**Reduced susceptibility to vancomycin in isogenic *Staphylococcus aureus* strains of sequence type 59: tracking evolution and identifying mutations by whole-genome sequencing**

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**Objectives:** Vancomycin-intermediate *Staphylococcus aureus* (VISA) and heterogeneous VISA (hVISA) phenotypes are increasingly reported in methicillin-resistant *S. aureus* (MRSA) strains of distinct genetic backgrounds. This study tracked genetic evolution during the development of vancomycin non-susceptibility in a prevalent Asian community-associated MRSA clone of sequence type (ST) 59.

**Methods:** ST59 strains were consecutively isolated from a patient who failed chemotherapy for a septic knee over 15 months. The genetic mutations associated with the VISA phenotype were identified by whole-genome sequencing of two strains, which had the vancomycin-susceptible *S. aureus* (VSSA) and VISA phenotypes. The mutations were subsequently screened in other strains. By correlating the accumulated mutations with vancomycin susceptibility, genetic evolution was tracked at the whole-genome scale.

**Results:** Nine non-synonymous mutations and two steps of genetic evolution were identified during the development of the VISA phenotype. The first step involved a nonsense mutation in agrC and point mutations at five other loci, which were associated with the VSSA-to-hVISA conversion. Mutations of rpoB and fusa following the use of rifampicin and fusidic acid were identified in the second step of evolution, which corresponded to the development of dual resistance to rifampicin and fusidic acid and the conversion of hVISA to VISA.

**Conclusions:** *In vivo* genetic evolution of *S. aureus* occurred in stepwise order during the development of incremental vancomycin non-susceptibility and was related to the use of antimicrobial agents.

**Keywords:** genetic evolution, ST59, vancomycin-intermediate *Staphylococcus aureus*

**Introduction**

*Staphylococcus aureus* strains with reduced susceptibility to vancomycin, including vancomycin-intermediate *S. aureus* (VISA) strains (vancomycin MIC 4–8 mg/L), heterogeneous VISA (hVISA) strains and vancomycin-susceptible *S. aureus* (VSSA) strains with a higher vancomycin MIC (1.5–2 mg/L), have been increasingly reported in the past decade and are associated with poor clinical outcomes in patients treated with glycopeptides.1–8 It is now clear that the mechanism of the VISA or hVISA phenotype is distinct from the vanA-mediated high-level resistance in vancomycin-resistant *S. aureus* (VRSA, vancomycin MIC ≥16 mg/L) and involves multiple different pathways.¹

Comparative genomics using whole-genome sequencing of isogenic strains with VSSA and VISA phenotypes has successfully identified several point mutations associated with the VISA phenotype. Frequently reported VISA-associated single-nucleotide polymorphisms (SNPs) have been identified in genes of two-component regulatory systems (graSR, vraSR and walKR),7–11 a proteolytic regulatory gene (cipP),10 a gene encoding membrane proteins (yvrF),12 the gene for the RNA polymerase β subunit (rpoB)13,14 and the global regulatory gene (agrC).15 Evidence further suggests that the mutations in the two-component regulatory systems vraSR and graSR are responsible for the phenotypic conversions of VSSA to hVISA and hVISA to VISA, respectively.8,16

Although these mutations were identified in VISA strains of limited genetic lineages, the VISA and hVISA phenotypes are not confined to strains with specific genetic backgrounds, but rather have emerged in many successful pandemic clones, including a predominant community-associated (CA) MRSA clone, USA300.¹,12,17,18
It appears that the known VISA-associated SNPs are differentially harboured by strains of distinct backgrounds. In this study, we aimed to identify VISA-associated SNPs in another CA-MRSA clone, sequence type (ST) 59. ST59 is among the most prevalent of the CA-MRSA clones in the Asia–Pacific region. By whole-genome sequencing of isogenic strains with the VSSA and VISA phenotypes, we were able to comprehensively track in vivo genetic evolution in this clone with incremental vancomycin non-susceptibility.

Materials and methods

Strains and growth conditions

The MRSA strains used in this study were consecutively isolated from wound, synovial and tissue cultures of a septic knee in a 67-year-old woman during a 15 month period from 13 October 2001 to 8 January 2003. The patient underwent surgical debridement and had several courses of glycopeptide treatment (including vancomycin and teicoplanin) for her septic knee. Seven MRSA strains (109, 1962, 2357, 2358, 2459, 2482 and 2516) were stored at −80°C after their first isolation from the patient. The strains used in this study were grown from the original stock cultures. For strains 1962, 2357 and 2358, the growth of the stock culture in broth medium and subsequently on blood agar revealed two distinct sizes of colonies (Figure S1, available as Supplementary data at JAC Online). The large colonies were haemolytic and yellow (designated as 1962A, 2357A and 2358A), whereas the small colonies were not haemolytic and were white (designated as 1962B, 2357B and 2358B). The 10 strains were multilocus sequence type ST59, staphylococcal chromosomal cassette mec (designated as 1962B, 2357B and 2358B). The 10 strains were multilocus sequence type ST59, staphylococcal chromosomal cassette mec (SCCmec) type IV, agr group I and spa type t437, and had indistinguishable PFGE patterns (Figure S2, available as Supplementary data at JAC Online). Bacteria were grown in liquid or on solid basal medium (1% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K$_2$HPO$_4$, and 0.1% glucose) or tryptic soy broth (TSB) (Sigma, St Louis, MO, USA) unless otherwise indicated. All of the isolates exhibited substantial (>2-fold) decreases in vancomycin MIC values compared with their values at first isolation (see Table 2).

5-Haemolysin production

5-Haemolysin production was measured according to the procedure described elsewhere. Briefly, the S. aureus isolates were streaked perpendicularly to RN4220 on Columbia agar plates (Oxoid, Cambridge, UK) with 5% sheep blood and incubated at 37°C in 5% CO$_2$ overnight. Synergistic haemolysis within the 5-haemolysin zone produced by RN4220 was evaluated. The presence of synergistic haemolysis indicates the production of 5-haemolysin. ATCC 25923 and Mu50 were used as positive and negative controls, respectively, for the production of 5-haemolysin.

Doubling time measurement

The measurement of growth rate was modified from a procedure described elsewhere. Overnight cultures of the strains were diluted to an optical density (OD) of 0.05 in 60 mL of brain heart infusion broth and grown at 37°C with shaking at 150 rpm. The OD was monitored every 30 min for 8 h. Doubling times were calculated as follows: \((t_2 - t_1)\cdot \log 2)/(\log \text{OD}_{600} \text{ at } t_1 - \log \text{OD}_{600} \text{ at } t_2)\), where \(t_1\) is the first sampling time and \(t_2\) is the second sampling time. The measurements were replicated three times for each strain.

Next-generation sequencing and mapping the short reads to a reference genome of the ST59 lineage using SHRIMP

The whole-genome sequencing of VISA strain 2482 and its isogenic vancomycin-susceptible parental strain 109 was performed using the Illumina Genome Analyzer Ix (Illumina, San Diego, CA, USA). A total of 11 million and 13 million reads were generated for strains 109 and 2482, respectively, with similar average read lengths of 96.37 and 96.45 bp. The software SHRIMP 2.2.2 was used to map the reads to a reference genome of M013 (GenBank accession number CP003166). M013 is a CA-MRSA strain with the same genetic lineage (ST59) as the two sequenced strains.

Identifying genetic alternations using Nesoni

Genetic alternations between the sequenced strain and the reference strain, including SNPs and insertions and deletions (INDELs), were identified using Nesoni 0.58 (http://cibioinformatics.com/nesoni.shtml). Nesoni is a high-throughput sequencing data analysis tool that tallies the raw base and counts at each mapped position in the reference strain and compares the raw counts of bases at each position using Fisher’s exact test. The VISA phenotype-associated SNPs and INDELs identified by whole-genome sequencing comparison were checked by PCR amplification and sequencing of the PCR products. The oligonucleotide sequences and conditions of each PCR are displayed in Tables S1 and S2 (available as Supplementary data at JAC Online).

Susceptibility tests

Susceptibility to fusidic acid was determined using the standard disc diffusion method according to the CLSI 2011 guidelines. The MICs of rifampicin and vancomycin were determined by Etest (bioMérieux) according to the manufacturer’s instructions. Briefly, S. aureus isolates were grown in TSB at 37°C to a cell density equivalent to that of a 0.5 McFarland standard. Bacteria were streaked on Mueller–Hinton agar. The plates were incubated at 37°C for 24 h before reading.

Modified population analysis profile/area under the curve (PAP–AUC) analysis

A modified PAP–AUC analysis was performed according to the procedure described previously. Briefly, bacteria were incubated in 5 mL of TSB at 37°C overnight with shaking at 150 rpm. The bacterial solution was diluted with sterile saline to 10$^{-3}$ and 10$^{-4}$. Aliquots of 100 μL of the diluted bacterial solution were spirally plated on brain heart infusion agar plates containing vancomycin at concentrations of 0, 0.5, 1, 2, 2.5 and 4 mg/L. The plates were incubated at 37°C for 48 h before counting the colony numbers. The PAP–AUC was calculated using GraphPad Prism 5.0 (GraphPad software, Inc., La Jolla, CA, USA). A PAP–AUC ratio of ≥0.9 between the tested strain and Mu3 indicated the presence of the hVISA phenotype.

Results

Absence of known VISA-associated mutations in the ST59 VISA strains

As the first step to explore the genetic determinants of the VISA phenotypes in the ST59 strains, we screened for known VISA-associated genetic mutations in selected regulatory alleles, including graSR/vraG, yvqF/vraSR, walkR and cipP, by PCR amplification of full-frame alleles and sequencing of the PCR products. The respective sequences of each allele were compared between the successive MRSA strains. No nucleotide differences in the screened alleles were identified in VISA strains compared with their parental VSSA strain. Additionally, we did not detect any VISA-associated amino acid substitutions in these alleles (Table S3, available as Supplementary data at JAC Online).
Comparative genomics of isogenic VSSA and VISA strains

We sequenced and compared the whole genomes of one VISA strain (strain 2482) and its parental VSSA strain (strain 109). Nesoni analysis identified 14 mutations in the genome of VISA strain 2482 compared with VSSA strain 109. All of the predicted mutations were single nucleotide substitutions, which were further confirmed by PCR sequencing. Except for 1 mutation in the non-coding region, the other 13 mutations, generating 4 synonymous and 9 non-synonymous amino acid substitutions, were identified in four and eight alleles, respectively (Table 1).

The eight genes with non-synonymous mutations included agrC, rpoB, fusA, pros, pnpA, prmA and a gene encoding a putative membrane peptidase (M013TW_1564).

The agrC gene is an important element in the quorum-sensing agr regulon in S. aureus, and mutation of the agr locus is frequently seen in VISA strains. The nucleotide substitution from T to A creates a stop codon at amino acid position 193, which prematurely terminates AgrC translation. The genetic change was supported by the finding that \( \delta \)-haemolysin production was significantly impaired in strain 2482 (Table 2).

Non-synonymous mutations were identified in the rpoB gene, which encodes the RNA polymerase \( \beta \) subunit; mutation of this gene Table 1. Genetic polymorphisms in a VISA strain (2482) of the ST59 lineage, identified by comparing the whole-genome sequence with its parental VSSA strain (109)

<table>
<thead>
<tr>
<th>Mutation in VISA (nucleotide no. in M013)</th>
<th>Locus(^a)</th>
<th>Gene</th>
<th>Product</th>
<th>Effect of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>T to A (2048894) M013TW_1990</td>
<td>agrC</td>
<td>histidine kinase of the competence regulon ComD</td>
<td>L193stop</td>
<td></td>
</tr>
<tr>
<td>C to T (571032) M013TW_0529</td>
<td>rpoB</td>
<td>DNA-directed RNA polymerase ( \beta ) subunit</td>
<td>A477V</td>
<td></td>
</tr>
<tr>
<td>C to T (571188) M013TW_0529</td>
<td>rpoB</td>
<td>DNA-directed RNA polymerase ( \beta ) subunit</td>
<td>S529L</td>
<td></td>
</tr>
<tr>
<td>T to G (579694) M013TW_0534</td>
<td>fusA</td>
<td>translation elongation factor G</td>
<td>V407G</td>
<td></td>
</tr>
<tr>
<td>T to A (1259968) M013TW_1203</td>
<td>pros</td>
<td>prolyl-tRNA synthetase</td>
<td>M488T</td>
<td></td>
</tr>
<tr>
<td>T to C (1275201) M013TW_1214</td>
<td>pnpA</td>
<td>polynucleotide nucleotidyltransferase</td>
<td>I164T</td>
<td></td>
</tr>
<tr>
<td>A to G (1630551) M013TW_1564</td>
<td>prmA</td>
<td>50S ribosomal protein L1 methyltransferase</td>
<td>S130N</td>
<td></td>
</tr>
<tr>
<td>T to C (165806) M013TW_1593</td>
<td>rpoB</td>
<td>DNA-directed RNA polymerase ( \beta ) subunit</td>
<td>C99Y</td>
<td></td>
</tr>
<tr>
<td>C to T (1891497) M013TW_1806</td>
<td>menE</td>
<td>( \alpha )-succinylbenzoic acid-CoA ligase</td>
<td>synonymous</td>
<td></td>
</tr>
<tr>
<td>A to G (417002) M013TW_0369</td>
<td>guaB</td>
<td>inosine-( 5' )-monophosphate dehydrogenase</td>
<td>synonymous</td>
<td></td>
</tr>
<tr>
<td>A to G (1584111) M013TW_0369</td>
<td>xerD</td>
<td>tyrosin recombinase XerD</td>
<td>synonymous</td>
<td></td>
</tr>
<tr>
<td>C to T (6963) M013TW_0006</td>
<td>gyrB</td>
<td>DNA gyrase subunit B</td>
<td>synonymous</td>
<td></td>
</tr>
<tr>
<td>A to G (1735742) M013TW_1677</td>
<td>valS</td>
<td>valyl-tRNA synthetase</td>
<td>synonymous</td>
<td></td>
</tr>
<tr>
<td>C to T (1395343)</td>
<td>non-coding region (between M013TW_1284 and M013TW_1285)(^b)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Gene identification number in M013. M013 is a whole-genome sequenced CA-MRSA strain of the ST59 lineage from Taiwan.

\(^b\)M013TW_1284 encodes 30S ribosomal protein S14p and M013TW_1285 encodes guanosine monophosphate reductase.

Table 2. Phenotypic changes, including drug susceptibilities, production of \( \delta \)-haemolysin and growth rates, in 10 successive MRSA strains of the ST59 lineage

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation profile</th>
<th>Vancomycin MIC(^a) (mg/L)</th>
<th>Fusidic acid MIC(^a) (mg/L)</th>
<th>Rifampicin MIC(^a) (mg/L)</th>
<th>Growth rate PAP–AUC (vs Mu3)</th>
<th>( \delta )-Haemolysin</th>
<th>Doubling time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td>A</td>
<td>2</td>
<td>0.793</td>
<td>0.008</td>
<td>S</td>
<td>+</td>
<td>37.9 ± 3.3</td>
</tr>
<tr>
<td>1962A</td>
<td>A</td>
<td></td>
<td>1.5</td>
<td>0.012</td>
<td>S</td>
<td>+</td>
<td>37.2 ± 6.0</td>
</tr>
<tr>
<td>1962B</td>
<td>B</td>
<td>4</td>
<td>0.921</td>
<td>0.008</td>
<td>S</td>
<td>−</td>
<td>38.9 ± 6.1</td>
</tr>
<tr>
<td>2357A</td>
<td>A</td>
<td>−</td>
<td>0.829</td>
<td>0.012</td>
<td>S</td>
<td>−</td>
<td>39.1 ± 3.8</td>
</tr>
<tr>
<td>2357B</td>
<td>C</td>
<td>8</td>
<td>1.172</td>
<td>32</td>
<td>R</td>
<td>−</td>
<td>56.6 ± 13.1</td>
</tr>
<tr>
<td>2358A</td>
<td>A</td>
<td>−</td>
<td>0.807</td>
<td>0.012</td>
<td>S</td>
<td>+</td>
<td>35.2 ± 10.3</td>
</tr>
<tr>
<td>2358B</td>
<td>C</td>
<td>8</td>
<td>1.168</td>
<td>32</td>
<td>R</td>
<td>−</td>
<td>55.7 ± 9.1</td>
</tr>
<tr>
<td>2459</td>
<td>C</td>
<td>8</td>
<td>1.301</td>
<td>32</td>
<td>R</td>
<td>−</td>
<td>55.9 ± 11.2</td>
</tr>
<tr>
<td>2462</td>
<td>C</td>
<td>8</td>
<td>1.315</td>
<td>32</td>
<td>R</td>
<td>−</td>
<td>58.3 ± 13.3</td>
</tr>
<tr>
<td>2516</td>
<td>C</td>
<td>8</td>
<td>1.350</td>
<td>32</td>
<td>I</td>
<td>−</td>
<td>56.1 ± 12.4</td>
</tr>
</tbody>
</table>

S, susceptible; R, resistant; I, intermediate.

\(^a\)Determined by Etest.

\(^b\)Determined by the disc diffusion method.
is a known mechanism of rifampicin resistance in *S. aureus*. Amino acid alterations at positions 477 (alanine to valine) and 529 (serine to leucine) in *rpoB* of strain 2482 were associated with a 4000-fold increase in the rifampicin MIC (Table 2). The gene *fusA* encodes translation elongation factor G, and mutation of this gene was reported to mediate resistance to fusidic acid. Consistently, the amino acid alteration from valine to glycine at position 407 in strain 2482 was accompanied by the development of resistance to fusidic acid (Table 2). The other five alleles with non-synonymous mutations encode a variety of enzymes with unknown roles in drug resistance.

### Genetic evolution in the development of vancomycin non-susceptibility

To trace the genetic evolution accounting for vancomycin non-susceptibility, the eight alleles containing the nine non-synonymous mutations were amplified and sequenced in the eight successive strains. The nine VISA-associated SNPs and their associated phenotypes, including the drug susceptibilities, production of ε-haemolysin and growth rates of the 10 successive strains, are shown in Tables 2 and 3. Strains 1962A, 2357A and 2358A harboured wild-type alleles found in the VSSA strain 109, which was designated as mutation profile A (Table 3). All strains of mutation profile A produced ε-haemolysin and exhibited similar growth rates and drug susceptibilities, indicating that these four isolates are of the same strain (Table 2). The patient had received three courses of glycopeptide therapy before the isolation of strains 2357A and 2358A (Figure 1). The results indicate that glycopeptide treatment in this patient had not eradicated the MRSA strain, although the strain remained susceptible to glycopeptide without undergoing major genetic evolution.

Strain 1962B was isolated 1 year after the isolation of its parental strain, 109, and contained six mutations in the eight alleles (mutation profile B, Table 3). The nonsense mutation in *agrC* was observed in this strain for the first time, resulting in dysfunction of the *agr* locus and impaired ε-haemolysin production (Table 2). The PAP–AUC ratio of the strain was also elevated to a level that fell within the range defining hVISA (ratio >0.9). However, the occurrence of the six point mutations was not associated with

<table>
<thead>
<tr>
<th>Strain</th>
<th>Date of isolation (dd/mm/yy)</th>
<th>Mutation profile</th>
<th>Locus with non-synonymous polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td>13/10/01</td>
<td>A</td>
<td>agrC193, rpoB477, rpoB529, fusA407, proC255, pnpA488, M013TW_1564164, prmA230, menE59</td>
</tr>
<tr>
<td>1962B</td>
<td>23/10/02</td>
<td>B</td>
<td>stop, A, S, V, N, T, T, N, Y</td>
</tr>
<tr>
<td>2357A</td>
<td>21/12/02</td>
<td>A</td>
<td>L, A, S, V, I, M, I, S, C</td>
</tr>
<tr>
<td>2357B</td>
<td>21/12/02</td>
<td>C</td>
<td>stop, V, L, G, N, T, T, N, Y</td>
</tr>
<tr>
<td>2358A</td>
<td>22/12/02</td>
<td>A</td>
<td>L, A, S, V, I, M, I, S, C</td>
</tr>
<tr>
<td>2358B</td>
<td>22/12/02</td>
<td>C</td>
<td>stop, V, L, G, N, T, T, N, Y</td>
</tr>
<tr>
<td>2459</td>
<td>31/12/02</td>
<td>C</td>
<td>stop, V, L, G, N, T, T, N, Y</td>
</tr>
<tr>
<td>2482</td>
<td>05/01/03</td>
<td>C</td>
<td>stop, V, L, G, N, T, T, N, Y</td>
</tr>
<tr>
<td>2516</td>
<td>08/01/03</td>
<td>C</td>
<td>stop, V, L, G, N, T, T, N, Y</td>
</tr>
</tbody>
</table>

Grey shading indicates non-synonymous amino acid substitutions.

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**Table 3.** VISA-associated genetic polymorphisms in 10 successive MRSA strains of the ST59 lineage

[Figure 1. Antibiotic treatment courses and the isolation of successive MRSA strains with incremental non-susceptibility to vancomycin in a patient with a septic knee who failed glycopeptide treatment.]
Increased vancomycin MIC and doubling time in 1962B compared with the strains of mutation profile A. This result suggests that the six genetic alterations, including agrC truncation, did not substantially affect the growth rate and had only a modest effect on vancomycin susceptibility in *S. aureus*.

Strain 2357B contained additional mutations in rpoB and fusA (mutation profile C, Table 3) 2 months after the isolation of strain 1962B. Compared with strain 1962B, the mutations in strains with mutation profile C were associated with increased rifampicin MIC and resistance to fusidic acid. The evolution from mutation profile B to mutation profile C occurred during the use of rifampicin and fusidic acid in combination and was accompanied by an increase in the vancomycin MIC from 1.5 to 3 mg/L (from 4 to 8 mg/L at initial isolation). This result suggests that use of the two antimicrobial agents corresponded to mutations in rpoB and fusA, which resulted in resistance to rifampicin and fusidic acid and further increased the non-susceptibility to vancomycin.

**Discussion**

The results of our comparative genomic study identified nine non-synonymous mutations that were potentially responsible for the development of vancomycin non-susceptibility in the ST59 strains. The mutations appeared in an ordered series and accumulated during the process of genetic evolution. By analysing 10 successive strains, we successfully identified two steps of genetic alterations during strain evolution. It was intriguing to learn that the two steps of evolution, from profile A to B and then from profile B to C, were accompanied by phenotypic conversions from VSSA to hVISA and hVISA to VISA, respectively. In the VSSA-to-hVISA conversion, the genetic change in the hVISA strain included agrC truncation and five mutations in alleles encoding proteins with a variety of enzyme activities. Loss of the agr locus has frequently been reported in strains with the VISA phenotype. However, the significance of this loss in vancomycin non-susceptibility remains undetermined. The other five mutations had not previously been reported to be associated with the hVISA or VISA phenotype. A follow-up study is currently under way to address the role of each mutation in vancomycin non-susceptibility in strains of the ST59 background.

The strain with mutation profile B and the hVISA phenotype was identified ~12 months after the isolation of its parental VSSA strain. With no other isolates available, we were unable to determine whether the six mutations occurred in a single step or in an ordered series during the multiple courses of antibiotic therapy (Figure 1). In addition to the two courses of vancomycin treatment, the patient also received *in vitro* susceptible antimicrobial agents, including fusidic acid and trimethoprim/sulfamethoxazole, before the isolation of the hVISA strain. Remarkably, the hVISA strain and its parental VSSA strain co-existed in the same specimen and were isolated at the same time. This finding indicated that the antibiotic regimen, though active against the VSSA strain *in vitro*, was unable to eradicate it *in vivo*, but was sufficient to drive the point mutations and the development of the hVISA phenotype. Inadequate dosage may be among the reasons for this treatment failure. We found that during the two courses of glycopeptide treatment, vancomycin was administered at a dosage of 500 mg at 12 h intervals, which is considered suboptimal in treating *S. aureus* infections with a vancomycin MIC of 2 mg/L.

Although the VSSA strain was completely eliminated by the addition of rifampicin and fusidic acid, the antimicrobial regimen selected an hVISA strain with dual resistance to rifampicin and fusidic acid and might have promoted the hVISA-to-VISA phenotypic conversion (Figure 1 and Table 2). Mutation of the rpoB gene is a known mechanism of rifampicin resistance in *S. aureus* and has been reported to contribute to the VISA phenotype. In addition, the point mutations A477V and S529L (Table 1) in rpoB have been reported to be associated with dual resistance to rifampicin and vancomycin in strains of the ST59 background. These data strongly suggest that the two point mutations in the rpoB gene resulted in rifampicin resistance and contributed to hVISA-to-VISA phenotypic conversion in the ST59 strain.

Mutation of the fusA gene encoding translation elongation factor G has been demonstrated to confer resistance to fusidic acid in *S. aureus*. The V407G polymorphism identified in the ST59 VISA strains was a novel mutation that has not been previously reported. It has been shown that fusA mutations spontaneously occur in *S. aureus* with a frequency of $10^{-6}$ to $10^{-8}$; however, the use of fusidic acid can significantly increase the incidence of mutation. The use of fusidic acid in our patient may have enhanced the selection of the fusidic acid-resistant strain. Mutation of the fusA gene also occurred during the hVISA-to-VISA conversion. Whether the simultaneous occurrence of the two events was a coincidence or the fusA mutation is related to vancomycin non-susceptibility requires further study.

When the VISA strains of mutation profile C (strains 2357B, 2358B, 2459, 2482 and 2516) were isolated (Figure 1), the glycopeptide treatment was discontinued and rifampicin and fusidic acid were administered. Intriguingly, without pressure from glycopeptide, the level of vancomycin non-susceptibility continued to increase in the VISA strains, as demonstrated by the elevation of the PAP–AUC ratio from 1.168 in strain 2358B to 1.350 in strain 2516 (Figure 1 and Table 2). Although the vancomycin MIC level remained stable during this period, the elevation of the PAP–AUC ratio suggested a continued effect of rifampicin and/or fusidic acid on the vancomycin non-susceptibility of the VISA strain. The use of the *in vitro* non-susceptible agents to treat VISA infections not only was unable to eradicate the strains, but also may have increased the non-susceptibility to glycopeptide. This finding highlights the importance of obtaining drug susceptibility data and the appropriate choice of adequate antibiotic for the management of hVISA or VISA infections.

This study has several limitations. First, whole-genome sequencing of the two isogenic VSSA and VISA strains has not been completed. An estimate of 30 unfilled gaps remains. The comparative genomics results are therefore incomplete, and mutations occurring in the unfilled sequence gaps were not identified in the current study. Second, not all of the successive strains were whole-genome sequenced. Mutations in alleles other than the eight screened genes might have occurred in strains other than 109 and 2482. However, if there were unidentified mutations, it is likely that they would have had little impact on vancomycin non-susceptibility because VISA-associated mutations tend to accumulate, and if they are critical to the VISA phenotype they will remain in the 2482 strain.

In conclusion, by whole-genome sequencing of isogenic VSSA and VISA strains, we successfully identified eight alleles containing nine SNPs that were potentially associated with the VISA phenotype in epidemic CA-MRSA strains of the ST59 background.
By screening the nine SNPs in other successive strains, we identified two steps of genetic evolution corresponding to the phenotypic conversion from VSSA to hVISA and hVISA to VISA. The use of antimicrobial agents appeared to have a substantial impact on the genetic evolution of S. aureus during the development of drug nonsusceptibility. Inadequate antibiotic regimens and suboptimal dosage can result in treatment failure and can promote the development of drug resistance during the management of S. aureus infections with reduced susceptibility to vancomycin.

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Transparency declarations
Conflicts of interest: none to declare.

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Supplementary data
Figures S1 and S2, and Tables S1, S2 and S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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