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# Ciprofloxacin Resistance in Coagulase-Positive and -Negative Staphylococci: Role of Mutations at Serine 84 in the DNA Gyrase A Protein of *Staphylococcus aureus* and *Staphylococcus epidermidis*

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***gyrA* mutations in quinolone-resistant pathogenic isolates of *Staphylococcus* spp. have been detected by the direct *Hin*fI digestion of polymerase chain reaction products. Homology among *gyrA* genes allowed rapid examination of both coagulase-positive and -negative isolates. DNA sequence analysis revealed that ciprofloxacin resistance in *Staphylococcus epidermidis* is associated with a novel Ser-84→Phe mutation in the DNA gyrase A protein, analogous to Ser-84→Leu changes observed in *Staphylococcus aureus*.**

Members of the genus *Staphylococcus* are major human pathogens (4). Coagulase-positive *Staphylococcus aureus* strains cause a variety of nosocomial infections, including postoperative septicemia. Recent experience has also emphasized the importance of coagulase-negative species. *Staphylococcus epidermidis* has risen to prominence through its frequent association with hemodialysis shunts, indwelling catheters, artificial heart valves, and other prosthetic implants that are used increasingly in modern medicine (17). Similarly, *Staphylococcus saprophyticus* is now recognized as a common cause of female urinary tract infections (12). Antibacterial fluoroquinolones such as ciprofloxacin have been effective in treating staphylococcal infections, especially those caused by methicillin-resistant strains (7, 9, 18). Unfortunately, widespread use of these agents has led to the marked emergence of resistance, notably among *S. aureus* and *S. epidermidis* isolates (3, 11, 21, 22).

The mechanisms of quinolone resistance in *Staphylococcus* spp. are poorly understood. Most studies have focused on *S. aureus*. One resistance pathway appears to involve alterations in DNA gyrase, an $A_2B_2$ complex encoded by the *gyrA* and *gyrB* genes that catalyzes ATP-dependent DNA supercoiling and is the intracellular target of the quinolones (5, 6). Thus, the *gyrA* genes from three ciprofloxacin-resistant *S. aureus* clinical isolates were found to carry Ser-84→Leu and/or Ser-85→Pro codon changes (23) analogous to the Ser-83→Leu and Ala-84→Pro gyrase A mutations known to confer resistance in *Escherichia coli* (16, 25). A second mechanism, conferring norfloxacin resistance, involves *norA*, a gene that most likely encodes a drug efflux pump (26). Studies of quinolone resistance in the coagulase-negative staphylococci are less advanced. Norfloxacin resistance genes (possibly similar to *norA*) have been cloned from *S. epidermidis* and *Staphylococcus haemolyticus* (24). However, the role of gyrase in resistance has yet to be examined. Here we describe a simple, rapid method based on the polymerase chain reaction (PCR) for detecting quinolone resistance mutations in *gyrA* that is applicable to both coagulase-positive and -negative strains, including *S. aureus*, *S. epidermidis*, and *S. saprophyticus*. We have partially characterized the *S. epidermidis gyrA* gene and identified a novel mutation associated with clinical resistance to ciprofloxacin.

To validate the method, we used three ciprofloxacin-resistant *S. aureus* strains, SA34R, SA31R, and SA42R, generated from the respective susceptible clinical isolates SA34, SA31, and SA42 (23) by incremental stepwise selection on ciprofloxacin plates in vitro (Table 1). Three *S. epidermidis* strains obtained from patients at the VA Center, Minneapolis, Minn., were also examined: ciprofloxacin-susceptible isolates SE190 and SE193 and the drug-resistant strain SE197 (Table 1). *S. saprophyticus* NCTC 7292 is a ciprofloxacin-susceptible clinical isolate from urine. Genomic DNA was isolated from each strain (13) and subjected to PCR as previously described, using two 24-mer oligonucleotide primers corresponding to *S. aureus gyrA* nucleotide positions −15 to +9 (primer 1) and 455 to 478 (primer 2) (Fig. 1A) (23). A 493-bp *gyrA* gene fragment was amplified from each isolate (Fig. 1B). The presence or absence of *gyrA* mutations at codon 83/84 was then determined by digesting PCR products with *Hin*fI (Fig. 1C and D). Mutations in *gyrA* that remove this *Hin*fI site result in a concomitant amino acid substitution at Asp-83/Ser-84 in the gyrase A protein. The presence of the *Hin*fI site at nucleotide 247 generates a pair of *Hin*fI DNA fragments 230 and 189 bp in size, whereas its absence through mutation yields a distinctive 419-bp product (23) (Fig. 1A). It can be seen that both SA31R and SA42R but not SA34R carry a mutation that eliminates the *Hin*fI site at nucleotide 247, indicating a mutation in the protein at residue 83 or 84 (Fig. 1C).

*Hin*fI digestion of PCR products from ciprofloxacin-susceptible *S. epidermidis* SE190 and SE193 and *S. saprophyticus* NCTC 7297 also generated the 189- to 230-bp doublet, showing that *gyrA* genes from these organisms share substantial homology with that of *S. aureus* (Fig. 1C and D). Interestingly, like SA31R and SA42R, ciprofloxacin-resistant *S. epidermidis* SE197 gave a 419-bp *Hin*fI product, revealing the presence of a mutation at residue 84 of the gyrase A protein. DNA sequence analysis was used to characterize the *S. aureus* and *S. epidermidis gyrA* genes and their mutations.

PCR products derived from *S. aureus* DNA were cut with *Eco*RI and *Cla*I, cloned into *Eco*RI-*Acc*I-cut M13mp18 and

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TABLE 1. Fluoroquinolone susceptibilities of *Staphylococcus* isolates

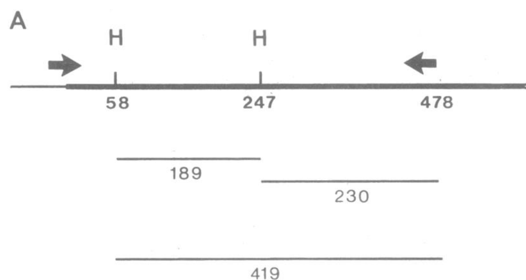
| Isolate | MIC (μg/ml)[a] | |
|---|---|---|
| | Ciprofloxacin | Norfloxacin |
| *S. aureus* | | |
| SA34 | 0.5 | |
| SA31 | 1.0 | |
| SA42 | 1.0 | |
| SA34R | >512 | |
| SA31R | >512 | |
| SA42R | >512 | |
| *S. epidermidis* | | |
| SE190 | 0.25 | <0.5 |
| SE193 | 0.5 | 2.0 |
| SE197 | 16 | 128 |
| *S. saprophyticus* | | |
| NCTC 7292 | 1.0[b] | |

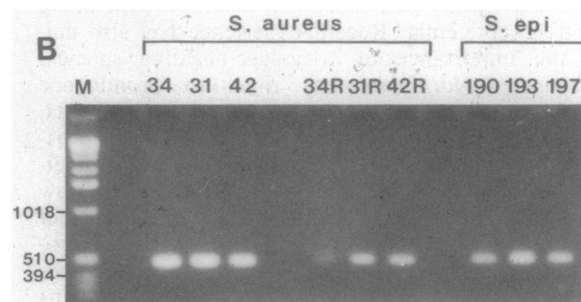
[a] Susceptibilities were determined by inoculating bacteria at $10^5$ CFU/ml into microdilution plates containing dilutions of the drug in divalent cation-supplemented Mueller-Hinton broth. The MIC is the lowest concentration showing no growth after overnight incubation at 35°C.

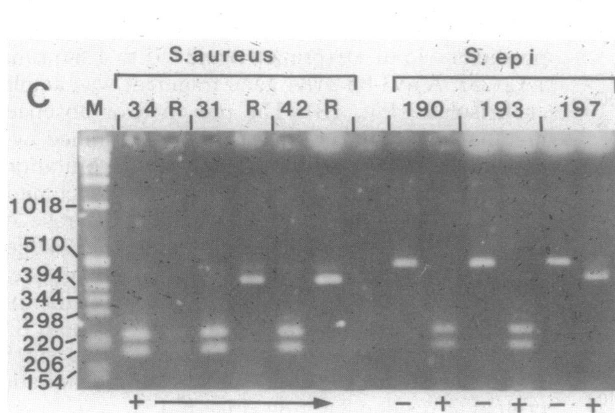
[b] Lowest concentration showing no growth on brain heart infusion plates after overnight incubation at 37°C.

M13mp19, and sequenced by the dideoxy chain termination method (20) with Sequenase version 2.0 and [α-$^{35}$S]dATP (United States Biochemicals), as described previously (23). *S. epidermidis* PCR fragments did not contain a *Cla*I site suitable for cloning purposes. Therefore, asymmetric PCR was carried out to permit direct sequence analysis; PCR reactions were in 10 mM Tris hydrochloride (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–0.01% gelatin containing 100 μM deoxynucleoside triphosphate, 2.25 ng of primer 1, 112.5 ng of primer 2, 1 to 1.5 U of *Taq* polymerase, and 5 ng of bacterial DNA. PCR conditions were as follows: 93°C, 1 min; 45°C, 2 min; 72°C, 2 min; 35 cycles. DNA products were sequenced directly with primer 1 and Sequenase as described above. A conveniently placed *Hae*III site was found, enabling double-stranded PCR fragments to be sequenced unambiguously following ligation into *Eco*RI-*Sma*I-cut M13 (data not shown). Sequence derived for complementary strands gave concurrent results.

*gyrA* fragments from *S. aureus* SA31R and SA42R differed at one nucleotide position compared with those of the sensitive precursors: both carried a C→T transition at position 251, resulting in a Ser-84→Leu substitution in the gyrase A protein. The same change was previously found in two



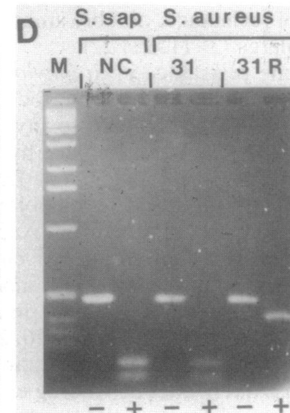




FIG. 1. Conservation of *gyrA* sequences in *S. aureus*, *S. epidermidis*, and *S. saprophyticus* allows detection of *gyrA* mutations in quinolone-resistant isolates by a direct PCR-*Hin*fI restriction fragment length polymorphism analysis. (A) Location of *Hin*fI sites (H) at the 5′ end of the wild-type *S. aureus gyrA* gene (shown by heavy line). Boldface numbers 58 and 247 identify the position of the first nucleotide in the *Hin*fI recognition sequence; 478 indicates the 3′ end of DNA to be amplified (10, 23). Arrows denote two oligonucleotide primers positioned next to their homologous sequences on complementary DNA strands. DNA fragment sizes arising from *Hin*fI digestion of PCR products are indicated. (B) PCR amplifies a 493-bp *gyrA* fragment from both *S. aureus* and *S. epidermidis* (S. epi) isolates (strains denoted by numbers; see Table 1). PCR was carried out on genomic DNA with *S. aureus gyrA* DNA primers (see panel A), and the DNA was analyzed by electrophoresis in 2% low-gelling agarose. M denotes marker DNA fragments. Molecular sizes (in base pairs) are indicated at left. (C) Detection of codon 83/84 *gyrA* mutations in ciprofloxacin-resistant strains of *S. aureus* and *S. epidermidis*. PCR products were incubated in the presence (+) or absence (−) of *Hin*fI and examined by gel electrophoresis. Susceptible *S. aureus* isolates and their resistant (R) derivatives were run in adjacent lanes. The presence of the *Hin*fI site at nucleotide 247 generates a 189- to 230-bp doublet. When the *gyrA* codon contains a mutation at positions 83 and 84, the doublet is replaced by a single 419-bp fragment. (D) PCR detection of *gyrA* mutations is applicable to *S. saprophyticus* isolates. A 493-bp *gyrA* fragment from susceptible strain NCTC 7292 (NC) was amplified by PCR with *S. aureus* primers (see above) and incubated in the presence (+) or absence (−) of *Hin*fI. Digestion products derived from *S. aureus* susceptible and resistant isolates are included for comparison.

```
                          a              t
ATGGCTGAATTACCTCAATCAAGAATTAATGAACGAAATATAACCAGTGAAATGCGTGAA    60
METAlaGluLeuProGlnSerArgIleAsnGluArgAsnIleThrSerGluMetArgGlu    20

     t     t     g               g          g  a      c t
TCATTCTTAGACTATGCTATGAGTGTTATCGTTTCTCGTGCATTACCTGATGTTAGAGAC   120
SerPheLeuAspTyrAlaMetSerValIleValSerArgAlaLeuProAspValArgAsp    40
                                (ala)

  t     a                 a  a     a                    a  g
GGATTAAAGCCAGTACATCGTCGTATTCTTTATGGTTTAAATGAACAAGGTATGACGCCC   180
GlyLeuLysProValHisArgArgIleLeuTyrGlyLeuAsnGluGlnGlyMetThrPro    60

      t a     a     a        c  t  t  c  a
GATAAACCTTATAAGAAATCTGCACGTATAGTCGGGGATGTCATGGGTAAATATCACCCT   240
AspLysProTyrLysLysSerAlaArgIleValGlyAspValMetGlyLysTyrHisPro    80
     (ser)

        c  a  t                  c t     t
CATGGTGATTCTTCAATTTATGAAGCAATGGTAAGAATGGCC  282   SE190,SE193
HisGlyAspSERSerIleTyrGluAlaMetValArgMetAla
          |
         PHE  SE197 (ciprofloxacin
         TTT         resistant)
```

FIG. 2. DNA sequence analysis of *gyrA* fragments obtained by PCR amplification of genomic DNA from *S. epidermidis* clinical isolates. Capital letters show the nucleotide sequence of ciprofloxacin-susceptible isolates SE190 and SE193, with the deduced protein sequence below. Sequence data for the ciprofloxacin-resistant isolate SE197 differed by a single C-to-T transition, leading to a Ser-84→Phe change in the gyrase A protein. The *S. aureus gyrA* nucleotide sequence and gyrase A protein sequence (10) are indicated, where different from those of SE190 and SE193, by lowercase letters and residues in parentheses, respectively.

ciprofloxacin-resistant clinical *S. aureus* isolates, SA47 (the matched posttherapy isolate to SA31) and SA146 (23). Thus, the Ser-84→Leu gyrase A mutation appears to be commonly associated with both clinical and in vitro resistance to ciprofloxacin in *S. aureus*. Interestingly, the *gyrA* gene sequence determined for SA34R was found to be wild type, indicating that ciprofloxacin resistance in this strain must arise from other mechanisms, e.g., mutations elsewhere in *gyrA* or in other genetic loci such as *norA* or *gyrB*. It is of course possible that mutations at other loci also contribute to high-level resistance, even in those strains with detectable codon 83/84 *gyrA* mutations (Table 1).

*S. epidermidis* SE190 and SE193 had identical *gyrA* sequences and showed very close homology with *S. aureus gyrA* (Fig. 2). Conserved *Hin*fI sites at positions 58 and 247 account for the similarity of the *Hin*fI digestion pattern to that of *S. aureus* (Fig. 1C). The *gyrA* gene fragment from ciprofloxacin-resistant isolate SE197 was identical in sequence to that from SE190 and SE193, except for a C→T transition at nucleotide 251 that abolishes the natural *Hin*fI site and generates a novel Ser-84→Phe substitution at the protein level.

These results show that ciprofloxacin resistance in both *S. epidermidis* and *S. aureus* is commonly associated with the mutation of the conserved serine 84 residue in the gyrase A protein. Previous studies with *E. coli* laboratory strain KL16 and *E. coli* clinical isolates have demonstrated that high-level quinolone resistance results from analogous Ser-83→Leu or Trp changes in GyrA protein (2, 16, 25). In other work, a genetically engineered Ser-83→Ala *gyrA* codon change conferred low-level quinolone resistance (8). In each case, these alterations replace Ser-83 with a hydrophobic residue lacking a hydroxyl side chain. The analogous Ser-84→Leu or Phe changes found in ciprofloxacin-resistant *Staphylococcus* spp. fall into the same category. Frequent association of gyrase A mutations at serine 83/84 with quinolone resistance in a range of clinically important organisms merits further analysis of the role of this residue in the interaction of quinolones with gyrase.

Our studies highlight the sequence conservation of *gyrA* genes among coagulase-positive and -negative staphylococci. We have found only 31 nucleotide differences (89% identity) between the 282-bp *S. epidermidis gyrA* sequence and that of *S. aureus* (Fig. 2). Most of these changes are silent, and the deduced 94-residue gyrase A protein sequence shows only two amino acid differences with the *S. aureus* protein: Ser-32 for Ala and Ser-63 for Pro (the same gyrase A residues found in *Bacillus subtilis*, another gram-positive organism [14]). This situation contrasts with the different morphological, biochemical, and physiological features exhibited by staphylococcal species (15, 17), e.g., coagulase response, cell wall structure (including different penicillin-binding proteins [1, 19]), and other properties that presumably account for their different pathological niches. Conservation of gyrase A protein sequence may reflect the critical biological importance of the protein and may also account for the broadly similar quinolone susceptibilities among the staphylococci (Table 1). By exploiting this homology, PCR techniques should allow alterations in gyrase to be readily detected in a variety of quinolone-resistant staphylococcal isolates.

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