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Incidence of Various *gyrA* Mutants in 451 *Staphylococcus aureus* Strains Isolated in Japan and Their Susceptibilities to 10 Fluoroquinolones

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Point mutations in the *gyrA* genes of 451 clinical strains of *Staphylococcus aureus* isolated in Japan were detected by a combination of nonradioisotopic single-strand conformation polymorphism analysis and restriction fragment length polymorphism analysis and by direct sequencing. Six types of *gyrA* mutations were observed in 149 of the 451 strains (33%), and ofloxacin MICs were greater than 6.25 μg/ml for 147 of the 149 strains (98.7%). These mutations were localized between codons 84 and 88, and they were associated with fluoroquinolone resistance. Two types of silent mutations were also found. Among these eight types of mutations, three types are novel, i.e., the serine at position 84 (Ser-84)→Val (TCA→GTA), Ser-84→Leu (TCA→TTA) plus Ile-86 (ATT→ATC, silent), and Phe-110 (TTT→TTC, silent). Among GyrA mutants, strains with a Ser-84→Leu alteration and strains with a Glu-88→Lys alteration were dominant. In contrast, few strains had Ser-84→Ala and Glu-88→Gly alterations. All fluoroquinolones tested showed greater than a fourfold decrease in their activities in terms of their MICs that inhibited 50% of strains tested for each GyrA mutant, in comparison with their MICs that inhibited 50% of strains tested for susceptible strains. Most of the currently available fluoroquinolones, such as norfloxacin, enoxacin, ofloxacin, ciprofloxacin, tosufloxacin, lomefloxacin, sparfloxacin, and fleroxacin, were ineffective against each mutant. Mutants containing a Ser-84→Leu or Val alteration showed high-level resistance to fluoroquinolones, and one containing a Ser-84→Ala alteration showed relatively low-level resistance. Double mutations were associated with a higher level of resistance than single mutations.

Fluoroquinolone antimicrobial agents are potent against gram-positive and -negative bacteria and are widely used clinically (8). Their primary target is considered to be DNA gyrase, an essential bacterial type II topoisomerase, which primarily catalyzes the introduction of negative supercoils into double-stranded DNA in the presence of ATP. DNA gyrase is a holoenzyme consisting of two A subunits (GyrA) and two B subunits (GyrB) encoded by the *gyrA* and *gyrB* genes, respectively (15, 20, 24).

Because fluoroquinolones have been used frequently, some bacteria, especially methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*, have developed resistance (1, 16, 17). In *S. aureus*, four quinolone resistance mechanisms have been reported. First, GyrA alteration leads to lower inhibitory activities of fluoroquinolones against DNA gyrase (13). Sites of point mutations in the *gyrA* gene showed close similarities to the sites in *Escherichia coli* (5, 6, 19). Second, a membrane protein, NorA, contributes to active efflux-mediated resistance. Yoshida et al. (26) proposed that mutants of this kind show higher levels of resistance to hydrophilic quinolones, such as norfloxacin and enoxacin, than to hydrophobic ones. Third, *cfxB-ofxC* (*flqA*) confers quinolone resistance on *S. aureus*; however, its mechanism is still unknown (23). Fourth, point mutations in the *gyrB* gene are also responsible for quinolone resistance (10).

Several methods for detecting point mutations in a specified gene have been attempted so far. Single-strand conformation polymorphism (SSCP) analysis is a rapid, simple, and effective method. It is based on the theory that denatured PCR fragments with different mutations migrate differently in a polyacrylamide gel because of their conformational changes by a base alteration(s) (7, 14). One study applied SSCP analysis for the detection of *gyrA* mutations in *S. aureus* and ascertained its validity (22).

In the study described here, we detected *gyrA* mutations in *S. aureus* by using a combination of SSCP and restriction fragment length polymorphism (RFLP) analyses. In the SSCP system, we amplified the region including codons 54 to 127, to encompass codons 68 through 107, which has been proved to be the quinolone resistance-determining region in *E. coli* (25). We examined more than 450 clinical isolates, the largest number to date, to study the incidence of various *gyrA* mutations in *S. aureus*. Relationships between the types of mutations and the susceptibilities of the mutants to 10 fluoroquinolones were also studied to determine if there are some differences among fluoroquinolones in their activities against *gyrA* mutants.

MATERIALS AND METHODS

Bacterial strains and antibacterial agents. Four hundred fifty-one strains of *S. aureus* were collected randomly from clinical specimens from individual patients at 15 hospitals in Japan in 1991 and 1992. Two *gyrA* mutants, SA35 (serine at position 85 [Ser-85]→Pro) and SA146 (Ser-84→Leu, Ser-85→Pro), were kindly provided by L. M. Fisher, University of London (19). Strains K-1 (Ser-84→Leu, Glu-88→Lys) and 3-2(288-3) (Ser-84→Leu, Glu-88→Gly) were used in a previous study (22). All fluoroquinolones used were synthesized by Ube Industries, Ltd., Ube, Japan. Methicillin was obtained commercially from Banyu Pharmaceutical Co., Ltd., Tokyo.

Susceptibility test. MICs were determined by the agar dilution method (11) in Mueller-Hinton II agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) with approximately 5×10^4 CFU. The MICs were read after 20 h

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of incubation at 35°C. In each determination, a standard strain, *S. aureus* FDA 209P JC, was used as a reference strain.

PCR. Two 20-mer primers, 5′-AATGAACAAGGTATGACACC (nucleotides 160 to 179) and 5′-TACGCGCTTCAGTATAACGC (complementary to nucleotides 363 to 382), were synthesized with a model 392 DNA synthesizer (Applied Biosystems Japan Co., Ltd., Urayasu-shi, Chiba, Japan), according to the sequences reported previously (2, 12). Genomic DNA was extracted by using Insta Gene Purification Matrix (Bio-Rad Laboratories, Hercules, Calif.), and 20 μl of the supernatant was used as template. The reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 200 μM (each) deoxynucleoside triphosphates, 500 μM (each) primers, 1 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and the template in a total volume of 50 μl. The samples were preheated at 94°C for 2.5 min; then, they were subjected to cycling of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min for 30 cycles; this was followed by incubation at 72°C for 9 min in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus). Production of the 223-bp PCR products that were aimed for was checked by 1% agarose gel electrophoresis and staining with 0.5 μg of ethidium bromide per ml.

SSCP analysis. PCR products (10 μl) were mixed with 10 μl of sample buffer containing 90% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. The mixtures were heated at 94°C for 5 min; then, they were immediately placed on ice. Samples were separated on a polyacrylamide gel (5% T [total monomer concentration], 2% C [crosslinker concentration]; 12 cm by 13.7 cm by 0.75 mm) containing 5% glycerol in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at a constant voltage of 100 for 3.5 h at 32°C. Gels were stained with a silver stain kit (2D-Silver Stain II "Daiichi"; Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) to detect DNA.

RFLP analysis. Five units of *Hin*fI (Toyobo, Tokyo, Japan) was added to 5 μl of PCR products in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The *Hin*fI site, which is present between nucleotides 247 and 251 in wild-type strains, generates a pair of 90- and 133-bp fragments. However, mutant DNA that lacks the site yields a single 223-bp fragment. The mixtures were incubated at 37°C for 2 h, and the fragments were separated on a 4% agarose gel. The gels were stained with ethidium bromide.

Direct sequencing. PCR-amplified DNA was directly sequenced by the dye terminator method in both the forward and the reverse directions. To remove the primers, electrophoresis was carried out on a 4% agarose gel. Portions containing the PCR products were cut from the gels, and the DNA fragments were electroeluted. The reaction was carried out in an automatic reaction apparatus, CATALYST (Applied Biosystems Japan) with 50 ng of DNA, 4.8 pmol of primers, and PRISM Ready Reaction Dye Terminator Cycle Sequencing Kit (Applied Biosystems Japan) according to the manufacturer's protocol. The products were automatically analyzed in an ABI model 373A DNA sequencer.

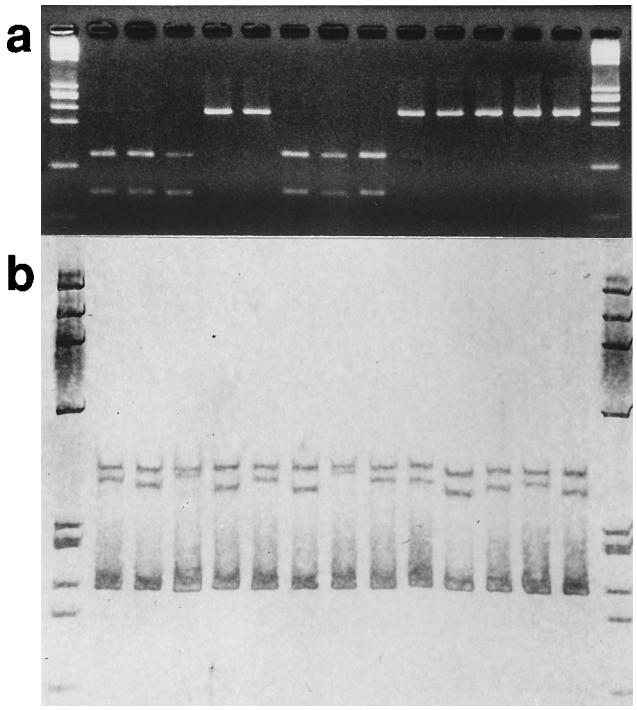

FIG. 1. (a) Agarose gel analysis of PCR-amplified DNA fragments digested with *Hin*fI. (b) Detection of *gyrA* mutations by SSCP analysis. Lane M, molecular size standard (ϕX174/*Hae*III digest); lane 1, wild type; lane 2, mutant with ATT→ATC at codon 86 (silent); lane 3, mutant with TTT→TTC at codon 110 (silent); lane 4, mutant with Ser-84→Leu; lane 5, mutant with Ser-84→Ala; lane 6, mutant with Ser-85→Pro; lane 7, mutant with Glu-88→Lys; lane 8, mutant with Glu-88→Gly; lane 9, mutant with Ser-84→Val; lane 10, mutant with Ser-84→Leu and Ser-85→Pro; lane 11, mutant with Ser-84→Leu and ATT→ATC at codon 86 (silent); lane 12, mutant with Ser-84→Leu and Glu-88→Lys; lane 13, Ser-84→Leu and Glu-88→Gly. Lanes 4 through 13, quinolone-resistant strains; lanes 6 and 10, strains generously donated by L. M. Fisher, namely, SA35 and SA147, respectively; lanes 12 and 13, mutants reported elsewhere (22), K-1 and 3-2(288-3), respectively.

RESULTS

Detection and analysis of polymorphism in the *gyrA* gene of *S. aureus*. The conditions of the SSCP analysis were optimized by using strains 209P, SA35, and SA146 and by changing pairs of primers and electrophoretic conditions, such as temperature and gel concentrations. Under the conditions provided, 451 clinically isolated strains and 4 other mutants reported previously yielded the expected PCR products and showed 10 different band patterns by SSCP analysis (Fig. 1b). If the banding pattern was distinct from others, PCR fragments from more than two strains were directly sequenced. The DNA sequences among the 10 patterns differed from each other except for the patterns for two pairs of mutants that were not well differentiated. The pairs were mutants with Ser-84→Leu plus Ile-86→Ile alterations and ones with a Ser-84→Val alteration and mutants with a Ser-84→Leu alteration and one with Ser-84→Leu and Glu-88→Gly alterations. These poor differentiations were solved by sequencing all 14 strains for the pattern for the first pair of mutants and by SSCP analysis under other conditions for the pattern for the second pair (22).

Table 1 shows the *gyrA* mutations in the 451 strains tested by SSCP and RFLP analyses. The incidence of GyrA mutants was 149 of 451 strains (33.0%). Among them, mutants with the Ser-84→Leu alteration were the highest in number; this was followed by mutants with Glu-88→Lys, Ser-84→Leu plus Ile-86→Ile, Ser-84→Val, Ser-84→Ala, and Glu-88→Gly alterations. Two types of silent mutants were also found; their DNA sequences were mutated at codons 86 and 110, but their GyrA sequences remained unchanged.

Susceptibilities of *gyrA* mutants to fluoroquinolones. Tables 2 and 3 show the susceptibilities of the 451 clinical strains and the 4 other mutants to 10 fluoroquinolones and methicillin.

Most of the strains with no GyrA mutation were susceptible to all fluoroquinolones tested. Among the drugs tested, CS-940

TABLE 1. Mutations in the *gyrA* gene in 451 *S. aureus* strains detected by SSCP and RFLP analyses

Codon(s)	Nucleotide mutation	Amino acid mutation	*Hin*fI[a]	No. of strains
84	TCA→TTA	Ser→Leu	−	96
	TCA→GCA	Ser→Ala	−	1
88	GAA→AAA	Glu→Lys	+	37
	GAA→GGA	Glu→Gly	+	1
84	TCA→GTA	Ser→Val	−	2
84 and 86	TCA→TTA, ATT→ATC	Ser→Leu, Ile (silent)	−	12
86	ATT→ATC	Ile (silent)	+	15
110	TTT→TTC	Phe (silent)	+	16
None			+	271

[a] +, *Hin*fI site present; −, *Hin*fI site absent.

TABLE 2. Susceptibilities of 447 *S. aureus* isolates with or without the GyrA mutation to 10 fluoroquinolones

Mutation(s), type of change (no. of strains)[a]	Drug	MIC (μg/ml)		
		Range	50%	90%
Wild or silent (302)	Norfloxacin	0.39–100	1.56	12.5
	Enoxacin	0.78–100	1.56	12.5
	Ofloxacin	0.39–6.25	0.78	1.56
	Ciprofloxacin	0.20–12.5	0.78	1.56
	Tosufloxacin	0.025–3.13	0.10	0.20
	Lomefloxacin	0.39–25	0.78	3.13
	Sparfloxacin	0.05–1.56	0.10	0.20
	Fleroxacin	0.39–25	0.78	3.13
	CS-940	0.025–0.78	0.10	0.20
	AM-1155	0.05–0.78	0.20	0.20
	Methicillin	0.39–3,200	3.13	1,600
Ser-84→Leu with or without silent mutation at Ile-86, P→lg NP (108)	Norfloxacin	50–1,600	100	1,600
	Enoxacin	12.5–>400	50	200
	Ofloxacin	6.25–200	25	100
	Ciprofloxacin	6.25–>800	50	400
	Tosufloxacin	1.56–>25	12.5	>25
	Lomefloxacin	25–400	100	200
	Sparfloxacin	6.25–50	12.5	25
	Fleroxacin	25–400	50	400
	CS-940	3.13–50	6.25	25
	AM-1155	1.56–25	6.25	12.5
	Methicillin	1.56–3,200	1,600	3,200
Glu-88→Lys, −1→+1 (37)	Norfloxacin	50–800	100	200
	Enoxacin	12.5–100	50	50
	Ofloxacin	6.25–25	12.5	25
	Ciprofloxacin	12.5–200	25	50
	Tosufloxacin	1.56–>25	6.25	25
	Lomefloxacin	25–200	50	100
	Sparfloxacin	3.13–6.25	6.25	6.25
	Fleroxacin	12.5–200	25	100
	CS-940	0.78–6.25	1.56	3.13
	AM-1155	1.56–6.25	3.13	3.13
	Methicillin	3.13–3,200	1,600	3,200

[a] P, polar; lg, large; NP, nonpolar; −1, negative charge; +1, positive charge.

was the most potent against them, inhibiting their growth when it was used at up to 0.78 μg/ml; this was followed by tosufloxacin and AM-1155. Norfloxacin and enoxacin MICs were greater than 12.5 μg/ml for approximately 12% of the strains.

When ofloxacin resistance was defined as an ofloxacin MIC of ≧6.25 μg/ml, the proportion of resistant strains was 8 of 302 (2.6%) for strains with no GyrA mutation. In contrast, the proportion was 147 of 149 (98.7%) for GyrA mutants, and it was significantly higher than that for strains with no GyrA mutation (χ^2 test, $P < 0.01$). Mutants with a Ser-84→Leu alteration showed high-level resistance to all 10 fluoroquinolones tested. The increases in the MICs that inhibited 50% of strains tested (MIC_{50}s) ranged from 32-fold with AM-1155 to 128-fold with lomefloxacin and sparfloxacin, in comparison

TABLE 3. Susceptibilities of seven isolates of *S. aureus* with other GyrA mutations

Drug	MIC (μg/ml) for strains with the following alterations (type of change)[a]:						
	Ser-84→Ala (P→NP)	Ser-85→Pro (P→NP)	Glu-88→Gly (−1→P)	Ser-84→Val (P→lg NP)	Ser-84→Leu and Ser-85→Pro	Ser-84→Leu and Glu-88→Lys	Ser-84→Leu and Glu-88→Gly
Norfloxacin	25	200	100	200	200	200	200
Enoxacin	25	100	50	100	100	100	100
Ofloxacin	3.13	6.25	3.13	12.5	12.5	200	200
Ciprofloxacin	6.25	50	12.5	50	50	100	100
Tosufloxacin	0.39	3.13	3.13	12.5	6.25	12.5	12.5
Lomefloxacin	6.25	50	25	100	100	200	200
Sparfloxacin	1.56	0.78	1.56	6.25	12.5	100	100
Fleroxacin	6.25	25	25	50	25	400	400
CS-940	0.78	1.56	0.78	6.25	12.5	400	200
AM-1155	0.78	1.56	0.78	6.25	6.25	100	50
Methicillin	1,600	50	3.13	1,600	50	800	1,600

[a] P, polar; NP, nonpolar; −1, negative charge; lg, large.

with the MIC_{50}s for susceptible strains. Mutants with a Glu-88→Lys alteration were also resistant to the 10 fluoroquinolones. The MIC_{50} of each fluoroquinolone rose 16- to 64-fold in comparison with the MIC_{50}s for susceptible strains. However, mutants of this type were one- to fourfold more susceptible to each fluoroquinolone than mutants with a Ser-84→Leu alteration, and few extremely highly resistant strains were in this group. Only CS-940 and AM-1155 were effective against mutants of this type. Although there were a few strains with Ser-84→Ala, Glu-88→Gly, and Ser-84→Val alterations, these *gyrA* mutants were also resistant to all of the fluoroquinolones tested by more than fourfold in comparison with the susceptible strains, among the GyrA mutants, MICs increased the least for a mutant with a Ser-84→Ala alteration. Mutants with double point mutations at Ser-84 and either Ser-85 or Glu-88 were more resistant than ones with a single point mutation, especially to newer fluoroquinolones.

When methicillin resistance was defined as an MIC of the drug of ≧12.5 μg/ml, the proportion of methicillin-resistant *S. aureus* was 133 of 149 (89.3%) among the GyrA mutants. In contrast, the proportion was 103 of 302 (34.1%) and was significantly lower (χ^2 test, $P < 0.01$) among strains with no GyrA mutation. The frequency of methicillin-resistant *S. aureus* was 36 of 37 (97.3%) among mutants with a Glu-88→Lys alteration, and it was significantly higher (χ^2 test, $P < 0.05$) than that among mutants with a Ser-84→Leu alteration and with Ser-84→Leu plus Ile-86 (silent) alterations (91 of 108; 84.3%). MICs of methicillin and those of each fluoroquinolone showed a low correlation coefficient (about 0.5) with every combination.

DISCUSSION

We attempted to detect *gyrA* point mutations in *S. aureus* by a new SSCP system and found that 10 band patterns could be differentiated. By combining *Hin*fI RFLP analysis with the SSCP analysis, two types (wild-type and a Ser-84→Ala alteration) could be differentiated, and each pattern corresponded to a distinct mutation, including three novel ones. The SSCP and RFLP analyses used in the present study, as well as one reported previously, was confirmed to be rapid and effective for detecting *gyrA* mutations in *S. aureus* strains.

Although SSCP analysis applied to bacteria (haploid) is theoretically supposed to bring about two bands, three bands were detected in every sample. The farthest-migrating band was considered to be the yield of reannealing of single-stranded DNA, as pointed out previously (22), and its molecular size was in accordance with the size of the aimed-for PCR fragment, 223 bp (Fig. 1b).

More than half of the fluoroquinolone-resistant *S. aureus* strains among the 451 strains used in the present study possessed a point mutation of a Ser-84→Leu alteration, as reported previously (6, 19, 22). This mutation was therefore considered to occur most frequently and to survive under clinical drug pressure. Point mutations were localized between codons 84 and 88, except for a silent mutation at codon 110, and they were closely related to fluoroquinolone resistance. The results support the fact that this restricted region would play a significant role in the quinolone-gyrase-DNA interaction, although a contribution of mutations in other regions of the *gyrA* gene was not completely ruled out.

Although strains with some single point mutations and ones with double mutations were rare, the following tendency was observed. Every type of GyrA mutant showed cross-resistance to all fluoroquinolones tested. GyrA mutants with a Ser-84→Leu or Ser-84→Val alteration showed the highest level of resistance to fluoroquinolones; a mutant containing a Glu-88→Lys alteration showed the second highest level of resistance. Mutations of Ser-84→Ala and Glu-88→Gly were associated with relatively low-level resistance. Strains with double point mutations developed higher levels of resistance than strains with a single point mutation; this was especially the case for resistance to newer fluoroquinolones. Thomson and Sanders (21) reported dissociated resistance to fluoroquinolones in *S. aureus*. Comparing the degree of increase in the MIC_{50}s of the fluoroquinolones tested by mutations, the deterioration of antibacterial activity was relatively small with ofloxacin, AM-1155, CS-940, and enoxacin; on the other hand, it was large with norfloxacin and lomefloxacin (Tables 2 and 3).

Shen et al. (18) proposed a cooperative quinolone-DNA binding model for the inhibition of DNA gyrase in which the binding of DNA gyrase to DNA creates the drug binding site at the active site of the enzyme: Tyr-122 in *E. coli* GyrA (9). Recently, Yoshida et al. (27) proposed a quinolone pocket model in which a quinolone-gyrase interaction is assumed. Because of the high degree of homology of DNA gyrases among bacterial species (2, 12), these models are probably applicable to *S. aureus*. Residues 84 to 88, which would be adjacent to Tyr-123 (this residue corresponds to Tyr-122 in *E. coli*), would be located in the pocket. There are two possible mechanisms for quinolone resistance by alterations of GyrA: one is a steric hindrance of quinolone entrance into the pocket by conformational change, and the other is perturbation of the quinolone-gyrase-DNA interaction by physicochemical changes. Although computer-aided prediction of the secondary structure of the DNA gyrase molecule by the method of Chou and Fasman (3) revealed that each mutation observed here would lead to minimal change on the whole enzyme (data not shown), these changes may be significant at the local site of quinolone binding. It is intriguing that substitution of Ser-84 (polar) was limited to nonpolar amino acids, i.e., Leu, Val, and Ala, and that the degree of resistance was well associated with the lengths of aliphatic chains and the hydrophobicities of these amino acids.

The MICs of a given fluoroquinolone sometimes varied 64-fold for strains with the same *gyrA* mutation. Diversity in the MICs of norfloxacin and enoxacin for strains with no GyrA mutation would be caused by another resistance mechanism, probably a *norA* mutation, and a similar percentage (approximately 12%) of the GyrA mutants would also possess other mechanisms of resistance. It is known that other genes and environmental factors are associated with DNA supercoiling in bacteria (4).

Clinical isolates have various genetic backgrounds. Elucidation of the definite relationship between types of GyrA mutations and the magnitude of resistance to quinolones requires use of syngeneic, spontaneous resistant mutants or the introduction of site-directed mutagenesis into the cloned *gyrA* gene. These approaches remain to be explained more fully.

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