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Intact *mutS* in Laboratory-Derived and Clinical Glycopeptide-Intermediate *Staphylococcus aureus* Strains

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The *mutS* gene of the methyl-directed mismatch repair system was sequenced in 10 parent and glycopeptide-intermediate *Staphylococcus aureus* strains. The *mutS* gene was intact in all strains studied. Hence, mutations in this gene had played no role in the development of vancomycin resistance in these strains.

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are common causes of hospital-acquired infections, and recently their prominence as causes of community-onset infections has increased (2, 5). For many years, the glycopeptide antibiotic vancomycin was the agent to which all MRSA strains were uniformly susceptible. However, since 1997, clinical isolates with reduced susceptibility to glycopeptide antibiotics (glycopeptide-intermediate *S. aureus* [GISA]) have arisen in various countries around the world (11). Treatment failure has been a common result of infections with such strains. A typical vancomycin MIC for GISA strains is 8 μg ml^{-1}. Several laboratories have reported on laboratory passage-selected GISA strains for which, for the most part, the MICs are similar (7, 20, 22). Recently, the first fully vancomycin-resistant *S. aureus* strain (for which the MIC is >128 μg ml^{-1}) was reported. Full resistance was due to the acquisition of *van* genes from an enterococcal source (4).

In contrast to *van*-mediated resistance, the mechanism of vancomycin resistance in GISA strains is not fully understood. A variety of alterations in peptidoglycan structure compared to that of susceptible *S. aureus* have been reported for GISA strains (3, 9, 23). The role of a thickened cell wall in vancomycin resistance in GISA strains has been emphasized (6). Vancomycin resistance is believed to evolve in multiple mutational steps (10, 20, 24). Recently Avison et al. (1) reported on an in silico investigation comparing the genomes of clinical vancomycin-intermediate strain Mu50 with those of several unrelated vancomycin-susceptible strains. Several loss-of-function mutations affecting cell wall biosynthesis and intermediary metabolism genes were identified in Mu50.

Antibiotic resistance can arise by mutations in different chromosomal loci, and this process is affected by the mutation rate (14). Mutator strains that show significantly increased mutation rates are present in many bacterial populations (15). Such strains can develop a variability of alleles that may allow them to survive better in the host environment (13) or in the presence of antibiotics (18). The methyl-directed mismatch repair systems of mutator strains are typically defective (13, 18). Methyl-directed mismatch repair is dependent on the MutS, MutL, and MutH proteins and corrects mismatched base pairs arising during replication (16). O'Neill and Chopra (19) insertionally inactivated the *mutS* gene of *S. aureus* strain RN4220 and observed increased mutation frequencies with rifampin, fusidic acid, norfloxacin, and mupirocin. Schaaff et al. (21) have recently reported on the development of vancomycin resistance in a mutator derivative of strain RN4220, created through a knockout of *mutS*. Vancomycin resistance evolved more rapidly and to a higher level in the *mutS* knockout strain than in the parent with an intact *mutS* gene.

Pfeltz et al. (20) subjected 12 *S. aureus* strains of various genetic backgrounds and methicillin resistance levels to selection for vancomycin resistance. Six strains acquired vancomycin resistance rapidly, four strains acquired it slowly, and two strains did not acquire it at all. MRSA strains tended to acquire the vancomycin resistance phenotype more rapidly and to a higher level than related susceptible strains. Schaaff et al. (21) suggested that mutator clones might be present in MRSA populations at a high frequency and that they may play a role in the ease of acquisition of vancomycin resistance.

We decided to test this hypothesis by determining the status of *mutS* in strains of *S. aureus* that gave rise to vancomycin-resistant strains rapidly, slowly, and not at all. The *mutS* gene sequence was determined by sequencing PCR products of the *mutS* gene. Second, we report on the status of *mutS* in GISA strains derived from these parent strains. In addition, we report the sequences of the *mutS* genes in two clinical GISA strains. The *mutS* gene was intact in all strains sequenced, and hence our results did not support a major role for *mutS* mutations in the development of GISA strains.

Strains and growth conditions. The strains used in the study and their relevant characteristics are given in Table 1 (20). The strains were stored at −80°C in 30% (vol/vol) glycerol and periodically streaked onto tryptic soy agar (Difco Laboratories, Detroit, Mich.) to provide working plates that were stored at 4°C. Cultures on tryptic soy agar were grown at 37°C. Tryptic soy broth (Difco Laboratories) was used for liquid cultures, which were grown at 37°C with shaking at 250 rpm.

DNA isolation. Overnight-grown 5-ml cultures were used to isolate genomic DNA with a Wizard genomic DNA isolation kit (Promega Corporation, Madison, Wis.). GISA strains which are less susceptible to lysostaphin (20) were lysed by

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TABLE 1. Strains used

Strain	Strain background[a]	Vancomycin MIC ($\mu g\ ml^{-1}$)	Acquisition of vancomycin resistance
RN450	Mc^s, 8325-4	1	Failed to acquire
COL	Mc^r (ho)	2	Slow
$COLV_5$	GISA	6	Slow; 5 cycles in 7 wk
$COLV_{10}$	GISA	8	Slow; 7 cycles in 11 wk
$13136p^-m^+$	Mc^r (he)	1.5	Rapid
$13136p^-m^+V_5$	GISA	8	Rapid; 2 cycles in 1 wk
$13136p^-m^+V_{20}$	GISA	16	Rapid; 5 cycles in 5 wk
$BB270V_{15}$	Mc^r (he), 8325 line	12	Rapid; 5 cycles in 5 wk
$BB568V_{15}$	Mc^r (ho), COL	12	Rapid; 5 cycles in 5 wk
$BB399V_{12}$	Mc^r (ho), DU4916 line	12	Slow; 7 cycles in 8 wk
HIP5836	GISA[b]	6	
HIP5827	GISA[b]	8	

[a] Abbreviations: Mc^s, methicillin susceptible; Mc^r, methicillin resistant; he, heterogeneous resistance expression; ho, homogeneous resistance expression.
[b] Clinical isolate.

increasing the concentration of lysostaphin from 100 to 500 $\mu g\ ml^{-1}$ in 0.05 M Tris-HCl (pH 7.5) containing 145 mM NaCl.

Amplification and sequencing of *mutS* in *S. aureus*. Primers MutS-1 (5′-TTCAAGACTTCTTCATTAAACCGTTAATG-3′) and MutS-9 (5′-GAGTTCTTTAATTTTCCCCATTTTG C-3′) (21) were constructed (Sigma-Genosys, TheWoodlands, Tex.) to amplify the complete *mutS* gene as well as its putative promoter and ribosomal binding site. PCR amplification was carried out with the GeneAmp amplification system (Applied Biosystems, Foster City, Calif.) by using *Taq* DNA polymerase (Promega Corporation). The amplified product was purified with a QIAquick PCR purification kit (QIAGEN Inc, Valencia, Calif.) for direct PCR product sequencing. The purified PCR product (~3.2 kb) was completely sequenced by using a primer walking strategy. The terminator cycle sequencing method with ABI Prism BigDye v3.0 chemistry was used to sequence the *mutS* PCR product in an ABI Prism310 genetic analyzer (Applied Biosystems).

Sequence analysis. Multiple alignment of the translated *mutS* gene sequences of the strains studied, including strains Mu50 (a well-studied clinical GISA strain), N315 (a hospital MRSA strain) (12), and MW2 (a community MRSA strain) (2), was carried out by using the CLUSTAL W 1.8 multiple-sequence alignment tool. The sequences of *mutS* genes from Mu50, N315, and MW2 were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov).

Only minor changes were observed in the nucleotide sequences of the *mutS* genes from the various strains (Table 2). At positions 1424 and 2423, the clinical GISA strains had G nucleotides, compared to T and A nucleotides, respectively, in the other strains. At position 2642, the clinical GISA strains had a C nucleotide instead of the T nucleotide found in the other strains studied. At position 2418, strain $BB399V_{12}$ had a C nucleotide instead of the A nucleotide in the other strains. At position 2820, an A was substituted for G in strains RN450 and $BB270V_{15}$. This substitution was responsible for the substitution of lysine (K) for glutamic acid (E) in these two strains.

The only difference in nucleotide sequence observed in the putative promoter sequence was a G nucleotide at position 235 in strains Mu50, N315, HIP5836, and HIP5827 instead of the A nucleotide in the other strains.

The translated *mutS* sequences were highly conserved. Only two amino acid changes were detected. First, a proline (P) residue was substituted for a threonine (T) residue at position 706 in strain HIP5836. This change results in what is considered to be a weaker conservation group (STPA) according to the Gonnet Pam250 matrix. Second, strains RN450 and $BB270V_{15}$ had a K residue instead of an E residue at position 840. This change also results in a weak conservation group (NDEQHK). The same amino acid substitution in strains RN450 and $BB270V_{15}$ is consistent with the fact that both strains are of the 8325 lineage. However, being of this lineage appears to play no role in the acquisition of vancomycin resistance, because strain RN450 failed to develop vancomycin resistance.

In none of the translated *mutS* sequences was a mutation that would lead to a nonfunctional MutS protein detected. It is possible that mutations in the *mutL* and *mutH* genes may be present in our strains, although in naturally occurring enteric bacterial mutators (13) and *Pseudomonas aeruginosa* (17), the preponderance of mutations is in the *mutS* gene. It was originally believed that *mutS* in GISA strain Mu50 was inactive, as the original published genome sequence of this organism indicated that a frameshift mutation was present (12). However, the resequencing of a portion of *mutS* in Mu50 indicated that the gene was intact (19). It is possible that there may be differences in levels of *mut* gene expression between different strains. Downregulation of MutS and, to a lesser extent, MutH is known to occur in stationary-phase and starved bacteria (8). However, O'Neill and Chopra (19) found no increase in mutation frequency in stationary-phase *S. aureus*. Thus, we have been unable to support the hypothesis that mutator clones are present in MRSA isolates at a high frequency and that they are

TABLE 2. Nucleotide sequence changes in *mutS* and its putative promoter

Nucleotide position	Nucleotide in *S. aureus* strain:														
	RN450	COL	$COLV_5$	$COLV_{10}$	$13136p^-m^+$	$13136p^-m^+V_5$	$13136p^-m^+V_{20}$	$BB270V_{15}$	$BB568V_{15}$	$BB399V_{12}$	HIP5836	HIP5827	Mu50	N315	MW2
mutS															
1424	T	T	T	T	T	T	T	T	T	T	G	G	G	G	G
2418	A	A	A	A	A	A	A	A	A	C	A	A	A	A	A
2423	A	A	A	A	A	A	A	A	A	A	G	G	G	G	A
2642	T	T	T	T	T	T	T	T	T	T	C	C	C	C	T
2820	A	G	G	G	G	G	G	A	G	G	G	G	G	G	G
mutS promoter															
235	A	A	A	A	A	A	A	A	A	A	G	G	G	G	A

responsible for the greater ease with which GISA strains arise from such parent strains (20, 21).

A further implication of our results is that mutators do not play a major role in the development of antibiotic resistance in *S. aureus*, which is in accord with the findings of O'Neill and Chopra (19). Those authors have speculated that gram-positive bacteria in general are less reliant on the methyl-directed mismatch repair system to ensure genetic fidelity. Recently, Willems et al. (25) found amino acid substitutions in MutSL of oxazolidinone-resistant or -susceptible strains of the gram-positive bacterium *Enterococcus faecium* but no evidence of hypermutable phenotypes.

Nucleotide sequence accession numbers. The *mutS* sequences from the strains used in this study have been deposited in the GenBank database under accession numbers AY324086 (RN450), AY324087 (COL), AY324088 ($COLV_5$), AY324089 ($COLV_{10}$), AY324090 ($13136p^-m^+$), AY324091 ($13136p^-m^+V_5$), AY324092 ($13136p^-m^+V_{20}$), AY324093 ($BB270V_{15}$), AY324094 ($BB568V_{15}$), AY324095 ($BB399V_{12}$), AY324096 (HIP5836), and AY324097 (HIP5827).

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