Lack of Evidence for Involvement of Hypermutability in Emergence of Vancomycin-Intermediate Staphylococcus aureus

In a recent publication, Schaff et al. demonstrate that selection of laboratory mutants resistant to vancomycin occurs more readily in a mutS knockout mutant of Staphylococcus aureus than in the parental strain from which it was derived (10). On the basis of these findings, the authors speculate that the emergence of vancomycin-intermediately resistant S. aureus (VISA) may also occur in clinical isolates that possess elevated mutation frequencies. Their arguments, in part, are also based on a report that the naturally occurring VISA strain Mu50 apparently contains a frame shift in mutS that would potentially confer a mutator phenotype (1). During the period that the manuscript by Schaaff et al. (10) was in preparation, we published a paper that does not support a role for mutators in the emergence of VISA (8).

Strains exhibiting elevated mutation frequencies have recently been reported among natural populations of pathogenic Escherichia coli, Salmonella enterica, Pseudomonas aeruginosa, Neisseria meningitidis, and Helicobacter pylori (6, 8). The majority of naturally occurring mutators contain defects in the methyl-directed mismatch repair (MMR) system, with mutations in mutS predominating (4, 7). MMR-deficient strains possess superior genetic backgrounds for the selection of some antibiotic resistance mutations, since mutation frequencies up to 1,000-fold higher than that of normal strains have been reported, and resistance levels achieved in mutators can be greater than those arising in nonmutator hosts (6, 7).

Intermediate resistance to vancomycin in S. aureus probably involves sequential mutations in a number of genes affecting peptidoglycan synthesis (1, 2). Since mutations in mutS are responsible for hypermutable phenotypes in other species, it was not unreasonable for Schaaff et al. (10) to propose that VISA strains might have emerged in staphylococcal mutators deficient in MutS activity. However, in our opinion, evidence for this theory is lacking.

Using an approach similar to that described by Schaaff et al. (10), we created a derivative of S. aureus RN4220 disrupted in mutS that displayed elevated mutation frequencies for resistance to a number of antibiotics (8). We agree that such a phenotype has the potential to accelerate evolution in S. aureus for adaptation to selective processes (in this case, antibiotics). Our work and that of Schaaff et al. (10) therefore indicate that if MutS-deficient strains arose in the clinical setting, they could contribute to the emergence of antibiotic resistance, including intermediate-level vancomycin resistance.

However, on the basis of quantitative mutation frequency determinations, none of the seven VISA strains we have examined, including Mu50, demonstrate any phenotypic evidence for mutator status (8, 9), and there is currently no experimental evidence to suggest that they have ever exhibited such a phenotype. Furthermore, rescuing a portion of mutS from Mu50 indicates that this gene is intact (8).

In support of their theory for involvement of mutators in emergence of VISA, Schaaff et al. (10) also note that vancomycin-resistant mutants selected in S. aureus RN4220ΔmutS exhibit higher levels of resistance to the antibiotic (MIC, 32 μg/ml) than mutants derived from the parental strain RN4220 (MIC, 4 μg/ml). However, resistant mutants for which vancomycin has high MICs (up to 100 μg/ml) have been selected in the laboratory from other S. aureus strains (3, 11). Since there is no reason to believe that these strains are mutators, it is apparent that mutator status is unlikely to be a requirement for the generation of high-level vancomycin resistance in S. aureus.

Whether VISA strains have passed through a hypermutable state during which increased accumulation of mutations has enabled strains to circumvent growth inhibition by vancomycin is unknown. However, our analysis of several VISA strains has demonstrated that none currently exhibits a mutator phenotype. Furthermore, the apparent frame shift in the mutS gene of VISA strain Mu50 is a sequencing artifact, and the mutation frequency of this strain is that of the wild type. In addition, other multiply resistant S. aureus strains we have tested show no increases (8) in their mutation frequencies relative to those of antibiotic-sensitive isolates or laboratory strains. Mutation frequencies in all the naturally occurring isolates that we examined, including strains recovered from cystic fibrosis patients, were significantly lower than those observed in a strain in which mutS had been disrupted (8). Our finding that no mutators were present in nearly 500 S. aureus isolates, including many methicillin-resistant S. aureus (MRSA) strains, argues against the authors’ suggestion that mutator clones may be highly prevalent among MRSA.

REFERENCES

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Authors’ Reply
In our recent publication by Schaaff et al. (6), we studied the hypothesis—which could be clearly demonstrated—that hypermutability can promote the evolution of vancomycin resistance in S. aureus. However, the impression that our intention was to imply that only hypermutators can reach high resistance is wrong. We cannot exclude that the control strain would have reached a high resistance level if the selection procedure had been further extended. Furthermore, we chose mutS for inactivation because it is one of the strongest mutator genes. We cannot and did not want to exclude the possibility that mutations in other mutator genes may be present in S. aureus and might enhance development of vancomycin resistance. Currently, it also cannot be excluded that a strain may lose its mutator status once the population has adapted to the antibiotic.

Our speculation in the discussion section was of course fueled by the paper by Avison et al. (1) which described the frame shift in mutS in the Japanese VISA strain Mu50. However, this frame shift was based on a sequencing mistake in the public database. It is owing to the work of O’Neill and Chopra (4) that this error has been cleared. Despite this, there is one observation that still points to hypermutability in VISA strains: vancomycin resistance seems to be a phenomenon that is sometimes very difficult to confirm, since many isolates that are suspected to be VISA, i.e., isolates that are positive in the initial tests, seem to lose this resistance upon subculture. These unstable strains are not the isolates that will be studied further or included in VISA collections, and this phenomenon could well be based on reversion of mutations that were acquired during treatment of the patient with vancomycin.

From a study which identified one weak mutator isolate among 493 Staphylococcus aureus strains, O’Neill and Chopra concluded that staphylococcal hypermutators are rare. Their survey included seven stable VISA strains, none of which showed a mutator status (4). We have checked a library of 33 multiresistant strains that were taken from an original screening of about 265 MRSA isolates (2) and included 3 stable VISA isolates. Nearly all of these strains had at least once shown ability to grow on Trypticase soy agar containing 4 mg of vancomycin per liter. Among these strains, one strong mutator phenotype was identified (Table 1 and unpublished work). S. aureus 1450-94 is the type strain of the northern German epidemic strain and harbors a very small subpopulation (1/108 cells) that is able to grow on Trypticase soy agar containing 4 mg of vancomycin per liter (5). All German VISA that were described so far are related to this clone (2, 3). A mutator phenotype is also displayed by one of our homogenous VISA strains that has lost sodM, one of the two superoxide dismutase genes, during cryogenic storage (5); however, a high mutation frequency is observed only in the presence of oxygen radicals. In another screen, we looked at about 20 strains and found a second, although weaker, mutator phenotype in S. aureus 1045-99 (Table 1). In conclusion, these results demonstrate that staphylococcal mutator strains do exist and are present among the lineages that give rise to VISA, yet a direct link between mutator status of clinical strains and development of vancomycin resistance remains to be established.

### Table 1. Mutation frequencies of S. aureus strains

<table>
<thead>
<tr>
<th>S. aureus strain</th>
<th>Rifampin at 4× MIC</th>
<th>Fusidic acid at 4× MIC</th>
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<tbody>
<tr>
<td>NCTC8325</td>
<td>0.65</td>
<td>4.7</td>
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<tr>
<td>RN4220</td>
<td>0.85</td>
<td>3.2</td>
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<tr>
<td>1450-94</td>
<td>124.2</td>
<td>40.3</td>
</tr>
<tr>
<td>1045-99</td>
<td>2.98</td>
<td>7.55</td>
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</tbody>
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References

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