Downregulation of RNAIII in vancomycin-intermediate *Staphylococcus aureus* strains regardless of the presence of *agr* mutation

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Reduced vancomycin susceptibility in *Staphylococcus aureus* can cause serious problems relating to treatment failure and persistent infection. We investigated vancomycin susceptibility, genetic relationships and transcriptional changes of the accessory gene regulator (*agr*) in vancomycin-intermediate *S. aureus* (VISA) strains isolated from South Korea compared with vancomycin-susceptible *S. aureus* (VSSA) strains. Molecular characterization, population analysis profiling, *agr* sequencing and transcriptional profiling of RNAIII by real-time RT-PCR were performed. Of 16 VISA strains tested, eight exhibited ST5, *agr* II and type II SCC*mec*. The others exhibited ST239, *agr* I and type III SCC*mec*. A point mutation in AgrA (Asp8Gly or Ile238Lys) was found in only five VISA strains; no mutations were detected in the other strains. However, RNAIII levels markedly decreased in all VISA strains (mean of 1.39-fold change) compared with the VSSA strains (31.51-fold change) in late-exponential phases (*P*<0.0001). The downregulation of RNAIII could be an important genetic event in the VISA strains, regardless of the presence or absence of the *agr* mutation.

**INTRODUCTION**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major concern in the treatment of infectious diseases worldwide. MRSA causes a variety of infections, ranging from benign skin and soft tissue infections to fatal invasive diseases, both in hospital settings and more recently in community settings (Archer, 1998; Chambers, 2001; Tristan et al., 2007; Vandenesch et al., 2003). Increasing antibiotic resistance of *S. aureus*, including reduced susceptibility or resistance to vancomycin, represents a continuing threat to human health. In particular, the emergence of vancomycin-intermediate *S. aureus* (VISA) and heterogeneous-VISA (hVISA) presents a challenge with regard to treatment failure and persistent infection (Hiramatsu *et al.*, 2002; Howden *et al.*, 2004, 2005; Tenover & McDonald, 2005; Walsh & Howe, 2002). In a survey conducted by the Asian Network for Surveillance of Resistant Pathogens study group in South Korea, hVISA strains comprised ~6.1% of the total resistant strains reported (Song *et al.*, 2004). Chung *et al.* (2010) recently reported a VISA/hVISA frequency of 0.09% among *S. aureus* isolates collected during 2001–2006 in a nationwide surveillance study and investigated their molecular epidemiology.

These rare vancomycin-resistant *S. aureus* (VRSA) strains have been reported to carry the Tn1546-linked resistance mechanism but the molecular mechanism conferring reduced susceptibility in VISA is still unclear. VISA/hVISA strains exhibit phenotypic changes, including cell-wall thickening and reduced autolytic activity (Cui *et al.*, 2003;
Pflanz et al., 2000; Reipert et al., 2003). Consistent upregulation or mutation of vraSR, graSR, rpoB, walK and clpP genes has been reported to be involved (Cui et al., 2009, 2010; Kuroda et al., 2000; McAleese et al., 2006; Mwangi et al., 2007; Shoji et al., 2011). A point mutation and gene dysfunction of the hld locus of the accessory gene regulator (agr), a quorum sensing regulatory system in S. aureus, have also been described (McAleese et al., 2006; Sakoulas et al., 2002).

However, Howden et al. (2008) reported little genetic difference between vancomycin-susceptible S. aureus (VSSA) and VISA strains from clinical pairs, concluding that the establishment of reduced susceptibility to vancomycin could be related to multiple factors, including attenuating host factors.

In the present study, we investigated the genetic and transcriptional differences in the agr gene between VISA and VSSA strains obtained in South Korea. The clonal relationships of the VISA strains were also investigated by comparing the molecular characteristics of the clinical VSSA strains available in a database.

**METHODS**

**Bacterial strains and growth conditions.** Sixteen VISA strains with reduced vancomycin susceptibility that had been isolated from Korea Centers for Disease Control and Prevention were used for population analysis profiling (PAP), agr gene sequencing and agr expression profiling. Strains Mu50 (ATCC 700699) and Mu3 (ATCC 700698) were used as standard VISA strains for PAP. Of the 137 MRSA strains used in our previous studies (Park et al., 2007, 2008), 11 clinical VSSA strains (PT3, R9, ES50 and YS24, representative of ST5-SCCmec II; YS17, PT2 and PT13 of ST239-SCCmec III; ES22 and YS2 of ST72-SCCmec IVA; and WS19 and PT26 of ST1-SCCmec IVA) were chosen for vancomycin susceptibility testing and PAP. Of these clinical strains, YS17 and YS24 exhibited agr gene sequence types identical to those of the strains with reduced vancomycin susceptibility and were used for agr gene sequencing and expression profiling. S. aureus strains ATCC 29213 and NCTC 8325 were used as standard strains for vancomycin susceptibility and agr gene expression profiling.

For expression profiling, bacterial cells from an overnight culture were inoculated into fresh medium and grown to early- (OD600 0.1, 1 h after inoculation), middle- (OD600 0.6, 2.5 h after inoculation) or late-exponential phase (OD600 2.5, 5 h after inoculation). OD600 was measured using a spectrometer (Spectronic 20D+; Thermo Fisher Scientific).

**Vancomycin susceptibility and population analysis profiling.** Vancomycin was purchased from Sigma Aldrich, Korea. Vancomycin susceptibilities were determined using the agar-dilution method as recommended by the CLSI (2010). PAP was performed using serial dilutions of a culture suspension (OD600 0.3) grown on brain heart infusion agar (BD) containing various concentrations of vancomycin (0–10 μg ml⁻¹) at 37 °C for 48 h (Berger-Bächi et al., 1986; Hiramatsu et al., 1997a). The experiments were repeated at least four times for each strain. The statistical significance of the difference between area under the curve (AUC) values was evaluated using paired t-tests or one-way analysis of variance with a Tukey test. The data were considered significant at P<0.05.

**Molecular epidemiological study of strains with reduced vancomycin susceptibility**

Multilocus sequence typing (MLST), protein A gene (spa) analysis, PFGE and SCCmec typing were performed as previously described (Aires-de-Sousa et al., 2006; Enright et al., 2000; Murchan et al., 2003; Oliveira & de Lencastre, 2002). Each sequence type (ST) and spa gene type was assigned using the S. aureus MLST (http://saureus.mlst.net/) and spa (http://www.ridom.de/spaserver/) databases, respectively. Pulsotypes were determined based on a similarity cut-off of 80% (analysed by the Dice coefficient and unweighted pair group method with arithmetic mean, with 1% tolerance and 0.5% optimization settings) and the criterion of six or fewer bands (Murchan et al., 2003). Pulsotypes were analysed using a previously generated database (Lee et al., 2008).

**RFLP and DNA sequence analyses of the accessory gene regulator (agr) locus.** The Agr group of each strain with reduced vancomycin susceptibility was identified by Draf-RFLP analysis as described previously (Papakyriacou et al., 2000). Amplification and DNA sequencing of the agr gene from strains YS17 and YS24 were performed using the primers listed in Table 1, as described previously (Renzoni et al., 2004). Sequence assembly, similarity searches and determination of the ORFs of the agr gene sequences were performed using BLASTN, BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ORF Finder (http://www.ncbi.nlm.nih.gov/orf/orf.html), respectively. Template sequences (accession nos DQ157958 and DQ157968) of agr I and II were used for multiple sequence alignments using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Larkin et al., 2007).

**RNAIII transcriptional profiling by real-time RT-PCR and delta- haemolysin expression.** For total RNA isolation, RNAProtect Bacteria Reagent (Qiagen) was added to bacterial cultures at the appropriate growth stage (early-, middle-, or late-exponential phase) before lysis in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) containing lysostaphin (200 μg ml⁻¹) for 5 min at 37 °C. Bacterial nucleic acids were isolated and purified using an Easy DNA kit (Invitrogen) and an RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. For real-time PCR, DNase I (Roche Applied Science) was added to the RNA preparations to remove contaminating DNA. Genomic DNA was used for DNA sequencing of the accessory gene regulator (agr) locus, spa analysis, PAP, and PFGE.

**Table 1. PCR primers and probes (Taqman) used for sequencing and expression analysis of the agr locus**

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer sequence (5’→3’)</th>
<th>Reverse primer sequence (5’→3’)</th>
<th>Probe sequence (5’→3’)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>agr</td>
<td>seq1 (CATACGACCTGAGTCCAGAGG)</td>
<td>seq2 (GGACCGATACGATGCAATTTG)</td>
<td>573T (CTTACGGCGCGCGCCAGCCA)</td>
<td>Renzoni et al. (2004)</td>
</tr>
<tr>
<td>16S</td>
<td>551F (GGCAAGGCGGATGCGGATGATT)</td>
<td>651R (GTTCGAATGACCCCTCAAGG)</td>
<td>388T (TTATTATGACG)</td>
<td>Vaudaux et al. (2002)</td>
</tr>
<tr>
<td>RNAIII</td>
<td>367F (TTCTACTTGATGTTGCAATTTCA)</td>
<td>436R (TGATTTCAATGACCCTCCG)</td>
<td>ATGTTGAATGA</td>
<td>Vaudaux et al. (2002)</td>
</tr>
<tr>
<td>RNAIII</td>
<td>34F (CTAAGGAGGCAATG)</td>
<td>135R (CTCAAGGAGCTCTCAT)</td>
<td>83T (AAAGGCTATGGAAA)</td>
<td>Vaudaux et al. (2002)</td>
</tr>
</tbody>
</table>

*5’-FAM- and 3’-TAMRA-labelled.
as the template for amplification of the real-time PCR target region and each PCR product was used as an internal standard for the quantification of transcriptional expression.

Real-time quantitative RT-PCR was performed using LightCycler probe master mix (Roche) and a LightCycler 480 (Roche) thermal cycler as described previously (Vaudaux et al., 2002). The primers and probes listed in Table 1 were used at concentrations of 0.2 and 0.1 mM, respectively. PCR efficiency was measured for each pair of primers and probes (Table 1) and was taken into account for the relative quantification. The level of each mRNA was normalized to the level of its 16S rRNA. RNAII and RNAIII levels were compared with those of the controls (ATCC 29213 and NCTC 8325) and VSSA strains YS17 and YS24. The statistical significance values of the normalized positivity cycle (Cp) of each transcript was evaluated by paired t-tests and data were considered significant at P < 0.05.

Using S. aureus strains RN4220, RN6607 (positive control) and RN9120 (negative control), δ-haemolysin expression of VISA strains was determined by the enhanced haemolysis zone as described previously (Sakoulas et al., 2002).

**Nucleotide sequence accession numbers.** The nucleotide sequences of the agr locus determined in the present study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank) under the accession numbers GU817317 (for strain V335), GU817318 (for strain V521), JN655161 (for strain V093), JN655162 (for strain V088), JN655163 (for strain V061) and JN655165 (for strain V023).

# RESULTS

**Genetic background of strains with reduced vancomycin susceptibility**

Almost all vancomycin-susceptible MRSA isolates from our previously established database grouped into major clonal types with combinational clustering based on MLST, spa cluster, PFGE and SCCmec typing analyses (Fig. 1) (Lee et al., 2008; Park et al., 2007). Out of sixteen VISA strains, eight exhibited ST5, agr II group and type II SCCmec, with the spa types t002 (r26-r23-r17-r34-r17-r20-r12-r16-r17) or t045 (r26-r17-r20-r17-r12-r17-r16) or t045 (r26-r17-r20-r17-r12-r17-r16) (strains V028, V061, V088, V093, V095, V222, V303 and V335). The others showed ST239, agr I group and type III SCCmec, with spa type t037 (r15-r12-r16-r02-r25-r17-r24) (V015, V018, V023, V063, V091, V518, V521 and V606). PFGE analyses of these strains, integrated with previously analysed PFGE types of clinical invasive VSSA strains, revealed that the genetic background of these strains grouped them into pulsotypes of ST5 and ST239, which are prevalent pulsotypes in multidrug-resistant hospital-associated infections (Fig. 1).
Population analysis profiling of VSSA and strains with reduced vancomycin susceptibility

The MICs of the VSSA strains ranged from 1 to 2 μg vancomycin ml⁻¹, whereas those of the VISA strains and strain Mu50 were 4 and 8 μg ml⁻¹, respectively. The Mu50 cell population began to decline at vancomycin concentrations >6 μg ml⁻¹ and the cell numbers of the VISA strains showed a rapid decrease at vancomycin concentrations >4 μg ml⁻¹ in the PAP study (Fig. 2). Nevertheless, a subpopulation of strains with reduced vancomycin susceptibility was capable of growth in the presence of 6–7 μg vancomycin ml⁻¹. In contrast, the growth of the VSSA strains was inhibited at 2–3 μg vancomycin ml⁻¹ (Fig. 2). Based on four experiments, the mean AUC of the VISA strains was 36.59 ± 6.01 (c.f.u.)micrograms, and that of strain Mu50 was 54.36 ± 2.87 (c.f.u.)micrograms. The AUC values of the VSSA strains was 13.99 ± 6.23 (c.f.u.)micrograms (P<0.001, with all pairwise multiple comparison procedures). The range of AUC ratios with strain Mu3 of the VISA strains was 0.9–2.2 (c.f.u.)micrograms.

Agr amino acid sequence analysis

The sequences of the entire agr locus of the VISA strains were compared with those of the two VSSA strains YS17 (agr I, ST239) and YS24 (agr II, ST5) and with two reference sequences (DQ157958 and DQ157964: agr I prototype; DQ157968: agr II prototype) from GenBank. The identical agr sequences grouped into six types accessioned with the numbers GU817317 (for strains V222 and V335), JN655163 (for strains V028, V061, V303 and Mu50), JN655161 (for strains V093 and V095), JN655162 (for strain V088), GU817318 (for strains V015, V018, V091, V518, V521 and V605) and JN655165 (for strains V023 and V063). The alignment of the deduced amino acid sequences of AgrA and AgrC were performed. Also the AgrC amino acid sequence of two VISA strains (agr I group) was identical to that of the reference sequence (DQ157964). Although residue Ser was replaced with Arg at position 6 in the AgrC amino acid sequence of six VISA strains including V521 (GU817318) (agr I group) compared with the reference sequences (DQ157958 and DQ157964), the sequence from them was identical to that from strain YS17 (VSSA).

δ-Haemolysin expression and evaluation of RNAIII transcriptional profiling by real-time RT-PCR.

Using real-time RT-PCR, the levels of RNAIII and RNAII (agrA) normalized to the level of 16S rRNA in the VISA strains represent at least four experiments with each strain. The AUC values for strains Mu50, V222, V335, V521 and V605 are 54.36 ± 2.87, 35.36 ± 3.65, 36.50 ± 7.13, 33.53 ± 3.13 and 37.45 ± 1.23 (c.f.u.)micrograms, respectively. The AUC values for the VSSA strains at vancomycin MIC 1 μg ml⁻¹ and vancomycin MIC 2 μg ml⁻¹ were 5.78 ± 3.13 and 17.07 ± 3.60 (c.f.u.)micrograms, respectively.
strains were compared with those in the reference strains (ATCC 29213 and NCTC 8325) and the VSSA strains (YS17 and YS24). The PCR efficiencies of 16S rRNA, RNAIII and RNAII obtained by using constructed standards were 1.955, 1.938 and 1.889, respectively. Fig. 3a shows that the levels of RNAIII in all VISA strains (1.39-fold change in late-exponential phase) were markedly lower than those in the reference strains (31.51-fold change in late-exponential phase) \((P<0.001; 95\% \text{ CI }; -32.677 \text{ to } -27.554)\). In contrast, there was little difference in the RNAII level between the VISA (1.60-fold change in late-exponential phases) and the VSSA strains (2.25-fold in late-exponential phases) \((P=0.2833; 95\% \text{ CI }, -0.582 \text{ to } 1.881)\) (Fig. 3b).

Also none of the VISA strains produce delta-haemolysin, as manifested by the absence of the enhanced haemolysis zone at the interface with RN4220.

**DISCUSSION**

Since 1997 when the first *S. aureus* strain with reduced susceptibility to vancomycin was reported in Japan (Hiramatsu *et al.*, 1997b), the emergence of hVISA/VISA strains has been a major threat in terms of therapeutic failure and persistent infection. Most of the geographically diverse hVISA/VISA strains belong to *agr* group I or II (Howe *et al.*, 2004), although strains of all *agr* groups (I–IV) could be induced to VISA status *in vitro* (Tsuji *et al.*, 2007).

In South Korea, the two major types of vancomycin-susceptible MRSA strains are known as multi-drug resistant and prevalent in hospital-acquired infection; they exhibit ST5-*agr* II-type II SCCmec and ST239-*agr* I-type III SCCmec, respectively (Kim *et al.*, 2007; Park *et al.*, 2007). Other major types common in community-associated infections are ST72-*agr* I-type IVA SCCmec (*tnp20* inserted into class B mec and carrying pUB110) and ST1-*agr* III-type IVA.
SCCmec clonal type (Park et al., 2007, 2009). PFGE analysis comparing the VISA strains in the present study with the vancomycin-susceptible strains reported in a previously established database (Lee et al., 2008) showed that the present strains belong to the major hospital-acquired infection clonal type with the multi-drug resistant nature (Fig. 1). These findings suggest that strains with reduced vancomycin susceptibility are clonally related to the major MRSA strains that are predominant in hospital-acquired infections in Korea.

The agr locus encodes an auto-inducing, quorum-sensing system and functions as a global regulator that can upregulate and downregulate adhesion molecules, toxins and other virulence factors (Ji et al., 1995). It is also capable of causing bacterial interference by auto-inducing peptides (Ji et al., 1997). An agr dysfunction has been shown to influence the establishment of VISA and hemB mutant small colony variants (Sakoulas et al., 2002; Vaudaux et al., 2002). In the alignment analysis of agr genes in this experiment, a point mutation was observed in only five of the VISA strains (V028, V061, V222, V303 and V335) and was not observed in the other 11 VISA strains. Previously, some studies have revealed marked genetic changes in hVISA/VISA strains but others have reported no agr sequence differences between clinical pairs (parental VSSA and hVISA/VISA strains) during persistent infection (Howden et al., 2006, 2008). These findings suggest that point mutations of the agr gene cannot always explain reduced vancomycin susceptibility.

However, the RNAIII transcript levels in all VISA strains, regardless of the presence of an agr mutation, markedly decreased during middle- and late-exponential phases (Fig. 3). As RNAIII plays key roles in global networking, decreased RNAIII levels may result in physiological changes in cell growth and may contribute to the reduced vancomycin susceptibility. Other studies have suggested that agr dysfunction, independent of point mutations, may cause decreased efficacy of vancomycin in MRSA infection and may be related to persistent bacteremia (Fowler et al., 2004; Sakoulas et al., 2006). Although diverse molecular and physiological factors have been reported, transcriptional changes in RNAIII and the consequent dysfunction of the agr gene are likely to be one of the important factors in the establishment of reduced vancomycin susceptibility and may be useful in identifying these strains.

In conclusion, the VISA strains should be clonally related to the major epidemiological groups (agr I and II) that are predominant in hospital-associated S. aureus infection. This study also suggests that the transcriptional downregulation of RNAIII could be one of important genetic clues in the development of reduced vancomycin susceptibility in addition to mutations in the agr locus and other genetic factors; other genetic events may also affect the regulation of RNAIII and not via agr mutation. Further studies are needed to elucidate other molecular mechanisms involved in agr dysfunction and the establishment of reduced vancomycin susceptibility. Additionally, other transcriptional changes that contribute to reduced vancomycin susceptibility should be investigated.

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REFERENCES


Staphylococcus aureus bacteremia. During persistent methicillin-resistant associated with the evolution of low-level vancomycin resistance expression patterns and attenuated host immune responses are in Korea.


