Reversion of the Glycopeptide Resistance Phenotype in 
*Staphylococcus aureus* Clinical Isolates

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The recent identification of glycopeptide intermediate-resistant *Staphylococcus aureus* (GISA) clinical isolates has provided an opportunity to assess the stability of the glycopeptide resistance phenotype by nonselective serial passage and to evaluate reversion-associated cell surface changes. Three GISA isolates from the United States (MIC of vancomycin = 8 μg/ml) and two from Japan (MICs of vancomycin = 8 and 2 μg/ml) were passaged daily on nutrient agar with or without vancomycin supplementation. After 15 days of passage on nonselective medium, vancomycin- and teicoplanin-susceptible revertants were obtained from each GISA isolate as determined by broth dilution MIC. Revertant isolates were compared with parent isolates for changes in vancomycin heteroresistance, capsule production, hemolysis phenotype, coagulase activity, and lysostaphin susceptibility. Several revertants lost the subpopulations with intermediate vancomycin resistance, whereas two revertants maintained them. Furthermore, although all of the parent GISA isolates produced capsule type 5 (CP5), all but one revertant tested no longer produced CP5. In contrast, passage on medium containing vancomycin yielded isolates that were still intermediate resistant to vancomycin, had no decrease in the MIC of teicoplanin, and produced detectable CP5. No consistent changes in the revertants in hemolysis phenotype, lysostaphin susceptibility, or coagulase activities were discerned. These data indicate that the vancomycin resistance phenotype is unstable in clinical GISA isolates. Reversion of the vancomycin resistance phenotype might explain the difficulty in isolating vancomycin-resistant clinical isolates from the blood of patients who fail vancomycin therapy and, possibly, may account for some of the difficulties in identifying GISA isolates in the clinical laboratory.

Recently, intermediate resistance to glycopeptides was identified in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates obtained from patients in Japan and the United States who failed treatment with vancomycin (3, 6, 7, 18, 21, 25). The isolation of these glycopeptide-intermediate-resistant *S. aureus* (GISA) isolates has raised concern since, after vancomycin and teicoplanin, few therapeutic options exist for treatment of MRSA infections (13).

The term GISA was used to describe these glycopeptide-intermediate isolates in a recent report by Tenover et al. (29) to reflect the various patterns of resistance to vancomycin and teicoplanin as determined by broth dilution MIC analysis performed and interpreted according to the recommendations from the National Committee on Clinical Laboratory Standards (NCCLS) (16). Using the broth MIC data published by Tenover et al. (29) and others (6, 8, 12, 18, 21, 25), we have grouped the clinical GISA isolates into three distinct classes of glycopeptide resistance and refer to them as classes A, B, and C. Class A isolates are intermediate for both vancomycin (MIC = 8 to 16 μg/ml) and teicoplanin (MIC = 16 μg/ml), class B isolates are intermediate for vancomycin but are teicoplanin susceptible (MIC ≤ 8 μg/ml). Class C isolates are susceptible to vancomycin (MIC ≤ 4 μg/ml) and are intermediate for teicoplanin. Clinical *S. aureus* strains from all three classes are heteroresistant for vancomycin since they contain minority subpopulations that can grow on agar medium containing >4 μg vancomycin/ml (6, 7, 21), a concentration above the NCCLS susceptibility breakpoint (17). The basis for heteroresistance, a phenomenon previously described for the methicillin resistance phenotype (30), is poorly understood for either methicillin or glycopeptides.

Prior to the identification of GISA clinical isolates, it was observed that staphylococci could persist in the blood of patients despite vancomycin therapy and that such isolates appeared to be susceptible to vancomycin (11, 19, 27). This paradox raised the possibility that GISA clinical isolates have been present for some time but have escaped detection. We hypothesized that vancomycin-resistant *S. aureus* might undergo a transient adaptation in the presence of vancomycin, which might be reversed upon withdrawal of the drug.

We evaluated the stability of the resistance phenotype as well as exoprotein phenotypes in GISA isolates by serially passaging them on nonselective medium and monitoring the glycopeptide resistance phenotype of the passaged isolates by broth dilution MIC determination and population analysis.

**MATERIALS AND METHODS**

*Culture conditions, GISA parent isolates, and passaging procedure.* *S. aureus* isolates were routinely cultured at 37°C and stored as frozen stocks in skim milk (Difco Laboratories, Detroit, Mich.) at −70°C as described previously (4). To evaluate hemolysis phenotype, strains were streaked onto sheep blood agar, incubated overnight at 37°C, and transferred to a refrigerator for 24 h. As shown in Table 1, parent isolates used in passaging belonged to the three classes of GISA phenotypes isolated from patients who did not respond to vancomycin treatment. Serial passaging was initiated by reviving frozen stocks of each GISA clinical isolate onto brain heart infusion (BHI) agar (Difco Laboratories) and incubating them overnight. Several colonies chosen randomly from each plate were passaged on BHI or BHI containing subinhibitory levels of vancomycin (Sigma, St. Louis, Mo.) (2 μg/ml for isolates Mu3 and PC and 4 μg/ml for isolates MI, NI, and Mu50). After overnight incubation several colonies were again randomly selected from each plate and passaged onto the identical medium. This procedure was repeated daily, and isolates were frozen for storage at 5-day
Table 1. Bacterial strains*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>MIC (μg/ml)</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI</td>
<td>Class A GISA clinical isolate</td>
<td>8 (I)</td>
<td>CDC isolate HIP5827, Fred Tenover (CDC)</td>
<td>2, 3, 25</td>
</tr>
<tr>
<td>NJ</td>
<td>Class B GISA clinical isolate</td>
<td>8 (S)</td>
<td>CDC isolate HIP5836, Fred Tenover (CDC)</td>
<td>3, 25</td>
</tr>
<tr>
<td>PC</td>
<td>Class B GISA clinical isolate</td>
<td>2–4 (S)</td>
<td>CDC isolate HIP 6297, Fred Tenover (CDC)</td>
<td>18, 21</td>
</tr>
<tr>
<td>Mu50</td>
<td>Class B GISA isolate</td>
<td>8 (I)</td>
<td>K. Hiramatsu (Juntendo University)</td>
<td>5–7</td>
</tr>
<tr>
<td>Mu3</td>
<td>Class C GISA clinical isolate</td>
<td>16 (I)</td>
<td>K. Hiramatsu (Juntendo University)</td>
<td>5–7</td>
</tr>
</tbody>
</table>

* Classes A, B, and C refer to glycopeptide resistance patterns described in the introduction which are based on previously published broth dilution MIC values of vancomycin and teicoplanin based on twofold dilutions of drug. The values shown for MI, NJ, Mu50, and Mu3 are reproduced from Tenover et al. (29).

RESULTS

PFGE of GISA parent strains and revertants. PFGE was performed to confirm that the Smal restriction pattern of each GISA strain was consistent with the published pattern and to confirm the clonality of each revertant to the respective parent. Although the Smal restriction patterns of all GISA isolates were published previously (6, 18, 21, 25), the PC isolate was not compared directly to the other GISA isolates in the same gel. Therefore, we confirmed that the Smal restriction patterns in our laboratory were similar to those previously published (6, 18, 21, 25). We also determined that the restriction patterns of the PC and MI GISA isolates were identical when compared on the same pulsed-field gel: a difference of just two bands could be discerned when these two isolates were compared with NJ (data not shown). The Smal restriction patterns of the GISA parent strains also confirmed the conclusions of others that the U.S. isolates MI, PC, and NJ, are closely related to each other and that they are more distantly related to the Japanese isolates. The Smal restriction patterns of each revertant was identical to its respective parent strain.

Serial passaging associated with decreased glycopeptide MICs. Table 2 compares the broth dilution MICs of vancomycin and teicoplanin for the GISA clinical strains, the susceptible revertant isolates, and the isolates passaged on medium containing vancomycin. GISA clinical strains MI, NJ, PC, and Mu50 each produced a revertant isolate with an MIC of vancomycin in the susceptible range after 15 days of passaging (as indicated by “[P15]”). However, isolate MI[P15] was less susceptible than the other P15 revertants. Whereas the MIC of vancomycin for isolate MI[P15] and MI[P25] was at the susceptibility breakpoint (4 μg/ml), the MICs of vancomycin for revertants NJ[P15], PC[P15], and Mu50[P15] were 2 to 3 μg/ml. Passaging of NJ[P15] until day 60 did not lead to a further decrease in the MIC of vancomycin; the MIC of vancomycin for isolates NJ[P15], NJ[P60], and MI[P60] were each measured at least three times, and the MIC was never greater than 3 μg/ml.

Although the MICs of teicoplanin for the parent GISA strains Mu50, MI, NJ, and PC were lower than those previously published (6, 18, 21, 25), we nevertheless detected a lower MIC of teicoplanin for the revertants of these strains than we did in the respective parent. For the vancomycin-susceptible class C isolate, Mu3, the MIC of vancomycin did not change with serial passaging; however, the MIC of teicoplanin decreased. The MICs of vancomycin and teicoplanin did not decrease intervals. At days 5, 15, and 25 of passage, vancomycin and teicoplanin broth dilution MICs were determined. Serial passaging was discontinued when it was determined the MIC of vancomycin or teicoplanin had decreased to the lowest possible limit or until 60 days. Isolates which had broth dilution MICs of vancomycin of >4 or teicoplanin of >8 were classified as resistant to vancomycin or teicoplanin of 4 μg/ml and were therefore classified as MRSA (4 g/ml).
TABLE 2. MICs of glycopeptides and capsule type

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)</th>
<th>Capsule type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vancomycin</td>
<td>Teicoplanin</td>
</tr>
<tr>
<td>MI</td>
<td>6 (I)</td>
<td>8 (S)</td>
</tr>
<tr>
<td>MI[P15]</td>
<td>4 (S)</td>
<td>5 (S)</td>
</tr>
<tr>
<td>MI[P25]</td>
<td>4 (S)</td>
<td>ND</td>
</tr>
<tr>
<td>MI[P60]</td>
<td>2 (S)</td>
<td>3 (S)</td>
</tr>
<tr>
<td>MI[VP15]</td>
<td>6 (I)</td>
<td>ND</td>
</tr>
<tr>
<td>MI[VP60]</td>
<td>6 (I)</td>
<td>ND</td>
</tr>
<tr>
<td>NJ</td>
<td>5 (I)</td>
<td>5 (S)</td>
</tr>
<tr>
<td>NJ[P15]</td>
<td>2–3 (S)</td>
<td>4 (S)</td>
</tr>
<tr>
<td>NJ[P60]</td>
<td>2–3 (S)</td>
<td>ND</td>
</tr>
<tr>
<td>NJ[VP15]</td>
<td>5 (I)</td>
<td>ND</td>
</tr>
<tr>
<td>PC</td>
<td>5 (I)</td>
<td>5 (S)</td>
</tr>
<tr>
<td>PC[P15]</td>
<td>2 (S)</td>
<td>3 (S)</td>
</tr>
<tr>
<td>PC[P30]</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PC[VP15]</td>
<td>5 (I)</td>
<td>ND</td>
</tr>
<tr>
<td>PC[VP30]</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mu50</td>
<td>5 (I)</td>
<td>5 (S)</td>
</tr>
<tr>
<td>Mu50[P15]</td>
<td>2 (S)</td>
<td>2 (S)</td>
</tr>
<tr>
<td>Mu50[VP15]</td>
<td>5 (I)</td>
<td>ND</td>
</tr>
<tr>
<td>Mu3</td>
<td>2 (S)</td>
<td>9 (I)</td>
</tr>
<tr>
<td>Mu3[P15]</td>
<td>2 (S)</td>
<td>6 (S)</td>
</tr>
<tr>
<td>Mu3[VP15]</td>
<td>2 (S)</td>
<td>ND</td>
</tr>
</tbody>
</table>

* [P15], passed on plain medium for 15 days; [P60], passed on plain medium for 60 days; [VP15], passed on vancomycin containing medium for 15 days; [VP60], passed on vancomycin containing medium for 60 days. Broth dilution MICs were determined as described in Materials and Methods with drug concentrations diluted in arithmetic increments. According to NCCLS criteria (17), isolates are susceptible (S) to vancomycin if their MIC of vancomycin is ≤4 μg/ml. Isolates are susceptible to teicoplanin if their MIC of teicoplanin is ≤8 μg/ml. Since arithmetic dilutions of glycopeptide were used, MICs of vancomycin of >4 and <32 μg/ml and MICs of teicoplanin of >8 and <32 μg/ml are intermediate (I). No clinical *S. aureus* isolate has been reported to have a resistant MIC of vancomycin or teicoplanin (≥32 μg/ml).

a The capsule serotyping was performed as described in Materials and Methods. N, nontypeable; ND, not determined. An asterisk indicates a weaker reaction with capsular antiserum compared with the control strain.

DISCUSSION

This report clearly shows that reversion of glycopeptide resistance can occur in all types of GISA clinical isolates described to date. Decreases in MICs of vancomycin and teicoplanin uniformly occurred after 15 days of serial passage on nonselective medium; the presence of vancomycin in the medium prevented such reversion. It is unclear why others did not observe reversion of GISA isolates (6, 21, 25). We can speculate that an insufficient number of generations of growth on nonselective medium were allowed before reversion was evaluated. The number of passages required to decrease the MIC of vancomycin for the MI GISA isolate to 2 μg/ml was higher than that required for the other GISA strains, an observation suggesting that the stability of the glycopeptide-resistance phenotype depends to some extent on the genetic background of the bacterial strain.

We found two classes of revertant phenotypes distinguishable by population analysis. Three revertants (MI[P60], NJ[P15], and PC[P15]) no longer had countable subpopulations recoverable from media containing >4 μg vancomycin/ml. The second class was represented by Mu50[P15] and MI[P15] revertants which still produced subpopulations with intermediate resistance (>4 μg/ml) to vancomycin that was detectable on agar medium. It should be emphasized, however, that population analysis indicated that none of the revertant isolates appeared to be as vancomycin susceptible as RN4220. These data suggest that the revertants could have a greater potential for “converting” to the resistance phenotype than would a typical vancomycin-susceptible *S. aureus* isolate. Accordingly, it has been proposed that class C GISA isolates such as Mu3 (Table 1) represent a pre-GISA evolutionary state (6). Conversely, our data raise the possibility that isolates from GISA classes B...
and C could represent revertants of isolates from GISA class A (vancomycin intermediate, teicoplanin intermediate).

All parent and revertant isolates had an MIC of oxacillin ranging from 16 to 1,024 μg/ml and were thus methicillin-resistant by NCCLS criteria (17). Thus, although Sieradzki and colleagues (21–23) previously observed an inverse relationship between the MICs of oxacillin and vancomycin, our data do not support such a view. We did not document an increase in the MIC of oxacillin with a decrease in the MIC of vancomycin in the revertants. Rather, the MICs of oxacillin for the revertant isolates tended to be one or two dilutions lower than those of the respective parent GISA strains (data not shown). Moreover, the MIC of oxacillin we measured for the PC isolate was 16 μg/ml, a value that was higher than the MIC of oxacillin of 0.8 μg/ml reported by Sieradzki et al. (21). We cannot explain this discrepancy; however, the MICs of oxacillin we documented for all our GISA isolates are consistent with other reports (1–3, 6, 7, 18, 25, 29) which documented the GISA isolates as MRSA.

The revertant derived from Mu3 (Mu3[P15]) was associated with a decrease in the teicoplanin broth dilution MIC with no change in the broth dilution MIC of vancomycin or vancomycin population analysis. These data may indicate there is a baseline MIC of vancomycin which cannot further decrease for each bacterial strain, a feature likely to be dictated by the strain-specific cell wall milieu.

It is unclear why CP5 was no longer produced in all revertant isolates except Mu50[P15]. Based on the observation that the U.S. isolates appear to be more closely related to each other than Mu50, we can speculate that the tendency to lose capsule production in GISA isolates depends to some extent on the genetic background. We recognize the possibility that the loss of CP5 production in the revertants could be due to in vitro passaging and that concomitant loss of capsule production and vancomycin resistance may be unrelated. However, historical evidence suggests that capsule production is not lost during in vitro passage (26). Moreover, in this study, in vitro-passaged GISA strains did not lose their ability to produce CP5 when vancomycin-selective pressure was present during the passaging. Additionally, isolate 523, a previously described vancomycin-susceptible clinical isolate (4), continued to produce CP8 after 15 days of serial passage on nonselective medium. Further studies are warranted to determine whether type 5 capsule is a cofactor in vancomycin resistance.

The reversion phenomenon we observed may explain why vancomycin-resistant *S. aureus* isolates are not usually isolated from the blood of patients with sustained *S. aureus* bacteremia even though they are receiving vancomycin therapy. We speculate that most *S. aureus* isolates revert rapidly in the absence of glycopeptides but that the identified GISA isolates may have developed a mechanism to stabilize the glycopeptide resistance phenotype for a longer period. Clinical microbiology labora-
tories routinely incubate isolates on nonselective media before susceptibility testing is performed, a situation which provides an opportunity for reversion to occur. Despite this apparent stability of resistance in the identified GISA isolates, susceptibility testing by disk diffusion and the Vitek System has proven unreliable in detecting glycopeptide resistance (29). Even MIC testing by broth dilution is not able to detect the vancomycin-intermediate bacteria in class C GISA isolates such as Mu3 and the Mu50 revertant. It is possible the insensitivity of these techniques in detecting GISA isolates is related to the decreased resistant subpopulation remaining after reversion.

Considering the tendency to revert, identified GISA isolates should be maintained on vancomycin-containing media to prevent reversion. Indeed, since we received all isolates growing on nonselective media, reversion of the teicoplanin resistance phenotype during transit may explain why the MICs of teicoplanin we documented are lower than were previously published for most isolates. Accordingly, reversion should be considered as a possible explanation when decreased glycopeptide resistance is observed in S. aureus mutants produced in the laboratory (24), especially when the procedures used to produce mutations involve incubations in nonselective medium for prolonged periods. Additionally, it might be recommended that S. aureus isolates should be cultured in clinical microbiology laboratories on medium containing a low level of vancomycin as well as on routine nonselective medium to ensure that glycopeptide-resistant isolates can be identified.

A further understanding of the cause of reversion and the revertant phenotype could lead to an understanding of the mechanism of glycopeptide resistance and possibly of the factors affecting the regulation of resistance. For instance, why did reversion occur in the absence of selective antimicrobial pressure? First, it is likely that the mutation(s) responsible for the glycopeptide-resistant phenotype may be relatively unifit and maintained only under continued selective glycopeptide pressure. Alternately, a genetic element mediating resistance in the clinical GISA isolates might be lost during serial passage. This latter possibility seems unlikely because glycopeptide MICs decreased in stepwise fashion in the serially passed isolates and resistance from the GISA isolates has not been shown to be transferrable to date (unpublished data). Thus, since the MIC decreased in stepwise fashion (e.g., the MI revertants on day 15 compared with those on day 60) the reversion phenomenon observed here likely involves multiple stepwise chromosomal mutations that together conserve energy in the bacterial population in the absence of glycopeptides. The stepwise nature of reversion also suggests that in vivo, the development of resistance occurred by stepwise mutations. Identification of the genetic loci that are altered in each revertant could lead to an understanding of the mechanism(s) of resistance.

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ADDITIONAL PROOF

During the revision of the manuscript, a decrease in vancomycin MIC was demonstrated after serial passage of three clinical S. aureus isolates with decreased susceptibility to vancomycin, including Mu50 (Aeschlimann et al., Antimicrob. Agents Chemother. 43:1914–1918, 1999).

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