of these 29 SNVs in *fmtC*, *htrA* and *upps* are novel. Since we only examined the sequences of these 12 genes in a limited number (87) of MRSA isolates, it is conceivable that other isolates may have other important sequence variations that were not detected in this study.

Of the 12 genes with SNVs, *graR, graS, vicK* and *vraR* are related to the two-component system.[9] The others (*mecA, pbp2, pbp4, srtA, tcaA, fmtC, htrA* and *upps*) are more closely associated with vancomycin resistance. The *mecA* gene encodes the penicillin-binding protein 2a (PBP2a), which plays a role in resistance to beta-lactam antibiotics. Previous studies have shown that vancomycin treatment triggers deletions in the *mecA* gene.[26,27] The N146K, N204K and G246E mutations in the MecA protein that we found in this study are located near or in the dimerization domain of PBP2a (KEGG database; http://www.kegg.jp/ssdb-bin/ssdb_motif?kid=sav:SAV0041) and would cause conformational changes of PBP2a. A recent study showed that these mutations (N146K, N204K and G246E) are highly associated with ceftaroline resistance,[28] suggesting that they are also related to vancomycin susceptibility.

The *pbp2* and *pbp4* genes encode other penicillin-binding proteins (PBPs), which are also major components of the cell wall. The N-terminal domain of these PBPs encodes a glycosyltransferase, which catalyzes glycan chain polymerization from lipid II. Their C-terminal domain encodes a transpeptidase, which cross-links glycan chains.[29] In addition

Table 4 Amino acid sequence variations in 16 MRSA isolates

Types	Amino acid	VSSA (5)					hVISA (9)									VISA (2)	
CGMH strain		43	46	234	49	64	33	250	236	241	10	50	60	215	246	36	80
VAN MIC		1	1	2	1.5	1	2	2	2	2	2	2	2	2	2	2	2
PAP/AUC		0.82	0.65	0.73	0.83	0.69	0.94	0.91	1.14	0.93	1.2	0.91	0.98	1	0.95	1.47	1.37
fmtC	Q692E	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+
graR	**D148Q**	−	−	−	+	+	−	−	−	+	+	+	+	+	+	+	+
graS	**L26F**	−	−	−	+	+	−	−	+	−	+	+	+	+	+	+	+
	I59L	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+
	T224I	−	−	−	+	+	−	−	+	+	+	+	+	+	+	+	+
htrA	T278I	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+
	P306L	−	−	+	+	+	−	−	−	+	+	+	+	+	+	+	+
	I311T	−	−	+	+	+	−	−	−	−	+	+	+	+	+	+	+
mecA	N146K	−	−	+	+	+	−	−	−	−	+	+	+	+	+	+	+
	N204K	−	−	+	+	+	−	−	−	−	+	+	+	+	+	+	+
	G246E	−	−	+	+	+	−	−	−	+	+	+	+	+	+	+	+
pbp2	C197Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	A420V	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+
	A557T	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+
pbp4	S189T	−	−	−	+	+	−	+	−	−	+	+	+	+	+	+	+
	S395C	−	−	−	+	+	−	+	−	−	+	+	+	+	+	+	+
	A409T	−	−	−	+	+	−	+	−	−	+	+	+	+	+	+	+
srtA	N57K	−	−	+	+	+	−	−	+	+	+	+	+	+	+	+	+
	E167G	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+
tcaA	**L218P**	−	−	+	+	+	−	+	−	+	+	+	+	+	+	+	+
	Y237H	−	−	−	+	+	−	+	−	+	+	+	+	+	+	+	+
	T262S	−	−	+	+	+	−	+	−	+	+	+	+	+	+	+	+
	R283H	−	−	+	+	+	−	+	−	−	+	+	+	+	+	+	+
	G312D	−	−	+	+	+	−	+	+	−	+	+	+	+	+	+	+
upps	I63V	−	−	+	+	+	−	−	+	+	+	+	+	+	+	+	+
	K101E	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+
vraR	**E59D**	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+
vicK	**R222K**	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+

Notes: Sequences in bold typeface have been reported in previous studies. Minus sign (−): sequence identical to that of N315; plus sign (+): sequence different from that of N315.
Abbreviations: CGMH, Chang Gung Memorial Hospital; hVISA, heterogeneous vancomycin-intermediate *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*; PAP/AUC, population analysis profile and area under the curve ratio; VISA, vancomycin-intermediate *S. aureus*; VSSA, vancomycin-sensitive *S. aureus*.

to the glycosyltransferase and transpeptidase domains, *PBP4* also has a domain encoding a carboxypeptidase, which hydrolyzes the C-terminal D-Ala-D-Ala peptide bond of the precursor of peptidoglycan.[30] Previous studies have shown that vancomycin treatment increases the expression of *pbp2*, but decreases the expression of *pbp4* in *S. aureus*.[31] It has been shown that *pbp4* mutations result in a decrease in its carboxypeptidase activity, leading to increased production of D-Ala-D-Ala termini in peptidoglycan and decreased vancomycin-binding affinity.[31] The sequence variation S189T in PBP4, we found in this study, is located in its carboxypeptidase domain (KEGG database; http://www.kegg.jp/ssdb-bin/ssdb_motif?kid=sav:SAV0642) and conceivably would reduce its activity.

Previous studies on PBP2 have found that mutations in the transpeptidase domain of PBP2 increase the susceptibility of *S. aureus* to ceftizoxime.[32] The C197Y, A420V and A557T mutations in PBP2 found in this study are located in its transpeptidase and transglycosylase domains (KEGG database; http://www.kegg.jp/ssdb-bin/ssdb_motif?kid=sav:SAV1450). The C197Y mutation has been shown to be associated with the susceptibility of *S. aureus* to ceftaroline,[33] which is effective for treatment of hVISA or VISA infections.[34] Although the other two mutations have not been investigated, it is conceivable that they will alter the activity of PBP2.

The *srtA* gene encodes sortase A, which is involved in the modification of cell surface proteins.[35] Transcriptome analysis revealed a decreased expression of *srtA* by vancomycin

Table 5 Amino acid sequence variations in 30 VSSA isolates

Strain ID	Amino acid sequence variations					
	FmtC	HtrA			Upps	
	Q692E	T278I	P306L	I311T	I63V	K101E
CGMH43	–	–	–	–	–	–
CGMH46	–	–	–	–	–	–
CGMH234	–	–	+	+	+	–
CGMH49	+	+	+	+	+	+
CGMH64	+	+	+	+	+	+
CGMH827	–	–	–	–	+	–
CGMH828	–	–	+	+	+	–
CGMH829	–	–	+	+	+	–
CGMH830	–	–	+	+	+	–
CGMH831	+	+	+	+	+	–
CGMH832	–	–	–	–	–	–
CGMH833	–	–	–	+	+	–
CGMH834	–	–	+	+	+	–
CGMH835	–	–	+	+	+	–
CGMH836	–	–	+	+	+	–
CGMH837	–	–	–	–	–	–
CGMH838	–	–	+	+	+	–
CGMH839	–	–	+	+	+	–
CGMH840	–	–	+	+	+	–
CGMH841	–	–	+	+	+	–
CGMH842	–	–	+	+	–	–
CGMH843	–	–	+	+	+	–
CGMH844	–	–	+	+	+	–
CGMH845	–	–	+	+	+	–
CGMH846	–	–	–	–	–	–
CGMH847	–	–	+	+	+	–
CGMH848	–	–	+	+	–	–
CGMH849	–	–	+	+	+	–
CGMH850	–	–	+	+	+	–
CGMH851	+	+	+	+	+	+
	13.3%	13.3%	86.7%	86.7%	76.7%	10.0%

Notes: Minus sign (–): sequence identical to that of N315; plus sign (+): sequence different from that of N315.
Abbreviations: CGMH, Chang Gung Memorial Hospital; *S. aureus, Staphylococcus aureus*; VSSA, vancomycin-sensitive *S. aureus*.

Table 6 Amino acid sequence variations in 41 hVISA isolates

Strain ID	Amino acid sequence variations					
	FmtC	HtrA			Upps	
	Q692E	T278I	P306L	I311T	I63V	K101E
P-value[a]	4.4×10^{-9}	4.4×10^{-9}	0.01	0.2	0.05	5.1×10^{-10}
CGMH33	–	–	–	–	–	–
CGMH250	–	–	–	–	–	–
NTUH9148	–	–	+	+	+	–
CGMH2	+	+	+	+	+	+
CGMH6	+	+	+	+	+	+
CGMH9	+	+	+	+	+	+
CGMH10	+	+	+	+	+	+
CGMH22	+	+	+	+	+	+
CGMH23	+	+	+	+	+	+
CGMH37	+	+	+	+	+	+
CGMH42	+	+	+	+	+	+
CGMH50	+	+	+	+	+	+
CGMH55	+	+	+	+	+	+
CGMH57	+	+	+	+	+	+
CGMH62	+	+	+	+	+	+
CGMH75	+	+	+	+	+	+
CGMH82	+	+	+	+	+	+
CGMH85	+	+	+	+	+	+
CGMH103	+	+	+	+	+	+
CGMH104	+	+	+	+	+	+
CGMH108	+	+	+	+	+	+
CGMH199	+	+	+	+	+	+
CGMH208	+	+	+	+	+	+
CGMH215	+	+	+	+	+	+
CGMH216	+	+	+	+	+	+
CGMH222	+	+	+	+	+	+
CGMH224	+	+	+	+	+	+
CGMH232	+	+	+	+	+	+
CGMH238	+	+	+	+	+	+
CGMH246	+	+	+	+	+	+
CGMH247	+	+	+	+	+	+
NTUH9095	+	+	+	+	+	+
TSGH5046	+	+	+	+	+	+
TSGH5047	+	+	+	+	+	+
CMMC2012	+	+	+	+	+	+
CMMC2086	+	+	+	+	+	+
NTUH9140	+	+	+	+	+	+
CGMH236	–	–	–	–	+	–
CGMH60	+	+	+	+	+	+
CGMH72	+	+	+	+	+	+
CGMH241	–	–	+	+	+	–
	87.8%	87.8%	92.7%	92.7%	95.1%	87.8%

Notes: Minus sign (–): sequence identical to that of N315; plus sign (+): sequence different from that of N315. [a]*P* values were generated by the Student's *t*-test by comparing the data from VISA and hVISA isolates.
Abbreviations: CGMH, Chang Gung Memorial Hospital; CMMC, Chi Mei Medical Center; hVISA, heterogeneous vancomycin-intermediate *Staphylococcus aureus*; NTUH, National Taiwan University Hospital; TSGH, Tri-Service General hospital; VISA, vancomycin-intermediate *S. aureus*.

treatment.[10] The N167K mutation identified in this study is located in the sortase domain (KEGG database; http://www.kegg.jp/ssdb-bin/ssdb_motif?kid=sav:SAV2528) and, thus, would decrease its activity.

The *tcaA* gene encodes a membrane protein that has been shown to be associated with glycopeptide resistance.[36] Structural analysis showed that amino acid residues 195–322 of the TcaA protein contain an OprB porin domain (http://www.kegg.jp/ssdb-bin/ssdb_motif?kid=sauf:X998_2340) of the ABC transporter, which plays a major role in carbohydrate uptake. Previous studies have shown that the ABC transporter is also involved in bacterial multidrug resistance.[37] Since the five amino acid variations (L218P, Y237H, T262S, R283H and G312D) in the TcaA protein we found are all located in its OprB porin domain, it is conceivable that these mutations will affect vancomycin resistance.

Table 7 Amino acid sequence variations in 16 VISA isolates

Strain ID	Amino acid sequence variations					
	FmtC	HtrA			Upps	
	Q692E	T278I	P306L	I311T	I63V	K101E
P-value[a]	4.2×10^{-5}	1.2×10^{-6}	0.01	0.01	0.1	1.8×10^{-5}
CGMH36	+	+	+	+	+	+
CGMH80	+	+	+	+	+	+
CMUH4022	+	+	+	+	+	+
CGMH852	+	+	+	+	+	+
CGMH853	−	−	+	+	+	−
CGMH854	+	+	+	+	+	+
CGMH855	+	+	+	+	+	+
CGMH856	+	+	+	+	+	+
CGMH857	−	+	+	+	−	−
CMUH2021	+	+	+	+	+	+
TSGH188	+	+	+	+	+	+
TSGH204	+	+	+	+	+	+
TSGH205	+	+	+	+	+	+
TSGH218	+	+	+	+	+	+
TSGH243	+	+	+	+	+	+
TSGH257	+	+	+	+	+	+
	87.50%	93.80%	100%	100%	93.80%	87.50%

Notes: Minus sign (−): sequence identical to that of N315; plus sign (+): sequence different from that of N315. [a]P values were generated by the Student's t-test by comparing the data from VSSA and VISA isolates.
Abbreviations: CGMH, Chang Gung Memorial Hospital; CMUH, China Medical University Hospital; TSGH, Tri-Service General Hospital; VISA, vancomycin-intermediate *Staphylococcus aureus*; VSSA, vancomycin-sensitive *S. aureus*.

In this study, six novel SNVs were found in *fmtC*, *htrA* and *upps* genes that have not been shown to be associated with vancomycin resistance. Three of them (Q692E in FmtC, T287I in HtrA and K101E in Upps) were correlated with vancomycin resistance (Tables 5–7). The *htrA* gene encodes a serine protease of the DegP family.[38] Previous studies have shown that HtrA can suppress the production and secretion of bacteriocin by *Streptococcus pneumonia*.[39] Bacteriocin-producing enterococci and staphylococci also have been described.[40–42] There have been no reports on the relationship between bacteriocin production and vancomycin resistance. Bacteriocin production has been shown to augment niche competition by enterococci in the gastrointestinal tract.[43] It is unknown whether any of the MRSA isolates examined in this study produces bacteriocin. Therefore, the mechanisms by which HtrA mediates vancomycin resistance remain to be investigated. The T278I mutation is predicted to be located in the coil structure of HtrA (http://www.ebi.ac.uk/interpro/protein/Q5HH63). It is possible that this mutation alters the secondary structure and thus the enzymatic activity of HtrA, leading to vancomycin resistance.

The *upps* gene encodes undecaprenyl pyrophosphate synthase, which catalyzes the formation of undecaprenyl pyrophosphate (UPP) by condensing farnesyl pyrophosphate with eight molecules of isopentenyl pyrophosphate.[44] UPP is a lipid carrier during peptidoglycan synthesis. Inhibition of Upps has been shown to suppress cell wall synthesis and decrease the susceptibility of bacteria to vancomycin.[11] Structural prediction (EMBL-EBL; http://www.ebi.ac.uk/interpro/protein/A0A1D4QTF1) revealed that Upps has a dimer interface residue at amino acid position 101. Thus, the K101E variation discovered in this study very likely would destabilize Upps, leading to vancomycin resistance.

The *fmtC* (also called *mprF*) gene encodes phosphatidylglycerol lysyltransferase that mediates lysinylation of phosphatidylglycerol.[45] Mutations in *fmtC* in *S. aureus* and *Bacillus subtilis* have been shown to cause a decrease in the production of lysyl-peptidoglycan, thus increasing negative charges of the cell membrane and reducing the susceptibility of bacteria to vancomycin and daptomycin.[46,47] The Q692E mutation, that we found in this study, is located in the IPR02430 domain (EMBL-EBL; http://www.ebi.ac.uk/interpro/entry/IPR024320), which is involved in the transfer of the lysyl group from l-lysyl-tRNA to membrane-bound peptidoglycan. This mutation very likely will disrupt this process, resulting in vancomycin resistance.

Limitations of this study include limited number of MRSA isolates examined and lack of detailed information of infections caused by the isolates, correlation between gene expression levels and vancomycin resistance, and functional studies of the SNVs. Therefore, the possibility that the SNVs discovered in this study may not completely correlate with vancomycin resistance of MRSA isolates still exists.

Conclusion

We have identified 29 SNVs that are more prevalent in hVISA and VISA than in VSSA isolates. Through sequence comparison of 87 isolates, we demonstrated that three novel SNVs in *htrA*, *upps* and *fmtC* genes are associated with vancomycin resistance. Since other environmental factors such as coinfections and patient's conditions may also affect vancomycin resistance, allelic replacement or complementation of these SNVs needs to be performed to confirm the roles of these mutations in vancomycin resistance.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Comparative analysis gene list

Accession number	Gene name	Location	Gene function	Gene mutation type	Reference
SA1843	agrC	2080353–2081468	Accessory gene regulator C	SNP (L193stop)	4
SA0366	ahpC	422549–423118	Alkyl hydroperoxide reductase subunit C	Transcriptome analysis data	22
SA1226	asd	1401012–1402001	Aspartate semialdehyde dehydrogenase	Transcriptome analysis data	16
SA1557	ccpA	1784194–1785138	Catabolite control protein A	Knock out deletion	15
SA0723	clpP	827630–828217	ATP-dependent Clp protease proteolytic subunit	Truncating deletion, SNP (M1V, H83R, R152H)	20
SA1096	clpQ	1242040–1242585	Heat shock protein HslV	Transcriptome analysis data	16
SA0480	ctsR	560629–561090	Transcription repressor of class III stress genes homologue	Transcriptome analysis data	20
SA0639	cydC	731989–733620	Hypothetical protein, similar to ABC transporter required for expression of cytochrome bd	Transcriptome analysis data	20
SA0640	cydD	733617–735290	Hypothetical protein, similar to ABC transporter required for expression of cytochrome bd	Transcriptome analysis data	20
SA1228	dapB	1402887–1403609	Dihydrodipicolinate reductase	Transcriptome analysis data	16
SA1206	fmtA	1379204–1380466	Factor essential for expression of methicillin	Transcriptome analysis data	21
SA1193	fmtC	1363612–1366134	Oxacillin resistance-related FmtC protein	Transcriptome analysis data	10
SA0309	geh	365458–367533	Glycerol ester hydrolase	Transcriptome analysis data	16
SA0430	gltB	490664–495163	Glutamate synthase large subunit	Transcriptome analysis data	16
SA0614	graR	708245–708919	Hypothetical protein, similar to two-component response regulator	SNP (T11A, E15K, S79F, D148Q, F151L, N197S)	9
SA0615	graS	708912–709952	Hypothetical protein, similar to two-component sensor histidine kinase	SNP (L26F, I59L, T224I)	9
SA0879	htrA	997117–999426	Serine protease HtrA	Transcriptome analysis data	10
SA1859	ilvB	2099308–2101077	Acetolactate synthase large subunit	Transcriptome analysis data	16
SA0980	isdE	1109815–1110693	Hypothetical protein, similar to ferrichrome ABC transporter	SNP (A48V)	17
SA1997	lacA	complement (2268598–2269026)	Galactose-6-phosphate isomerase LacA subunit	Transcriptome analysis data	16, 20
SA1996	lacB	complement (2268067–2268582)	Galactose-6-phosphate isomerase LacB subunit	Transcriptome analysis data	16, 20
SA1995	lacC	complement (2267122–2268054)	Tagatose-6-phosphate kinase	Transcriptome analysis data	16, 20
SA1994	lacD	complement (2266138–2267118)	Tagatose-1,6-diphosphate aldolase	Transcriptome analysis data	16, 20
SA2103	lytR	2365947–2366894	Hypothetical protein, similar to lyt divergon expression	Transcriptome analysis data	10
SA0038	mecA	complement (45031–47037)	Penicillin-binding protein 2 prime	Transcriptome analysis data	10
SA0344	metE	complement (401175–403403)	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	Transcriptome analysis data	10
SA0641	MgrA	complement (735417–735860)	Hypothetical protein; regulatory protein involved in autolytic activity	Deletion	10
SA0997	murI	1130843–1131643	Glutamate racemase	Transcriptome analysis data	10
SA1926	murZ	complement (2174362–2175621)	UDP-N-acetylglucosamine 1-carboxylvinyl transferase 2	Transcriptome analysis data	10
SA0847	oppD	960028–961110	Oligopeptide transport system ATP-binding protein OppD homolog	Transcriptome analysis data	10
SA2237	opuCA	complement (2511766–2512998)	Glycine betaine/carnitine/choline ABC transporter opuCA	Transcriptome analysis data	10
SA2236	opuCB	complement (2511134–2511769)	Glycine betaine/carnitine/choline ABC transporter opuCB	Transcriptome analysis data	10
SA2235	opuCC	complement (2510176–2511117)	Glycine betaine/carnitine/choline ABC transporter opuCC	Transcriptome analysis data	10

(Continued)

Table S1 (*Continued*)

Accession number	Gene name	Location	Gene function	Gene mutation type	Reference
SA2234	opuCD	complement (2509481–2510176)	Glycine betaine/carnitine/choline ABC transporter opuCD	Transcriptome analysis data	10
SA1283	pbp2	1486656–1488839	Penicillin-binding protein 2	Overexpression	10
SA0598	pbp4	complement (690688–691983)	Penicillin binding protein 4	Overexpression/allelic replacement inactivation	10
SA1024	pbpA	1158054–1160288	Penicillin-binding protein 1	Transcriptome analysis data	10
SA1659	prsA	1892718–1893680	Peptidyl-prolyl *cis/trans* isomerase homolog	Deletion frameshift mutation	10
SA0963	pycA	1091242–1094694	Pyruvate carboxylase	Transcriptome analysis data	10
SA0500	rpoB	579620–583171	RNA polymerase beta chain	SNP (G171D, A447V, D471Y, H481Y)	4
SA1872	rsbU	complement (2119821–2120822)	SigmaB regulation protein RsbU	Transcriptome analysis data	14
SA2094	SA2094	complement (2353833–2355233)	Hypothetical protein, similar to Na$^+$/H$^+$ antiporter	SNP (A94T)	17
SA0573	SarA	666347–666721	Staphylococcal accessory regulator A	Transcriptome analysis data	10
SA1691	sgtB	complement (1938571–1939380)	Hypothetical protein, similar to penicillin-binding protein 1A/1B	Transcriptome analysis data	10
SA0111	sirA	complement (127549–128541)	Iron-regulated ABC transporter	Transcriptome analysis data	20
SA0110	sirB	complement (126538–127533)	Iron-regulated ABC transporter siderophore permease protein SirB	Transcriptome analysis data	20
SA0109	sirC	complement (125543–126460)	Iron-regulated ABC transporter siderophore permease protein SirC	Transcriptome analysis data	20
SA0456	SpoVG	526376–526702	Stage V sporulation protein G homolog	Deletion	19
SA2316	srtA	complement (2600886–2601506)	Sortase	Transcriptome analysis data	10
SA0592	tagA	685072–685836	Teichoic acid biosynthesis protein	Transcriptome analysis data	23
SA2146	tcaA	complement (2411587–2412969)	TcaA protein	SNP (M202T, L218P, T279I, R283H, G312D)	9
SA0857	trfA	971601–972320	Hypothetical protein, similar to negative regulator of genetic competence MecA	Insertion inactivation	18
SA0858	trfB	972441–973427	Hypothetical protein, similar to transcription factor	Insertion inactivation	18
SA1103	upps	1248834–1249604	Undecaprenyl pyrophosphate synthetase	Point mutation on ribosome binding site	11
SA0018	vicK	25648–27474	Two-component response regulator	SNP (L10F, N48K, R222K/I, G275V, R282C, F330S, V380I, S437F, A468T, T492K, D496N, V494L, A567D)	20
SA0017	vicR	24928–25635	Two-component response regulator	Transcriptome analysis data	16
SA1700	vraR	complement (1946742–1947371)	Two-component response regulator	SNP (E59D, A113V, S164P, S329L)	9, 20
SA1701	vraS	complement (1947361–1948404)	Two-component response regulator	SNP (I5N, G9V, G88D, T104A, L123H, S167N, F243S, A260V, K272I, A314V, L315M, I317T, F321L, P327S)	9, 20
SA1095	xerC	1241147–1242043	Site-specific recombinase XerC homolog	Transcriptome analysis data	16
SA1702	SA1702	1948401–1949102	Conserved hypothetical protein	SNP (E56G, L86I, Q136H)	9