Selection of Heterogeneous Vancomycin-Intermediate

Staphylococcus aureus by Imipenem

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Vancomycin (VAN)-intermediate Staphylococcus aureus (VISA) and heterogeneous VISA (hVISA) isolates are considered to have emerged from VAN-susceptible S. aureus (VSSA) by spontaneous mutation during VAN exposure. We previously reported that laboratory mutant H14, obtained from VSSA strain ΔIP by exposure to imipenem (IPM), showed overexpression of the vraS two-component system and a typical hVISA phenotype. In the present study, to elucidate the mechanism of VSSA conversion to hVISA, we further characterized strain H14 by determining its whole-genome sequence, morphology, cell wall synthetic activity, and gene expression. Genome sequencing revealed that H14 harbored a mutated vraS (designated vraS_H14) that caused an amino acid substitution (S329→L). This mutation is different from the VraS mutation (N2→I) identified in representative clinical hVISA strain Mu3. However, H14 exhibited a phenotype similar to that of Mu3, including heterogeneous resistance to VAN, enhanced cell wall synthetic activity, and vraSR overexpression. Replacement of the vraS gene of ΔIP with the mutated vraS_H14 gene confirmed that the S329→L substitution was responsible for both the upregulation of vraSR and conversion to the hVISA phenotype. This conversion was also achieved by using the vraS gene of Mu3, which carries a mutation (N2→I), but not with the native vraS gene of strain N315. Finally, we carried out a study to analyze the appearance of hVISA from VSSA by exposure of ΔIP to selective concentrations of VAN and beta-lactam antibiotics. A total of 8 and 5 hVISA isolates were detected among 50 isolates selected with VAN and IPM, respectively. Among the 13 hVISA mutants, mutation in vraSR was detected only in mutant strain H14, suggesting that additional mutational mechanisms can be responsible for evolution to the hVISA phenotype. We conclude exposure not only to VAN but also to beta-lactams may select for reduced glycopeptide susceptibility in S. aureus.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth condition. The Staphylococcus strains and plasmids used in the present study are listed in Table 1. The cloning and transformation of Escherichia coli JM109 were carried out by standard techniques (http://catalog.takara-bio.co.jp/en/PDFFiles/9052_e.pdf; Takara-Bio Co., Ltd., Shiga, Japan). All S. aureus strains were cultivated in brain heart infusion broth (CMI) with or without 1 mg/liter of RIF, ampicillin-sulbactam (SAM), or IPM. DNA methods. DNA manipulations were performed by standard methods (11). Restriction enzymes were used as recommended by the manufacturer (Takara). Routine PCR amplification was performed with an Expand High Fidelity system (Roche, Mannheim, Germany).

Transmission electron microscopy. The preparation for and the examination of S. aureus cells by transmission electron microscopy were performed as described previously (4).

Incorporation of 14C-labeled D-glucose or 3H-labeled N-acetyl-D-glucosamine into cells. The preparation of the cells and the purification of peptidoglycan were performed as described previously (8), and the rate of incorporation was determined as described previously (8). These assays were performed in triplicate.

Whole-genome sequencing of H14 and identification of SNPs compared to the sequence of parent strain ΔIP. Sequencing of the strain H14 genome was performed with a Genome Sequencer 20 system, a recently introduced highly par-
Characterization of laboratory-derived hVISA mutant H14, which originated from VISA strain ΔIP. To investigate the phenotypes associated with reduced susceptibility to VAN, we analyzed the cell wall thickness and cell wall biosynthetic activity of H14, along with those of vraSR null mutant strain H14-KVR and parent strain ΔIP, which were used as controls. Electron microscopy analysis showed that H14 had a significantly thicker cell wall and a rougher cell surface than ΔIP, while H14-KVR had a cell wall much thinner than that of the parent strain (Fig. 1A). Student’s t test demonstrated that the increase in cell wall thickness was statistically significant in all cases (P < 0.0001).

To test for cell wall biosynthetic activity, the rates of incorporation of [14C]-labeled d-glucose and N-acetyl-d-glucosamine into the cell wall peptidoglycan fraction were evaluated by using a fixed number of nondividing cells. As shown in Fig. 1B, the incorporation of N-acetyl-d-glucosamine and d-glucose by H14 was significantly enhanced compared to that by ΔIP and H14-KVR. These data indicate that the vraSR mutation could cause enhanced cell wall biosynthesis activity.
The *vraS* mutation is the only genetic event responsible for hVISA phenotype acquisition in H14. To clarify the genetic mechanism of hVISA phenotype acquisition, genome sequencing of H14 was carried out. The 2,795,992-bp-long whole-genome sequence of H14 was determined. Since H14 is an in vitro derivative of N315, the sequence was practically identical to that of N315 (14), with the exceptions being 42 SNPs and 5 insertions or deletions between the two chromosomes (data not shown). PCR amplification and sequencing of /H9004 IP chromosomal DNA for the 45 differences found no additional mutations except for the previously reported *vraS* mutation, which causes the replacement of a single amino acid, Ser329 with Leu, in H14. This result confirms that the *vraS* mutation causing the replacement of Ser329 with Leu was the only genetic event responsible for the acquisition of the hVISA phenotype in H14.

**Phenotypic change conferred by *vraS* gene replacement.** To see if the single amino acid substitution in the sequence encoding VraS114 is responsible for both the phenotypic expression of hVISA and the overexpression of *vraSR*, we constructed two *vraS* mutants from ΔIP and its RIF-resistant strain, ΔIP-rifR. The native *vraS* gene of the parent strains was replaced by *vraS*H14 to obtain ΔIP:*vraS*H14 and ΔIP-rifR:*vraS*H14 (VraS114 mutant strains) by the Etest method and population analysis. As shown in Table 2, the MICs of glycopeptides for the *vraS*H14 mutant were higher than those for the parent strain. The population analysis of ΔIP:*vraS*H14 and ΔIP-rifR:*vraS*H14 showed an increase in the proportion of VAN-resistant subpopulations compared with that for parent strain ΔIP (Fig. 2). The patterns of the population curves for H14 and ΔIP:*vraS*H14 were almost superimposable on each other. The same result was obtained by using ΔIP with the RIF resistance marker (the *rpoB* mutation), ex-
cept that the population curve for ΔIP-rifR::vraS<sub>H14</sub> was slightly deflected with 4 mg/liter of VAN. These data clearly demonstrate that the vraS mutation alone can confer the phenotypic expression of heterogeneous VAN resistance.

The MICs were then determined, and the MICs of IPM, fosfomycin, bacitracin, and daptomycin for ΔIP::vraS<sub>H14</sub> and ΔIP-rifR::vraS<sub>H14</sub> were found to be increased compared with the MICs for ΔIP for which the MICs were similar to those for H14 (Table 2). Recently, Muthaiyan et al. reported that the inactivation of vraSR significantly increased susceptibility to daptomycin (18). It was concluded that the vraS mutation (Ser<sub>329</sub>→Leu) alone was responsible for the altered antibiogram of H14 compared to that of its parent, strain ΔIP.

Assessment of vraS activation among VISA strains carrying a mutation in the vraSR operon. By Northern blot analysis, we examined the transcriptional level of vraSR in the hVISA and VISA strains in which a single amino acid substitution in the vraSR operon has already been identified (12). The positions of mutations in the vraSR operons of different strains are shown in Fig. 3A. Northern blot analysis demonstrated the overexpression of vraS in ΔIP-rifR::vraS<sub>H14</sub>, which was indistinguishable from the level of expression by original mutant strain H14 (Fig. 3B). The fact that the whole genome of H14 has only a singe mutation, in the vraS gene, compared to the sequence of ΔIP suggests that the VraS<sub>H14</sub> S329→L substitution (on the ATP-binding domain [amino acids 248 to 340]) affects the activation of the VraS sensor histidine kinase by modulating its autophosphorylation (3). The overexpression of vraS was also observed in VISA strains NJ and Mu50 and hVISA strain Mu3 (Fig. 3B). The VraS A260→V substitution of NJ may also be associated with the activation of histidine kinase activity. These data are consistent with the findings of this study that implicate the increased expression of the vraSR system in the conversion of VSSA to hVISA. It has not previously been reported that amino acid mutations in a global regulator such as the vraSR TCS are involved in heterogeneous VAN resistance in S. aureus. Further investigation is needed to clarify the relationship between the function of TCS and the role of a mutation in vraS.

Recently, evidence of the evolution from hVISA to VISA in vivo during infection has been reported (5, 7, 21, 24, 25). However, the present study described for the first time that a mutation in vraS is responsible for the increased level of transcription of vraS and the phenotypic expression of hVISA by the gene replacement experiment. It was also confirmed that this mutation could cause cell wall thickening and increase the

### TABLE 2. Antibiotic susceptibility profile of ΔIP and its derivative strains determined by Etest

<table>
<thead>
<tr>
<th>Strain</th>
<th>RIF (mg/liter)</th>
<th>Oxacillin</th>
<th>IPM (mg/liter)</th>
<th>VAN (mg/liter)</th>
<th>Teicoplanin</th>
<th>Fosfomycin</th>
<th>Bacitracin</th>
<th>Daptomycin</th>
<th>Gentamicin</th>
</tr>
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<tbody>
<tr>
<td>ΔIP</td>
<td>0.004</td>
<td>&gt;6</td>
<td>0.75</td>
<td>1</td>
<td>1</td>
<td>32</td>
<td>0.75</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>H14</td>
<td>0.004</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>2</td>
<td>8</td>
<td>96</td>
<td>96</td>
<td>1.75</td>
<td>0.75</td>
</tr>
<tr>
<td>ΔIP-KVR</td>
<td>0.004</td>
<td>1.5</td>
<td>0.19</td>
<td>0.75</td>
<td>0.25</td>
<td>0.38</td>
<td>4</td>
<td>0.5</td>
<td>0.75</td>
</tr>
<tr>
<td>ΔIP-rifR</td>
<td>&gt;32</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.5</td>
<td>24</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>ΔIP::vraS&lt;sub&gt;h14&lt;/sub&gt;</td>
<td>0.004</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>2</td>
<td>8</td>
<td>96</td>
<td>96</td>
<td>1.75</td>
<td>0.75</td>
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<tr>
<td>ΔIP-rifR::vraS&lt;sub&gt;h14&lt;/sub&gt;</td>
<td>&gt;32</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>3</td>
<td>12</td>
<td>96</td>
<td>96</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mu3</td>
<td>0.006</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>3</td>
<td>24</td>
<td>&gt;1,024</td>
<td>256</td>
<td>2</td>
<td>384</td>
</tr>
</tbody>
</table>

![FIG. 2. Resistant subpopulation profiles of ΔIP, its derivative vraSR mutants, and hVISA Mu3. The numbers of cells (log<sub>10</sub> CFU per milliliter) growing on BHI agar containing VAN are shown on the y axis; the VAN concentrations are shown on the x axis. The number of colonies that grew was counted after incubation at 37°C for 48 h.](http://aac.asm.org/)
rate of incorporation of N-acetyl-D-glucosamine and D-glucose into cells (Fig. 1B). Although the location of the VraS mutation identified in H14 is different from that identified in clinical hVISA strain Mu3, both mutations had the same effect on conferring VAN resistance in gene replacement experiments (data not shown).

**Beta-lactam antibiotics can select hVISA mutants from VSSA strain ΔIP.** We exposed VSSA strain ΔIP to selective concentrations of three beta-lactams, IPM, SAM, and CRO, to determine the ability to select mutants with reduced VAN susceptibility. ΔIP is a derivative of N315 in which the mecI repressor was inactivated and from which the β-lactamase-carrying plasmid was cured (Table 1). The genotype of inactivated mecI and the absence of the β-lactamase-carrying plasmid are characteristic of hVISA strain Mu3 and VISA strain Mu50, as reported previously (9). Therefore, for this study we used ΔIP as a representative Japanese health care-associated MRSA strain from which VISA has emerged. We spread 10⁸ CFU from an overnight culture on BHI agar plates containing several concentrations of antibiotics. As a control for this assay, we also spread them onto the agar plate containing VAN. We then picked 50 putative mutant colonies from each plate containing a selective concentration of antibiotic (VAN, 2 mg/liter; IPM, 8 mg/liter; SAM, 8 mg/liter; or CRO, 128 mg/liter) and established them as mutant strains by a colony purification procedure. A selective concentration was defined as the concentration which resulted in a reduction of the initial bacterial population by approximately 6 log units (with VAN) or 4 log units (with IPM, CRO, and SAM) (Fig. 4).

To detect the presence of hVISA isolates among the selected isolates, all isolates were investigated for their VAN susceptibilities by population analysis. Eight and 5 hVISA isolates were obtained from among the 50 isolates selected with VAN and IPM, respectively. No hVISA isolate was detected among the isolates selected with CRO or SAM (Fig. 4).

We determined the nucleotide sequence of vraSR from 13 hVISA isolates, and a mutation was only in mutant strain H14. This suggests that different genetic mechanisms, other than
vraS mutations, are used to acquire hVISA phenotypic expression. From these results, we also confirmed that hVISA can be selected not only by VAN but also by IPM. Since IPM was frequently used for the treatment of MRSA infections before the clinical introduction of VAN in 1991 in Japan, we suspect a possible role of IPM in the early emergence of hVISA in Japan, ahead of its emergence in other countries in 1996.

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REFERENCES


FIG. 4. Profiles of antibiotic-resistant subpopulations. The numbers of cells (log_{10} CFU per millilter) growing on antibiotic-containing BHI agar are shown on the x axis. The concentrations of VAN, IPM, CRO, and SAM are shown on the y axis. The number of colonies of VSSA strain JIP growing was counted after incubation at 37° C for 72 h. The concentrations of VAN, IPM, CRO, and SAM are shown on the x axis. The numbers after the antibiotic designations indicate selective antibiotic concentrations (in mg/liter). SBT/ABPC, sulbactam-ampicillin.


