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Topoisomerase Mutations in Fluoroquinolone-Resistant and Methicillin-Susceptible and -Resistant Clinical Isolates of *Staphylococcus aureus*

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The incidence of the various mutations in the genes encoding topoisomerase IV and DNA gyrase in fluoroquinolone-resistant clinical isolates of *Staphylococcus aureus* is not known. Using restriction fragment length polymorphism analysis and DNA sequencing, we found that in fluoroquinolone- and methicillin-resistant strains, mutations in *grlA* and *gyrA* are quite likely to be present together. For fluoroquinolone-resistant but methicillin-susceptible strains, mutations in *grlA* alone are more common.

Much evidence has accumulated indicating that topoisomerase IV and not DNA gyrase is the primary target of fluoroquinolone antimicrobial agents in *Staphylococcus aureus*. Using strains in which fluoroquinolone resistance was produced in a stepwise fashion, *grlA* (topoisomerase IV A subunit gene) mutations have been found to occur prior to mutations in *gyrA* (2, 3, 8).

There is significant homology between the *gyrA* and *grlA* genes, especially in the so-called quinolone resistance-determining region (QRDR) near their 5′ ends. This nucleotide homology translates into notable amino acid homology; between residues 71 and 140 of GyrA and the homologous region of GrlA (residues 67 to 136), there is 63% identity. The degree of identity approaches 100% for the amino acid residues surrounding sites at which substitutions correlating with fluoroquinolone resistance have been identified. The positions in question include Ser80, Glu84, and Ala116 of GrlA and Ser84, Ser85, and Glu88 of GyrA (2, 3, 5, 8, 10).

The incidence of the various *gyrA* and *grlA* mutations correlating with fluoroquinolone resistance in clinical isolates of *S. aureus* is not known. We addressed this issue by examining the QRDR regions of *gyrA* and *grlA* of such strains with restriction fragment length polymorphism (RFLP) analysis and DNA sequencing. Fluoroquinolone-resistant (FQR), methicillin-susceptible (MS), and methicillin-resistant (MR) clinical isolates of *S. aureus* were obtained from eight different U.S. cities between 1989 and 1996. MICs of norfloxacin (Sigma Chemical Co., St. Louis, Mo.) were determined by using an agar dilution technique in accordance with the guidelines of the National Committee for Clinical Laboratory Standards (7). Pulsed-field gel electrophoresis of genomic DNA was carried out as previously described, but modified by the use of a rapid method for insert preparation (4, 6). To reduce the possibility of clonality, a pulsed-field restriction pattern categorized as unrelated to those of other study strains, based on the criteria established by Tenover et al., was required for a strain to be included in this study (11). Genomic DNA for use in PCRs was obtained from isolated bacterial colonies by using the InstaGene Matrix procedure as described in the manufacturer's guidelines (Bio-Rad Laboratories, Hercules, Calif.). Primer sequences and PCR parameters for use in amplification of the QRDR regions of *gyrA* and *grlA* were those described by Sreedharan et al. and Ferrero et al., respectively (2, 10). Primers were synthesized at the Macromolecular Core Facility, Wayne State University. PCR fragments were purified by ammonium acetate precipitation prior to RFLP analysis (1).

RFLP analysis of PCR products was carried out by digesting them with *Hin*fI (recognition site, GANTC), *Bsr*GI (TGTA CA), or *Fnu*4HI (GCNGC) as described in the manufacturer's guidelines (New England Biolabs, Inc., Beverly, Mass.). Undigested (control) and digested fragments were separated in agarose gels and visualized following staining with ethidium bromide.

For *grlA*, the size of the PCR product is 771 bp. Digestion with *Hin*fI produces fragments of 326, 234, 130, and 81 bp. Loss of the recognition site that includes codons 79 and 80 ([GAC TC]C; recognition site in brackets), which takes into account the codon 80 mutations that correlate with fluoroquinolone resistance (TCC→TAC or TCC→TTC, resulting in a Ser→Tyr or Ser→Phe substitution), results in fragments of 560, 130, and 81 bp. Any nucleotide alteration in position 1 or 2 of either codon will be detected and will result in an amino acid substitution or the introduction of a stop codon. *Bsr*GI normally will not cut the PCR product, but the occurrence of a G→A mutation in codon 84 (GAA→AAA, resulting in the substitution of Lys for Glu, which correlates with fluoroquinolone resistance) produces a recognition site and results in fragments of 526 and 245 bp. *Fnu*4HI normally cuts the PCR product once, producing fragments of 428 and 323 bp. Loss of its recognition site that includes codons 115 and 116 ([GCG GC]A) will leave the 771-bp PCR fragment intact. Mutations correlating with fluoroquinolone resistance have been described for codon 116 (GCA→GAA or GCA→CCA, resulting in an Ala→Glu or Ala→Pro substitution). Any change in the first two positions of either codon will be detected by RFLP and will result in an amino acid substitution. Changes occurring at the N position of the recognition site will not be detected but are silent with respect to amino acid substitutions.

For *gyrA*, the size of the PCR product is 493 bp. *Hin*fI cuts the product twice, producing fragments of 231, 189, and 73 bp.

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TABLE 1. MICs, RFLP analysis results, and QRDR sequencing data for MS strains

Strain	MIC[a] (μg/ml)	RFLP analysis[b]		*gyrA* sequencing data[c]
		grlA	*gyrA*	
1	6.3	79/80	None	Wild type
2	12.5	79/80	None	Wild type
3	12.5	79/80	None	Wild type
4	12.5	79/80	None	Wild type
5	25	79/80	None	Wild type
6	25	79/80	None	Wild type
7	12.5	84	None	TTT_{110}→TTC; silent
8	25	84	None	TCA_{112}→TCG; silent
9	25	84	None	Wild type
10	50	79/80	83/84	
11	50	79/80	83/84	
12	>100	79/80	83/84	
13	>100	79/80	83/84	

[a] MIC of norfloxacin.
[b] Codon(s) in which a mutation(s) resulting in loss or acquisition of recognition sites was found.
[c] This sequencing was done only for strains lacking RFLP-detected *gyrA* mutations.

Loss of the recognition site that includes codons 83 and 84 ([GAC TC]A) results in fragments of 420 and 73 bp. Any nucleotide alteration in position 1 or 2 of either codon will be detected by RFLP, and all lead to amino acid substitutions. The most common mutations in codon 84 that correlate with fluoroquinolone resistance are TCA→TTA and TCA→GCA (Ser→Leu and Ser→Ala). Codons 85 and 88 of *gyrA* cannot be examined by RFLP since there is no restriction endonuclease that includes either position in its recognition site.

The nucleotide sequence of the QRDR region of *gyrA* (codons 2 to 146) was determined by use of the dideoxy chain termination method (9). Strains selected for sequencing included those found to have *grlA* but not *gyrA* mutations by RFLP analysis.

MICs of norfloxacin for MR strains ranged from 12.5 to >100 μg/ml, with 11 of the 16 strains requiring an MIC of 100 μg/ml or greater (data not shown). The norfloxacin MICs for only two of the MS strains were in this range (see Table 1). All MR strains were found to have mutations in both *grlA* at codons 79 and 80 [*grlA*(79/80)] and in *gyrA* at codons 83 and 84 [*gyrA*(83/84)] by RFLP analysis and thus were not subjected to DNA sequence determination (data not shown).

Results for MS strains are shown in Table 1. Unlike their MR counterparts, the majority of these isolates (9 of 13) were found to have single topoisomerase mutations. Results from RFLP analysis and DNA sequencing correlated for all strains in which both approaches were utilized; no strain was found to have any mutation(s) in the QRDR region of *gyrA* known to correlate with fluoroquinolone resistance that was undetected by RFLP analysis.

All MR strains had the same RFLP results, suggesting the likely presence of identical topoisomerase mutations. However, the variance in norfloxacin MICs observed for these strains suggests that other fluoroquinolone resistance mechanisms are at play (see below). For MS strains, the presence of a single *grlA* mutation resulted in norfloxacin MICs of ≤25 μg/ml, whereas the presence of mutations in both topoisomerase A subunit genes resulted in norfloxacin MICs of ≥50 μg/ml. The geometric mean MICs for MS strains having *grlA*(79/80), *grlA*(84), or *grlA*(79/80) plus *gyrA*(83/84) mutations were 14, 20, and >71 μg/ml, respectively.

These data indicate that clinical isolates of FQR and MR *S. aureus* are likely to have mutations in both *grlA* and *gyrA*. In contrast, FQR but MS strains often are single *grlA* mutants. Mutations in *grlA*(115/116) occur rarely, if at all, in clinical isolates of FQR *S. aureus*.

It has been shown that fluoroquinolone MICs increase sequentially as an *S. aureus* strain accumulates topoisomerase mutations (3). This phenomenon was observed in the MS strains used in this study; of these strains, the geometric mean norfloxacin MICs were approximately fourfold greater for those possessing double topoisomerase mutations than for those with single mutations. However, MICs that varied up to fourfold could be observed for strains with topoisomerase mutations in the same locations. For MR strains, this variation was equal to or greater than eightfold. This observation indicates the likely presence of additional undetected mechanisms of fluoroquinolone resistance. Possibilities include other undetected target mutations, at sites not amenable to detection by RFLP analysis, such as *gyrA* position 85 or 88 in the MR strains included in this study, mutations at *grlA* position 84 other than GAA→AAA, and mutations at as-yet-undescribed locations within the genes encoding topoisomerases. Mutations in *grlB* or *gyrB*, which were not analyzed in this study, also could contribute to differences in norfloxacin MICs. Finally, efflux-mediated resistance contributed by NorA also may be a factor (6).

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