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DNA Gyrase *gyrA* Mutations in Ciprofloxacin-Resistant Strains of *Staphylococcus aureus*: Close Similarity with Quinolone Resistance Mutations in *Escherichia coli*

SANTHA SREEDHARAN,[1] MARK ORAM,[1] BARBARA JENSEN,[2] LANCE R. PETERSON,[2] AND L. MARK FISHER[1]*

Molecular Genetics Group, Department of Cellular and Molecular Sciences, Division of Biochemistry, St. George's Hospital Medical School, University of London, Cranmer Terrace, London SW17 0RE, United Kingdom,[1] and Infectious Disease Section, Veterans Administration Medical Center, Minneapolis, Minnesota 55417[2]

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The *gyrA* genes isolated from three ciprofloxacin-resistant clinical isolates of *Staphylococcus aureus* carried codon 84 (serine→leucine) and/or codon 85 (serine→proline) mutations that were absent in pretreatment susceptible strains. These substitutions occur in a region of the gyrase A protein wherein directly analogous mutations of serine 83→leucine and alanine 84→proline in *Escherichia coli* confer quinolone resistance. Thus, DNA gyrase A subunit mutations are implicated in resistance to ciprofloxacin in *S. aureus*.

Fluoroquinolone inhibitors of DNA gyrase (e.g., ciprofloxacin) are potent antibacterial agents that have been widely used in the treatment of difficult bacterial infections, including those involving methicillin-resistant *Staphylococcus aureus* strains (10, 14, 18). However, similar to experience with earlier quinolones such as nalidixic acid, the isolation of *S. aureus* strains resistant to fluoroquinolones is now commonplace (8, 11, 12; L. R. Peterson, J. N. Quick, B. Jensen, S. Homan, S. Johnson, J. Tenquist, C. Shanholtzer, R. A. Petzel, L. Sinn, and D. N. Gerding, Arch. Intern. Med., in press). The emergence of fluoroquinolone resistance is of particular concern given that relatively few antimicrobial agents are effective against methicillin-resistant staphylococcal infections.

The molecular basis of quinolone resistance in *S. aureus* is not understood. In *Escherichia coli*, resistance commonly arises through mutation of the *gyrA* and (less often) *gyrB* genes, encoding the A and B subunits of the A_2B_2 gyrase complex which catalyzes ATP-dependent DNA supercoiling (references 3, 4, and 19 to 21 and references therein). Analysis of the *S. aureus gyrB-gyrA* locus, recently isolated from ciprofloxacin-susceptible clinical isolate 81231, revealed that the N-terminal region of the *S. aureus* gyrase A subunit is highly homologous to that of its *E. coli* counterpart (6). In particular, Ala-67, Ser-83, and Gln-106, residues whose substitution leads to quinolone resistance in *E. coli* (1, 2, 20, 21; our unpublished data), are all conserved in the *S. aureus* gyrase A protein as residues 68, 84, and 107 (6). This observation led us to examine whether ciprofloxacin resistance in *S. aureus* is associated with mutations similar to those found in *E. coli*.

We focused on six methicillin-resistant clinical isolates of *S. aureus* obtained from patients at the Veterans Administration Medical Center, Minneapolis, Minn. (Table 1). Two pairs of strains, 34-35 (from patient 1) and 31-47 (from patient 2), were matched isolates, being ciprofloxacin-susceptible pretreatment and ciprofloxacin-resistant posttreatment strains (Table 1). Chromosomal DNAs prepared from strains 31 and 47 gave identical patterns of *Hae*III fragments, suggesting persistence of the same strain throughout treatment (Peterson et al., in press). Strains 42 and 146 were isolated from different patients. Whereas the susceptible isolates all exhibited ciprofloxacin MICs of 0.5 to 1.0 μg/ml, the MICs for resistant strains were 16- to >256-fold higher (Table 1). By analogy with *E. coli*, these high levels of resistance suggested that the strains carry mutations in *gyrA*, conceivably at codon 84. This possibility was tested by using a restriction fragment length polymorphism (RFLP) analysis that we originally developed for mutations in *E. coli gyrA* (2).

The *S. aureus* gyrase A protein carries conserved Asp-Ser residues at positions 83 and 84 whose coding sequence in the *gyrA* gene forms a *Hin*fI restriction site (GANTC) beginning at nucleotide position 247 (Fig. 1a; also see sequence in Fig. 2) (6). Inspection of codon usage (2) shows that any mutation that removes this *Hin*fI site will necessarily cause an amino acid substitution in the gyrase A protein either at position 83 or at position 84. To detect polymorphism at the 247 *Hin*fI site, genomic DNA was isolated from clinical isolates (9), digested with *Hin*fI, and hybridized on Southern blots to an *Rsa*I-*Cla*I *gyrA* probe (nucleotide positions 271 to 439) (Fig. 1a). The presence or absence of the 247 site should result in the detection of a 241- or 430-bp *Hin*fI fragment, respectively (Fig. 1a). In this assay, Hybond-N filters were hybridized overnight at 65°C in 5× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1.5% dried milk. Filters were washed successively in 2× SSC–0.1% sodium dodecyl sulfate (SDS) (two 10-min washes at room temperature), then in 1× SSC–0.1% SDS (two washes as before), and finally in 0.1× SSC–0.1% SDS (two 15-min washes at 65°C). DNAs from ciprofloxacin-susceptible strains 34, 31, and 42 gave the smaller 241-bp fragment (Fig. 1b). In contrast, resistant isolates 47 and 146 produced the larger *Hin*fI fragment, indicating loss of the 247 *Hin*fI site and thus an amino acid substitution at position 83 or 84 of the gyrase A subunit. Strain 35 had the normal 247 *Hin*fI site even though it was 120-fold more resistant than isolate 34, suggesting that the putative resistance mutation must reside at a location other than codon 83 or 84 in *gyrA*. Such a change was found at codon 85.

To identify the *gyrA* mutations at the nucleotide level, the

* Corresponding author.

TABLE 1. Ciprofloxacin susceptibility of *S. aureus* clinical isolates[a]

Isolate	MIC (μg/ml)
34 (pretreatment)	0.5
35 (posttreatment)	64
31 (pretreatment)	1
47 (posttreatment)	16
42 (susceptible)	1
146 (resistant)	>256
81231 (susceptible)	0.5

[a] Strains 34-35 and 31-47 are matched pairs of isolates obtained from patients pre- and posttreatment. Susceptibility testing was done by inoculation of *S. aureus* at 10^5 CFU/ml into microdilution plates containing dilutions of ciprofloxacin in divalent cation-supplemented Mueller-Hinton broth. The MIC is the lowest concentration showing no growth after overnight incubation at 35°C (Peterson et al., in press).

5′ ends of *gyrA* genes from clinical isolates were amplified from chromosomal DNA by polymerase chain reaction (PCR), allowing subsequent cloning and DNA sequence analysis. Two 24-mer oligonucleotide primers were synthesized for PCR: 5′-AAGGAGGAAGAATTCATGGCTGAA (*gyrA* positions −15 to +9, with an artificial *Eco*RI site at position −6) and 5′-AGACTGACGGCTCTCTTTCATTAC (complementary to positions 455 to 478). PCR reactions were carried out in 10 mM Tris hydrochloride (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–0.01% gelatin containing 200 μM each deoxynucleoside triphosphate, 1 μM each primer, 1 to 1.5 U of *Taq* DNA polymerase, and 5 ng of bacterial DNA (total volume, 50 μl). PCR conditions were as follows: 92°C, 1 min; 45°C, 1 min; 74°C, 3 min; 30 cycles. The amplified 493-bp *gyrA* fragments were isolated and digested with *Eco*RI and *Cla*I, and the resulting 439-bp fragments were ligated into *Eco*RI-*Acc*I-cut M13mp18 and M13mp19 replicative-form DNA prior to transformation into *E. coli* XL1 *recA*. Single-stranded recombinant M13 DNA was sequenced by using T7 DNA polymerase and [α-^{35}S]dATP according to the Amersham Multiwell protocol. Two independent clones of each PCR product in M13mp18 and M13mp19 were sequenced in full, and the resulting complementary strand sequences gave concurrent results (Fig. 2).

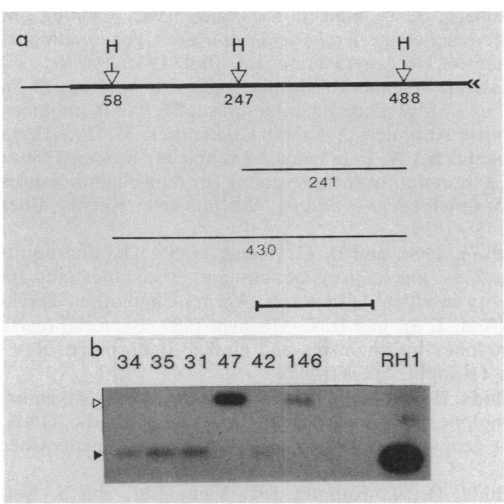

FIG. 1. Detection of mutations in the *gyrA* gene of ciprofloxacin-resistant clinical isolates of *S. aureus*, using a *Hin*fI RFLP analysis. (a) Location of *Hin*fI sites at the 5′ end of the *S. aureus gyrA* gene (shown by the heavy line). H denotes *Hin*fI sites present in the wild-type gene, and bold numbers identify the position of the first nucleotide in the *Hin*fI recognition sequence. The presence of the *Hin*fI site at 247 or its absence as a result of mutation in codon 83 or 84 generates a 241- or 430-bp *gyrA* fragment on *Hin*fI digestion that can be identified by hybridization to an *Rsa*I-*Cla*I *gyrA* probe (heavy bar). (b) Southern hybridization of *Hin*fI-digested chromosomal DNA from *S. aureus* clinical isolates to the radiolabeled *Rsa*I-*Cla*I *gyrA* probe. Numbers identify clinical isolates used; plasmid pRH1, carrying the 5′ end of the *gyrA* gene (6) cloned from a ciprofloxacin-sensitive *S. aureus* strain, was digested with *Hin*fI and used as a control (right). Open and filled arrowheads denote 430- and 241-bp *Hin*fI *gyrA* fragments, respectively. DNA fragments were sized by running DNA markers alongside (not shown).

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STRAIN                          GYR A GENE

                80          83              87          90
E. coli      .His Gly Asp SER Ala Val Tyr Asp Thr Ile Val.
(K12)         CAT GGT GAC TCG GCG GTC TAT GAC ACG ATT GTA
                81          84  85                      91
S. aureus    .His Gly Asp SER SER Ile Tyr Glu Ala Met Val.
(81231)       CAT GGT GAC TCA TCT ATT TAT GAA GCA ATG GTA
                             |   |
  34 (s)      --- --- --- --- --- --- --- --- --- --- ---
  35 (r)      --- --- --- --- C-- --- --- --- --- --- ---
                             (Pro)

  31 (s)      --- --- --- --- --- --- --- --- --- --- ---
  47 (r)      --- --- --- -T- --- --- --- --- --- --- ---
                         (Leu)

  42 (s)      --- --- --- --- --- --- --- --- --- --- ---

 146 (r)      --- --- --- -T- C-- --- --- --- --- --- ---
                         (Leu Pro)
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FIG. 2. Summary of *gyrA* sequences from ciprofloxacin-susceptible (s) and -resistant (r) *S. aureus* strains. DNA sequence is shown for the coding strand in the region corresponding to gyrase A codons 81 to 91 and is compared with the sequence of the coding strand of the strain 81231 *gyrA* gene (6). Base changes and the codon changes (in parentheses) are indicated. The corresponding *E. coli* K-12 *gyrA* sequence is shown for comparison (15). *Hin*fI restriction sites are underlined.

The *gyrA* gene fragments obtained from ciprofloxacin-susceptible isolates 34, 31, and 42 were identical in sequence to that reported previously for *gyrA* in *S. aureus* 81231 (Fig. 2) (6). DNA sequences obtained for resistant isolates 35 and 47 were identical to that for strain 81231 *gyrA* except for single-base changes: a T→C transition in strain 35 *gyrA* (nucleotide 253), producing a Ser-85→Pro substitution in the gyrase A protein, and a C→T transition in strain 47 *gyrA* (position 251), resulting in a Ser-84→Leu substitution at the protein level. Interestingly, resistant isolate 146 carried both of these *gyrA* mutations. Since only the Ser-84→Leu codon change abolishes the 247 *Hin*fI site (Fig. 2), the DNA sequence results are in accord with those of the RFLP analysis (Fig. 1).

Two lines of evidence strongly suggest that the *gyrA* mutations reported here are responsible for or at least contribute to ciprofloxacin resistance in *S. aureus*. First, the Ser-85→Pro and Ser-84→Leu changes were present in the gyrase A protein of resistant *S. aureus* strains isolated posttherapy but were absent in the corresponding susceptible pretreatment isolates (Table 1 and Fig. 2). Strain 146, carrying both mutations, was more resistant than either of the single mutants 35 and 47. Second, the Ser-84→Leu and Ser-85→Pro substitutions in *S. aureus* gyrase A protein are directly equivalent to the Ser-83→Leu and Ala-84→Pro changes known to confer quinolone resistance in *E. coli* (20). The direct analogy with the *E. coli* system strongly indicates that we have identified ciprofloxacin resistance mutations in *S. aureus*. This assignment can ultimately be tested by site-directed mutagenesis and expression of the mutant

protein. The genetics of quinolone resistance in *S. aureus* are currently not well developed. However, Ubukata et al. have isolated a gene from *S. aureus* termed *norA* that confers resistance to the quinolone norfloxacin (17). The *norA* gene has a restriction map different from that of our *S. aureus* gyrase clones and unlike resistant gyrase genes appears to act in a genetically dominant fashion (5, 17). The data suggest that *norA* is not a gyrase allele.

Understanding the mechanism by which quinolone resistance mutations act on DNA gyrase has been hindered by the absence of structural information on the drug-gyrase-DNA complex. However, Shen and colleagues have proposed a model in which the quinolone binds both to gyrase and to the single-stranded DNA regions resulting from transient DNA breakage by the A subunits (13). Mutation of specific gyrase A residues close to catalytic Tyr-122 (7) (e.g., Ser-83 [84 in *S. aureus*] to hydrophobic residue Leu or Trp) presumably disrupts key interactions involving the gyrase-quinolone complex. Although highly conserved, gyrase A proteins from different species also exhibit sequence differences that may modulate quinolone action. For example, *S. aureus* gyrase A protein has the sequence Asp-Ser-Ser at positions 83 to 85 instead of the Asp-Ser-Ala in *E. coli* (Fig. 2). Interestingly, we find that substitution of Ser-85 with proline, which could produce a major structural alteration, is also associated with high-level ciprofloxacin resistance in *S. aureus*. It may be that serine in the *S. aureus* A protein either preserves or can take part in a critical interaction necessary for quinolone action. Such considerations will be important in assessing the different quinolone sensitivities of DNA gyrases from different susceptible and resistant bacterial sources (16).

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