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Genetic alterations responsible for reduced susceptibility to vancomycin in community-associated MRSA strains of ST72

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Objectives: We previously reported the first case of vancomycin treatment failure due to development of vancomycin-intermediate resistance in a patient with an MRSA of ST72, a community genotype in Korea. We investigated two isogenic MRSA strains from this patient, who experienced treatment failure with vancomycin and rifampicin.

Methods: We tracked the genetic alterations that confer reduced susceptibility to vancomycin on those two isogenic MRSA strains by WGS.

Results: Five non-synonymous mutations were identified, including *rpoB* (H481Y), *dprA* (G196C), *femA* (F92C), *vraR* (E127K) and *agrC* (E391stop). We further studied the role of a mutation of *vraR* in reduced susceptibility to vancomycin. Introduction of the mutated *vraR* (E127K) into a vancomycin-susceptible *Staphylococcus aureus* strain resulted in an increase in *vraSR* mRNA expression and vancomycin MIC and development of the hetero-VISA phenotype, which was confirmed by the population analysis profile (PAP)/AUC. Electron microscopy showed increased cell wall thickness in the strains with mutated *vraR*.

Conclusions: Based on the genomic data, molecular experiments and PAP and cell wall analyses, we propose that a single mutation of *vraR* is associated with the reduced susceptibility to vancomycin in MRSA and further treatment failure.

Introduction

Staphylococcus aureus is the leading cause of both healthcare-associated (HA) and community-associated (CA) infections. In the past decade, CA infections with MRSA have emerged throughout the world. CA-MRSA clones vary, and there are geographical differences among strains. The major CA-MRSA clone in Korea, ST72, with staphylococcal cassette chromosome *mec* (SCC*mec*) type IV (ST72-MRSA-IV), has distinct features, such as lack of Panton-Valentine leucocidin (PVL) genes in those that have spread throughout the Asian region or internationally.[1]

Vancomycin has been the mainstay of treatment for patients with MRSA infections and thus reduced susceptibility to vancomycin is becoming a worldwide problem. Vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA) strains are increasingly reported in MRSA strains throughout the world.[1,2] With the exception of USA300, there have been few reports describing reduced susceptibility to vancomycin among CA-MRSA clones.[3] Vancomycin-resistant *S. aureus* (VRSA) strains have been shown to occur due to the transfer of the *vanA* gene from vancomycin-resistant enterococci.[4] In VISA, on the other hand, the mechanism of reduced susceptibility to vancomycin is based on an accumulation of spontaneous chromosomal mutations:[5] SNPs in genes of two-component regulatory systems (*vraSR*, *graRS* and *walKR*), a gene encoding membrane proteins (*yvqF*), transcription regulators (*agrA*, *agrC* and *rpoB*), PBPs (*pbp4* and *pbpB*) and a proteolytic regulatory gene (*clpP*).[5–10] Although the mutations in these genes were identified in VISA strains of a limited number of genetic lineages, the molecular mechanisms of VISA and hVISA phenotypes may not be confined to strains with specific genetic backgrounds.

We previously reported the first case of a CA-ST72-MRSA-IV strain with vancomycin-intermediate resistance in Korea.[11]

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Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics[a]	Reference or source
E. coli		
DH5α	F^- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44 λ- thi-1 gyrA96 relA1*; general-purpose host strain for molecular cloning	[34]
S. aureus		
RN4220	8325-4, r− m+, restriction-defective laboratory strain	[35]
VSSA303	VSSA clinical strain from blood; ST72-CA-MRSA-SCC*mec* IVA/PVL-negative, *spa* t324	this study
VISA072	VISA clinical strain from blood; VSSA303 carrying *dprA*$_{G196C}$, *femA*$_{F92C}$, *vraR*, *argC* and *rpoB* mutations	this study
S303- *dprA*$_{G196C}$	VSSA303 with p*dprA*$_{G196C}$ (DNA processing protein, *dprA*)	this study
S303-*femA*$_{F92C}$	VSSA303 with p*femA*$_{F92C}$ (UDP-*N*-acetylmuramoylpentapeptide-glycine glycyltransferase, *femA*)	this study
S303-*vraR*$_{E127K}$	VSSA303 with p*vraR*$_{E127K}$ (two-component response regulator, *vraR*)	this study
S303-*vraSR*$_{E127K}$	VSSA303 with p*vraSR*$_{E127K}$ (entire *vraSR* operon with *vraR*$_{E127K}$)	this study
RN-*dprA*$_{G196C}$	RN4220 with p*dprA*$_{G196C}$	this study
RN-*femA*$_{F92C}$	RN4220 with p*femA*$_{F92C}$	this study
RN-*vraR*$_{E127K}$	RN4220 with p*vraR*$_{E127K}$	this study
RN-*vraSR*$_{E127K}$	RN4220 with p*vraSR*$_{E127K}$	this study
Plasmids		
pMK4	*E. coli-S. aureus* shuttle vector; Ap^r, Cm^r	[20]
p*dprA*$_{G196C}$	*dprA*$_{G196C}$ from VISA072 cloned into pMK4; Cm^r	this study
p*femA*$_{F92C}$	*femA*$_{F92C}$ from VISA072 cloned into pMK4; Cm^r	this study
p*vraR*$_{E127K}$	*vraR*$_{E127K}$ from VISA072 cloned into pMK4; Cm^r	this study
p*vraSR*$_{E127K}$	entire *vraSR*$_{E127K}$ operon from VISA072 cloned into pMK4; Cm^r	this study

[a]Cm^r, chloramphenicol resistance; Ap^r, ampicillin resistance.

In addition, ST72-MRSA-IV strains with reduced susceptibility to vancomycin and daptomycin were also described in Japan.[12] ST72 is a PVL-negative CA-MRSA clone identified specifically in Korea,[13] and it has recently become an important clone in hospitals, like the USA300 clone in the USA.[14] Reduced susceptibility to vancomycin in this clone would be of great concern. The present work aimed to investigate the genetic alterations that contribute to reduced susceptibility to vancomycin by tracking the mutations in ST72-MRSA-IV strains with reduced susceptibility to vancomycin using WGS and molecular techniques.

Materials and methods

Bacterial strains and plasmids

WGS drafts were obtained from a pair of vancomycin-susceptible *S. aureus* (VSSA) and VISA strains (VSSA303 and VISA072, respectively), which were isolated consecutively from a single patient, a 79-year-old man who experienced treatment failure with vancomycin and rifampicin during a 2 month period from August to November 2011. Both strains belonged to ST72, showing SCC*mec* type IV, *agr* group I and *spa* type t324, and were identical by PFGE. While the MICs of vancomycin and rifampicin for the VISA072 strain were 4 and ≥32 mg/L, those of VSSA303 were 1 and ≤0.06 mg/L, respectively. A detailed clinical history of the patient and preliminary characterization of the strains have been reported previously.[11] 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 a blood agar plate [Becton, Dickinson and Company (BD), NJ, USA] for 18–24 h before being used in an experiment. All *S. aureus* strains were routinely grown in brain heart infusion (BHI) broth (BD) and *Escherichia coli* was grown in LB broth or agar (BD). Chloramphenicol (12.5 mg/L) and ampicillin (100 mg/L) were used for the selection and maintenance of *S. aureus* and *E. coli* transformants, respectively.

Antimicrobial susceptibility testing

In vitro susceptibility testing was performed using the broth microdilution method according to the CLSI.[15] *In vitro* susceptibility to vancomycin, rifampicin, oxacillin, teicoplanin and daptomycin was tested. *S. aureus* ATCC 29213, *Enterococcus faecium* ATCC 29212 and *E. coli* ATCC 25922 were used as control strains. All tests were repeated with three independent cultures, each tested in duplicate.

WGS

To identify the genetic changes that confer the VISA phenotype, WGS of VSSA303 and VISA072 was completed using a next-generation sequencing technique. WGS was performed at Macrogen Inc. (Seoul, Korea) with Illumina Hiseq2000 Preliminary Performance Parameters. A total of 19 million and 24 million reads were generated for strains VSSA303 and VISA072, respectively. Paired-end read sequencing was performed with read lengths of 101 bp. Software BWA v0.5.9-r16[16] was used for assembly with the reference genome of *S. aureus* HL1 (GenBank accession number CP003979), which is a CA-MRSA strain of ST72, isolated in Korea.[17]

Genetic alterations between the strain VSSA303 and VISA072, including SNPs and insertions and deletions (indels), were identified using SAMtools v0.1.18 (http://samtools.sourceforge.net/) and GATK v1.4.11 (http://www.broadinstitute.org/gsa/wiki/index.php/The_Genome_Analysis_Toolkit). The VISA phenotype-associated SNPs and indels identified by WGS comparison were confirmed by PCR amplification and sequencing of the PCR products. To predict whether the identified amino acid substitutions were likely to affect protein function, sorting intolerant from tolerant (SIFT) scores were

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calculated (http://sift.jcvi.org). Based on the amino acids appearing at each position, SIFT calculates the probability that an amino acid substitution at a position is tolerated, depending on the most frequent amino acid being tolerated. If the normalized value is <0.05, the substitution is predicted to be deleterious.[18,19]

Cloning, transformation and overexpression of target genes

The cloning and transformation of *E. coli* were performed using standard techniques. All restriction endonuclease and T4 DNA ligase (New England BioLabs, MA, USA) reactions were carried out according to the manufacturer's instructions. The mutated genes of *S. aureus* VISA072 were amplified using PCR with designed primers with restriction endonuclease SmaI sites (Table S1, available as Supplementary data at *JAC* Online). All PCR fragment products were purified and ligated into a cloning site of *E. coli–S. aureus* shuttle vector pMK4.[20] These constructs were moved into *E. coli* DH5α, *S. aureus* RN4220 and the VSSA303 strain by electroporation, respectively. To improve transformation efficiency into the *S. aureus* strain, a modified electroporation method for *S. aureus* was performed as previously described.[21] Competent cells of *S. aureus* strain VSSA303 were concentrated to 1–3×10 cfu/mL. To test the impact on vancomycin susceptibility of the single-base mutations in each target gene, we overexpressed target genes cloned into pMK4 in a VSSA strain. All constructs were verified by both sequence and restriction enzyme analysis, followed by screening of successful transformants using a chloramphenicol marker on the pMK4 vector. To better understand the effect of each mutation on acquisition of reduced susceptibility to vancomycin, antimicrobial susceptibility testing, population analysis profile (PAP)/AUC analysis and transmission electron microscopy (TEM) were performed for WT and mutated strains.

Quantitative real-time RT–PCR (qRT–PCR)

vraSR mRNA expression was evaluated for VSSA303 and VISA072 under no vancomycin exposure and for VISA072 and a strain with a mutated *vraR* gene (S303-*vraSR*$_{E127K}$) under vancomycin exposure. RNA extraction for qRT–PCR was performed as previously described.[22] Total RNA was extracted using an RNeasy isolation kit (Qiagen); all RNA samples were analysed by A_{260}/A_{280} spectrophotometry and gel electrophoresis to assess concentration and integrity. qRT–PCR analysis was done using an AccuPower GreenStar qPCR Master mix (Bioneer, Daejeon, Korea) according to the manufacturer's protocol. Gene expression was compared according to threshold cycle (C_T) values converted into fold changes with respect to the reference strain (VSSA303, value = 1) using $\log_2 - (\Delta\Delta C_T)$. The change (*n*-fold) in the transcript level was calculated using the following equations: $\Delta C_T = C_{T(\text{test DNA})} - C_{T(\text{reference cDNA})}$, $\Delta\Delta C_T = \Delta C_{T(\text{target gene})} - \Delta C_{T\ (16\text{S rRNA})}$ and ratio $= 2^{-\Delta\Delta CT}$. The quantity of cDNA for each experimental gene was normalized to the quantity of 16S cDNA in each sample determined in a separate reaction. Each RNA sample was run in triplicate; values represent the means of at least three separate RNA samples.

Modified PAP/AUC

A modified PAP/AUC analysis was carried out according to the procedure described previously.[23] In brief, single colonies were inoculated in tryptic soy broth (TSB) and grown overnight at 37°C with shaking at 150 rpm. The bacterial solution was diluted with sterile saline to 10^{-3} and 10^{-5}. Aliquots of 100 μL of the diluted bacterial solution were spirally plated on BHI agar plates with the following concentrations of vancomycin: 0, 0.5, 1, 2, 3, 4, 6 and 8 mg/L. Colonies were counted after the plates had been incubated at 37°C for 48 h. PAP/AUC was calculated using Graphpad Prism 5.0 (Graphpad software, Inc., CA, USA). The AUC_{test}/AUC_{Mu3} ratio was calculated and strains were categorized as VSSA, hVISA and VISA according to the ratio as follows: VSSA, <0.9; hVISA, 0.9–1.3; and VISA, >1.3. Mu3 and Mu50 strains served as hVISA and VISA controls, respectively.[23]

TEM

The preparation of *S. aureus* cells for TEM was performed using standard procedures described previously.[24] Briefly, cells suspended in growth medium were fixed with an equal volume of 5% glutaraldehyde and stored for at least 24 h at 4°C until they were further processed for TEM. Thirty cells of each strain with nearly equatorial cut surfaces were measured for the evaluation of cell wall thickness.

Results

Non-synonymous mutations in the VISA strain (VISA072) compared with VSSA303

We sequenced and compared the whole genomes of VISA strain VISA072 and its susceptible, parental strain VSSA303. VISA072 and VSSA303 had a genome of 2753954 and 2756914 bp, respectively. We were able to map reads from the two strains to 98.3% and 98.1% of the *S. aureus* HL1 reference genome, respectively. The overall G + C contents were 32.7% for both. Only five mutations (four SNPs and a deletion) were identified by comparison of genome sequence data, including *rpoB* (H481Y), *dprA* (G196C), *femA* (F92C), *vraR* (E127K) and a 64 bp deletion of *agrC* (E391stop) (Table 2). All predicted mutations were non-synonymous and were further confirmed by PCR and sequencing. SIFT scores were calculated for four amino acid substitutions to predict whether these mutations affect protein function; all mutations were predicted to be deleterious (SIFT score <0.05) (Table 2). A mutation in the *dprA* gene encoding DNA processing protein has not been identified previously as associated with antibiotic resistance. Even though mutations of *vraR*, *agrC* and *femA* genes have been found in the hVISA or VISA phenotype, the amino acid alterations detected in this study have not been identified. In the present study, we focused on the mutations in three genes (*dprA*, *femA* and *vraR*) to investigate the effect of genetic alterations on VSSA-to-VISA conversion.

Effect of genetic alterations on antibiotic susceptibility

To examine the functional influence of specific mutations found in the VISA strain, we cloned three mutated genes from VISA072, *dprA* (G169C), *femA* (F92C) and *vraR* (E127K), into multi-copy plasmid pMK4. Subsequently, the constructs were transformed into a vancomycin-susceptible clinical strain, VSSA303, and a vancomycin-susceptible laboratory strain, RN4220, by electroporation, generating transformed strains, designated S303-*dprA*$_{G169C}$, S303-*femA*$_{F92C}$, S303-*vraR*$_{E127K}$, RN-*dprA*$_{G169C}$, RN-*femA*$_{F92C}$ and RN-*vraR*$_{E127K}$, respectively (Tables 1 and 2). Because the two-component regulatory system VraSR is co-transcribed and the components work together, we constructed a mutated *vraR* gene (*vraR*$_{E127K}$) alone (S303-*vraR*$_{E127K}$ and RN-*vraR*$_{E127K}$) and mutated *vraR* plus intact *vraS* (S303-*vraSR*$_{E127K}$ and RN-*vraSR*$_{E127K}$), respectively, to confirm the role of the two-component regulatory system in vancomycin resistance. Transformed strains with a mutated *vraR* gene, S303-*vraR*$_{E127K}$ and RN-*vraR*$_{E127K}$, showed increased vancomycin MICs, compared with their parental strains VSSA303 and RN4220 (from 1 to 2 mg/L) (Table 3). Similar results were observed in S303-*vraSR*$_{E127K}$ and RN-*vraSR*$_{E127K}$, in which the *vraSR*$_{E127K}$ operon was introduced (Table 3). Simultaneously, the susceptibilities to teicoplanin of transformed strains with a mutated *vraR* gene also decreased (0.25 to 1 mg/L). However,

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Table 2. Non-synonymous mutations in a VISA strain (VISA072) of the ST72 lineage, identified by comparing the WGS with its parental VSSA strain (VSSA303)

Locus ID[a]	Gene	Mutation in VISA072 (nucleotide no. in HL1)	Product	Effect of mutation	SIFT score[b]
SAKOR_00528	*rpoB*	C to T (555020)	DNA-directed RNA polymerase β chain	H481Y	0.02
SAKOR_01176	*dprA*	G to T (1206081)	DNA processing protein	G196C	0.01
SAKOR_01311	*femA*	T to G (1350745)	UDP-*N*-acetylmuramoylpentapeptide-glycine glycyltransferase	F92C	0.01
SAKOR_01840	*vraR*	C to T (1906311)	histidine kinase two-component response regulator	E127K	0.01
SAKOR_02002	*agrC*	64 bp deletion (2035974)	sensory transduction histidine kinase	E391stop	–

[a]Gene identification number in *S. aureus* HL1 (GenBank accession number CP003979), which is a CA-MRSA strain with the same genetic lineage (ST72) as the two sequenced strains, VISA072 and VSSA303.
[b]Significant difference in gene function as predicted by the SIFT score.

Table 3. Antibiotic susceptibilities and PAP/AUC analysis of RN4220 and VSSA303 strains carrying mutated gene

Strain[a]	MIC[b] (mg/L)					PAP/AUC (ratio to Mu3)
	VAN	RIF	TEI	DAP	OXA	
VSSA303	1	<0.06	0.25	1	32	VSSA (0.53)
VISA072	4	>64	4	2	64	VISA (1.43)
RN4220	1	<0.06	0.5	0.5	0.12	VSSA (0.56)
RN-pMK4	1	<0.06	0.25	0.5	0.12	VSSA
RN-$dprA_{G196C}$	1	<0.06	0.25	0.5	0.12	NT
RN-$femA_{F92C}$	1	<0.06	0.5	0.5	<0.06	NT
RN-$vraR_{E127K}$	2	<0.06	1	0.5	0.12	hVISA (0.92)
RN-$vraSR_{E127K}$	2	<0.06	1	0.5	0.12	hVISA (0.92)
S303-$dprA_{G196C}$	1	<0.06	0.25	0.5	8	NT
S303-$femA_{F92C}$	1	<0.06	0.25	0.5	4	NT
S303-$vraR_{E127K}$	2	<0.06	1	1	16	hVISA (0.94)
S303-$vraSR_{E127K}$	2	<0.06	1	1	32	hVISA (0.96)

VAN, vancomycin; RIF, rifampicin; TEI, teicoplanin; DAP, daptomycin; OXA, oxacillin; NT, not tested.
[a]$vraSR_{E127K}$ operon carrying mutated *vraR* (E127K) and intact *vraS* was introduced into RN4220 and VSSA303 strains.
[b]MICs were determined using the broth microdilution method according to the CLSI.[15]

no MIC changes were observed for the other transformed mutants with mutated *dprA* and *femA* genes. The introduction of a mutated *femA* gene into VSSA303 and RN4220 decreased the oxacillin MIC from 32 to 4 mg/L and from 0.12 to ≤0.06 mg/L, respectively, which was a phenomenon similar to that reported for other *vraSR* mutants in MRSA strains.[25] In addition, a decrease in oxacillin MIC from 32 to 8 mg/L was observed for S303-$dprA_{G169C}$. The susceptibility to rifampicin of all transformants was not changed.

vraR mutation (E127K) confers acquisition of hVISA phenotype

To validate the impact on vancomycin susceptibility of the single-base mutation in *vraR* (E127K), we performed qRT–PCR and population analysis. VISA072 exhibited overexpression of the *vraSR* regulon without respect to vancomycin exposure and both VISA072 and a strain with a mutated *vraR* gene (S303-$vraSR_{E127K}$) also showed *vraSR* mRNA overexpression under vancomycin exposure (Figure 1). As shown in Figure 2, all mutants with mutated *vraR* (S303-$vraR_{E127K}$ and RN-$vraR_{E127K}$) showed a typical hVISA curve, as seen in the Mu3 strain (Figure 2a). Notably, similar results were observed in the RN4220 strain, indicating that mutated *vraR* ($vraR_{E127K}$) expression might not be effective in the limited physiological milieu of the VSSA cell to achieve an hVISA phenotype (Figure 2b). Additionally, no differences were found between transformants with $vraR_{E127K}$ and $vraSR_{E127K}$, which is consistent with the observation of vancomycin MICs (Table 3).

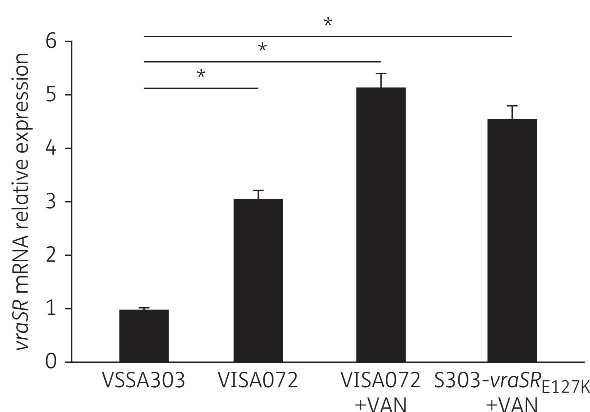

Figure 1. Quantification of *vraSR* mRNA by qRT–PCR. Relative fold change values of specific *vraSR* mRNAs are shown on the vertical axis; 16S rRNA was used as an internal control. VSSA303 was included as a reference strain (relative value = 1). *Significantly greater than strain VSSA303 ($P < 0.05$). The transcription level of VISA072 grown in the presence of vancomycin was significantly greater than that for VISA072 grown in the absence of vancomycin ($P < 0.05$). VAN, vancomycin.

Expression of mutated *vraR* (E127K) increases cell wall production

TEM revealed that S303-$vraR_{E127K}$ showed a remarkable change in cell wall thickness, compared with VISA072 and parental VSSA303 (Figure 3). The cell wall thickening, which prevents the diffusion of vancomycin to its active site in the cytoplasmic membrane in the

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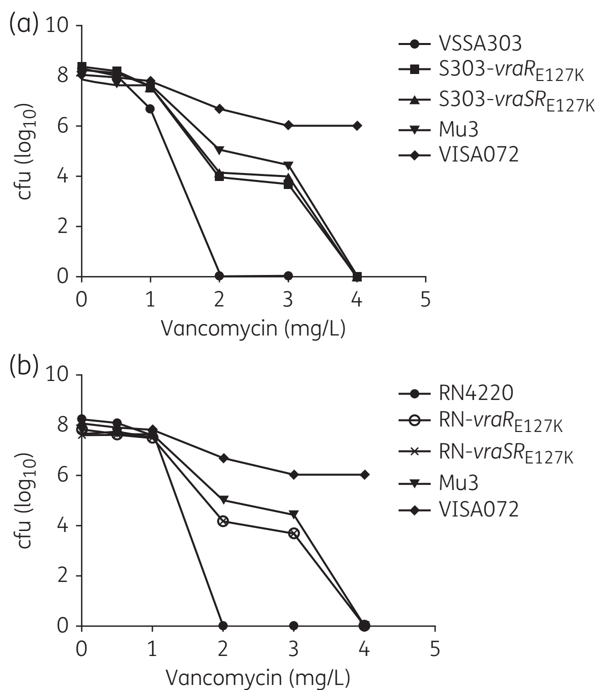

Figure 2. Vancomycin PAP of (a) VSSA303 and (b) RN4220 strains carrying mutated *vraR* and *vraSR*. All strains with mutated *vraR* (*vraR*$_{E127K}$ and *vraSR*$_{E127K}$) showed typical hVISA curves according to the growth rate of the Mu3 strain, indicating that mutated *vraR* contributed to VSSA-to-hVISA conversion.

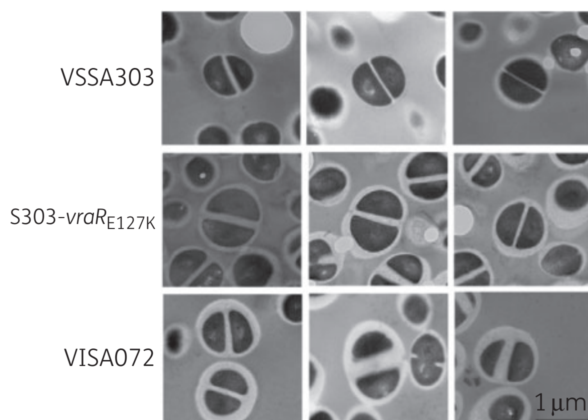

Figure 3. Cell wall thickness of *S. aureus* VSSA303, S303-*vraR*$_{E127K}$ and VISA072. Representative TEM revealed that S303-*vraR*$_{E127K}$ showed a remarkable change in cell wall thickness, compared with VISA072 and parental VSSA303, indicating the increased vancomycin MIC due to *vraR* mutation.

division septum, is one of the consistently observed features of hVISA or VISA strains.[26] The cell wall in S303-*vraR*$_{E127K}$ was thicker than that in the parental strain VSSA303, which might be due to the effect of *vraR* mutation on the cell wall. However, it was less thick than the phenotype observed in VISA072 strains, suggesting the existence of additional factors in the development of a full VISA phenotype.

Discussion

We tracked genetic alterations during the development of reduced susceptibility to vancomycin in a prevalent CA-MRSA clone in Korea, ST72-MRSA-IV. VISA or hVISA phenotypes have been reported mostly among HA-MRSA strains, rather than CA-MRSA strains, because selective pressure leading to vancomycin non-susceptibility may be much greater in the hospital environment. Several studies showed that the PVL-negative community strain ST72-MRSA-IV accounted for a substantial proportion of HA infections in the hospitals, suggesting that CA-MRSA strains are emerging as major causes of HA infections in Korea.[14] Recently, ST72-MRSA-IV isolates that developed reduced susceptibility to key drugs, such as vancomycin and rifampicin, have been described.[11,27]

The genetic mechanism of reduced susceptibility to vancomycin in VISA and hVISA is still not understood fully. Diverse mutations in a small number of staphylococcal regulatory genes associated with reduced susceptibility to vancomycin have been experimentally proven to confer the resistance phenotype.[2] Herein, we identified five non-synonymous mutations by WGS analysis, including E127K in *vraR*, H481Y in *rpoB*, G196C in *dprA*, F92C in *femA* and a 64 bp deletion of *agrC*. Introduction of mutated *vraR* into the susceptible strains VSSA303 and RN4220 resulted in an increase in vancomycin MIC, suggesting that the *vraR* mutation E127K might influence VSSA-to-hVISA conversion regardless of genetic background. In addition, introduction of mutated *vraR* and *vraSR* showed the same elevated vancomycin MICs and results in PAP, implying that the effect of introduced *vraR*$_{E127K}$ was not dependent on the presence of VraS in the cell. The conformational change of the VraR protein by genetic alteration might have activated its response regulator function, independently of its signal transducer, VraS. A remarkable increase in cell wall thickness in the VSSA303 strain carrying a mutated *vraR* was also shown. Taking these results together, it is proposed that the genetic alteration *vraR*$_{E127K}$ may partially contribute to development of the VISA phenotype.

In addition to glycopeptides such as vancomycin and teicoplanin, the VISA072 strain developed non-susceptibility to rifampicin and daptomycin during treatment. Genetic alterations in VISA072 might contribute to the reduced susceptibility to these antibiotics. The *rpoB* gene encodes the RNA polymerase β-subunit and mutations in the *rpoB* gene, including H481Y in this study, are known to be exclusively associated with rifampicin resistance.[28] Recently, it has been reported that mutations in the *rpoB* gene might confer dual hetero-resistance to daptomycin and vancomycin in *S. aureus* and promote hVISA-to-VISA conversion of a strain Mu3.[6,28] The *agrc* gene is an essential part of the quorum-sensing *agr* locus in *S. aureus*, the 64 bp deletion in *agrc* gene (encoding sensory transduction histidine kinase) that created a stop codon at amino acid position 391, prematurely terminating *agrc* translation. Loss of function in *agr* has frequently been found in VISA strains.[29] The exact mechanism of how reduced *agr* expression affects reduced antibiotic susceptibility is not well known. Because the *agr* locus may be associated with other regulatory genes in *S. aureus*, it is speculated that changes in *agr* expression may invoke cell wall changes, as seen in VISA strains. The dysfunction of *agr* has been shown to reduce toxin release and virulence *of S. aureus* in a murine arthritis model.[30] Thus, increased

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vancomycin resistance may be linked to reduced virulence in *S. aureus*, although we did not investigate it. FemA is involved in the formation of UDP-*N*-acetylmuramoylpentapeptide, a staphylococcal cell wall precursor.[31] Although the introduction of an altered *femA* gene into VSSA strains did not change the vancomycin MIC, it might affect cell wall synthesis and partially contribute to the reduced susceptibility to vancomycin.[32] The *femA* gene and *vraSR* are well known as additional genetic determinants of the oxacillin resistance phenotype.[33] Thus, the genetic alteration in the *femA* gene may be associated partly with the change in oxacillin resistance.

Evolution to full VISA appears to be a step-wise process in many cases, where a number of unique and highly diverse mutations, each having an additional impact on resistance accumulation over time.[2] Although it was revealed that a mutation in the *vraR* gene mainly affected the development of the VISA phenotype in our strain, genetic alterations in the *rpoB* and *agrC* genes may potentially contribute to the reduced susceptibility to vancomycin. A recent study described four SNPs involved in reduced susceptibilities to vancomycin and daptomycin based upon complete genome data on the ST72 background.[12] Interestingly, there was no consistency with SNPs in our results, except a mutation in the *rpoB* gene, despite the same genetic background as that in our study, implying there are diverse paths to the acquisition of antibiotic resistance.

There are some limitations in our study. First, we transformed and overexpressed mutated genes in VSSA strains without knockout of the corresponding gene in recipients to examine the effect of alteration in VISA. Other factors might have affected increased vancomycin MIC. Second, the effect of mutation in the *rpoB* gene was not examined in this study, even though the genetic alteration of the gene was well known to confer vancomycin resistance. In addition, we did not identify the reversion of VISA to the VSSA phenotype by inactivating *vraR*. However, we aimed to explore the role of a mutation of the *vraR* gene in reduced vancomycin susceptibility in our VISA strain, not the role of the whole gene.

In summary, we identified five amino acid mutations potentially associated with reduced susceptibility to vancomycin using isogenic VSSA and VISA strains of ST72. In addition to mutations in the *rpoB* gene, a genetic alteration in the *vraR* gene, E127K, is revealed to contribute to the development of the VISA phenotype, which was supported by cell wall thickness and PAP analysis.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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