

Detection of *grlA* and *gyrA* Mutations in 344 *Staphylococcus aureus* Strains

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Mutations in the *grlA* and *gyrA* genes of 344 clinical strains of *Staphylococcus aureus* isolated in 1994 in Japan were identified by combinations of single-strand conformation polymorphism analysis, restriction fragment length analysis, and direct sequencing to identify possible relationships to fluoroquinolone resistance. Five types of single-point mutations and four types of double mutations were observed in the *grlA* genes of 204 strains (59.3%). Four types of single-point mutations and four types of double mutations were found in the *gyrA* genes of 188 strains (54.7%). Among them, the *grlA* mutation of TCC→TTC or TAC (Ser-80→Phe or Tyr) and the *gyrA* mutation of TCA→TTA (Ser-84→Leu) were principal, being detected in 137 (39.8%) and 121 (35.9%) isolates, respectively. The *grlA* point mutations of CAT→CAC (His-77 [silent]), TCA→CCA (Ser-81→Pro), and ATA→ATT (Ile-100 [silent]) were novel, as was the GAC→GGC (Asp-73→Gly) change in *gyrA*. A total of 15 types of mutation combinations within both genes were related to ciprofloxacin resistance (MIC ≥ 3.13 µg/ml) and were present in 193 mutants (56.1%). Strains containing mutations in both genes were highly resistant to ciprofloxacin (MIC at which 50% of the isolates are inhibited [MIC₅₀] = 50 µg/ml). Those with the Ser-80→Phe or Tyr alteration in *grlA* but wild-type *gyrA* showed a lower level of ciprofloxacin resistance (MIC₅₀ ≤ 12.5 µg/ml). Levofloxacin was active against 68 of 193 isolates (35.2%) with mutations at codon 80 of *grlA* in the presence or absence of a concomitant mutation at codon 73, 84, or 88 in *gyrA* (MIC ≤ 6.25 µg/ml). The new fluoroquinolone DU-6859a showed good activity with 186 of 193 isolates (96.4%) for which the MIC was ≤ 6.25 µg/ml.

Staphylococcus aureus infections, particularly those caused by methicillin-resistant *S. aureus*, pose serious therapeutic difficulties, because few antimicrobial agents are effective against this pathogen. Fluoroquinolones, broad-spectrum and potent antimicrobial agents, have been effective in the treatment of these infections. With the increasing use of fluoroquinolones, resistance in *S. aureus*, especially methicillin-resistant *S. aureus* has become widespread in recent years (1, 12).

Three mechanisms involved in fluoroquinolone resistance have been proposed. One is topoisomerase IV gene mutations (4, 18), a second is DNA gyrase gene mutations (13, 16), and a third is an active efflux pump (8, 19). Recent studies have demonstrated that the primary target of fluoroquinolones in *S. aureus* is DNA topoisomerase IV, which is composed of the GrIA and GrIB subunits, encoded by the *grlA* and *grlB* genes,

respectively. DNA gyrase is considered a secondary target (3, 4, 10, 15). In *S. aureus* clinical strains, mutations in either the *grlA* or *gyrA* gene lead to quinolone resistance. *grlA* mutations are associated with both high- and low-level resistances, while *gyrA* mutations are responsible for increases in ciprofloxacin (CPFX) resistance in *grlA* mutants. A combination of mutations in both genes can cause high-level quinolone resistance (4, 5, 13, 15, 16, 18).

Several methods have been used for detection of point mutations of genes. Single-strand conformation polymorphism (SSCP) analysis is a rapid, simple, and effective method in which a mutated sequence is detected by a change in mobility during polyacrylamide gel electrophoresis caused by its altered folded structure (6). It has been applied in detection of DNA gyrase gene mutations in *S. aureus* (14, 17) and *Escherichia coli*

TABLE 1. Mutations in the *grlA* gene in 344 *S. aureus* strains

Codon(s)	Mutation(s)	No. of isolates	Method(s)
77	His [silent] (CAT→CAC)	7	SSCP, sequencing
80	Ser→Phe (TCC→TTC)	94	SSCP
	Ser→Tyr (TCC→TAC)	43	RFLP, sequencing
84	Glu→Lys (GAA→AAA)	5	RFLP
100	Ile [silent] (ATA→ATT)	4	SSCP, sequencing
80 + 81	Ser→Phe (TCC→TTC), Ser→Pro (TCA→CCA)	1	SSCP, sequencing
80 + 84	Ser→Phe (TCC→TTC), Glu→Lys (GAA→AAA)	8	SSCP, sequencing
	Ser→Tyr (TCC→TAC), Glu→Lys (GAA→AAA)	41	RFLP, sequencing
81 + 84	Ser→Pro (TCA→CCA), Glu→Lys (GAA→AAA)	1	SSCP, sequencing
None		140	SSCP

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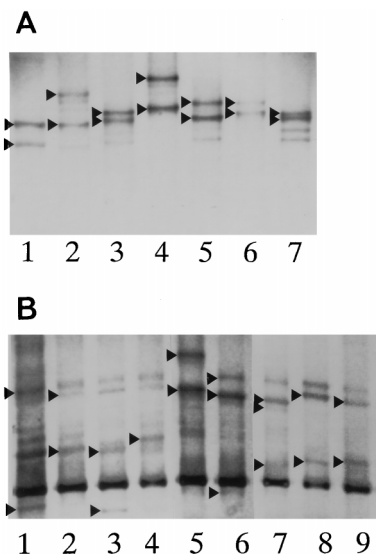


FIG. 1. Detection of mutations in the *grlA* gene (A) and the *gyrA* gene (B) by SSCP analysis. Mutations in the genes cause amino acid changes as follows. (A) Lane 1, His-77 (CAT→CAC [silent]); lane 2, Ser-80→Phe; lane 3, Ile-100 (ATA→ATT [silent]); lane 4, Ser-80→Phe and Ser-81→Pro; lane 5, Ser-80→Phe and Glu-84→Lys; lane 6, Ser-81→Pro and Glu-84→Lys; lane 7, none (wild type). (B) Lane 1, none (wild type); lane 2, Ser-84→Leu; lane 3, Ser-84→Leu and Ile-86 (ATT→ATC [silent]); lane 4, Ile-86 (silent); lane 5, Ser-84→Leu and Ser-85→Pro; lane 6, Asp-73→Gly; lane 7, Asp-73→Gly and Ser-84→Leu; lane 8, Ser-84→Leu and Glu-88→Lys; lane 9, Glu-88→Lys. Arrowheads indicate the bands used to distinguish differences.

(11). In SSCP analysis in our study, proper conditions and a new system were used for detection of *grlA* and *gyrA* mutations. By using combinations of SSCP analysis, restriction fragment length polymorphism (RFLP) analysis, and direct sequencing, we examined *grlA* and *gyrA* mutations in 344 *S. aureus* strains and studied the relationship between combinations of mutations of both genes and susceptibility of the various mutants to three fluoroquinolones.

MATERIALS AND METHODS

Antimicrobial agents and bacterial strains. CPF, levofloxacin (LVFX), and DU-6859a were synthesized at the New Product Research Laboratories I, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan. A total of 344 clinical *S. aureus* strains (one strain per patient) were collected by LVFX surveillance groups from 24 hospitals all over Japan. These were isolated from June to November 1994 and included 215 methicillin-resistant (32 CPF-resistant and 183 CPF-resistant) and 129 methicillin-susceptible (122 CPF-resistant and 7 CPF-resistant) strains. *S. aureus* FDA 209-P, 891185 (Ser-80→Phe), and 900165 (Glu-84→Lys) were used as controls for detection of *grlA* mutations, while FDA 209-P, 900165 (Ser-84→Leu), 6859a-r (Glu-88→Lys), 87-53 (Ile-86 [silent]), and 87-20 (Ser-84→Leu, Ile-86 [silent]) were used for detection of *gyrA* mutations (15, 16).

Determination of MICs. The MICs were determined by standard agar dilution methods (9) with Mueller-Hinton agar (Difco, Detroit, Mich.). Drug-containing agar plates were incubated with one loopful (5 μ l) of an inoculum corresponding to about 10^4 CFU per spot and were incubated at 37°C for 18 h. The MIC was defined as the lowest drug concentration which prevented visible growth of bacteria.

PCR experiments. Chromosomal DNA was prepared from 2 μ l of an overnight culture heated at 98°C for 5 min. PCR was performed with cycling at 94°C for 30 s, 52°C for 30 s, and 70°C for 1 min for 30 cycles by using 2.5 U of recombinant *Taq* DNA polymerase (Takara, Shiga, Japan). Oligonucleotides 5'-TTCCGTA AAAGTGC GAAAACAG (nucleotides 178 to 199) and 5'-CGCATTGCCGCT GGCGGATCCTTATCGATAC (complementary to nucleotides 323 to 353) were used for amplification of a 176-bp *grlA* fragment. For *gyrA* gene amplification, 5'-CATATAAAAAATCAGCAGCATCGTT (nucleotides 188 to 213) was used as the sense primer, and 5'-TGAGCCATACGTACCATTGC (complementary to nucleotides 265 to 284) (97-bp fragment) and 5'-CGCCATCTC CATCCATTGAACCAAA (complementary to nucleotides 328 to 352) (165-bp fragment) were used as antisense primers for SSCP analysis and direct sequencing, respectively. The amplified products were checked by agarose gel electrophoresis.

SSCP analysis. PCR products were mixed with denaturant solution (95% formamide, 20 mM EDTA [pH 8.0], 0.05% bromophenol blue, 0.05% xylene cyanol). The mixtures were heated at 80°C for 5 min and then were cooled on ice immediately. Samples were then separated by 12.5% polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) electrophoresis at 17°C, and DNA bands were visualized by using a silver stain kit (Daiichi Pure Chemicals).

RFLP analysis. For detection of mutations in the *grlA* gene, we used restriction endonuclease *Hinf*I (Toyobo, Tokyo, Japan) for the Ser-80→Tyr alteration and *Bsr*GI (New England Biolabs, Beverly, Mass.) for the Glu-84→Lys alteration. The reaction mixtures (1 U of enzyme added to 10 μ l of each PCR product) were incubated at 37°C for more than 1 h, and the sizes of the resulting fragments were ascertained by agarose gel electrophoresis.

DNA sequencing. The PCR-amplified DNA from *grlA* was cloned into the vector pGEM-T (Promega, Madison, Wis.) and sequenced with an ALFred DNA sequencer (Pharmacia). The amplified fragment from *gyrA* was sequenced directly with an AutoLoad solid-phase sequencing kit (Pharmacia).

RESULTS

Detection of mutations in the *grlA* gene. By using a combination of SSCP, RFLP analysis, and sequencing, five single-point mutations and four double mutations were observed in the *grlA* gene in 204 *S. aureus* strains (59.3%) (Table 1). Among them, the TCC→TTC or TAC (Ser-80→Phe or Tyr) single-point mutation was principal; it was detected in 137 isolates (39.8%). The double mutation of TCC→TTC or TAC and GAA→AAA (Ser-80→Phe or Tyr and Glu-84→Lys) was found in 49 mutants (14.2%). The CAT→CAC (His-77) and ATA→ATT (Ile-100) single-point mutations were silent mutations, i.e., they led to no amino acid alteration. The TCA→CCA (Ser-81→Pro) was a novel point mutation in the *grlA* gene. Double-codon alterations of Ser-80→Tyr and Glu-84→Lys, Ser-80→Phe and Ser-81→Pro, and Ser-81→Pro and Glu-84→Lys were also novel *grlA* mutations and were identified in 41, 1, and 1 isolates, respectively. As shown in Fig. 1A, six of nine types of *grlA* mutations were distinguishable from the wild type by SSCP analysis.

Detection of mutations in the *gyrA* gene. As shown in Table 2, 188 *S. aureus* strains (54.7%) contained mutations in the

TABLE 2. Mutations in the *gyrA* gene in 344 *S. aureus* strains

Codon(s)	Mutation(s)	No. of isolates	Method(s)
73	Asp→Gly (GAC→GGC)	1	SSCP, sequencing
84	Ser→Leu (TCA→TTA)	121	SSCP
86	Ile [silent] (ATT→ATC)	8	SSCP
88	Glu→Lys (GAA→AAA)	36	SSCP
84 + 73	Ser→Leu (TCA→TTA), Asp→Gly (GAC→GGC)	3	SSCP, sequencing
84 + 85	Ser→Leu (TCA→TTA), Ser→Pro (TCT→CCT)	6	SSCP, sequencing
84 + 86	Ser→Leu (TCA→TTA), Ile [silent] (ATT→ATC)	9	SSCP
84 + 88	Ser→Leu (TCA→TTA), Glu→Lys (GAA→AAA)	4	SSCP, sequencing
None		156	SSCP

TABLE 3. Susceptibilities of 344 *S. aureus* strains with or without mutations in the *grlA* and *gyrA* genes

Codon(s)		No. of strains	Drug	MIC ($\mu\text{g/ml}$)		
<i>grlA</i>	<i>gyrA</i>			Range	50%	90%
80	73	1	CPFX LVFX DU-6859a	6.25 1.56 0.2		
80	84	75	CPFX LVFX DU-6859a	12.5–800 3.13–50 0.2–6.25	25 12.5 0.78	100 25 1.56
80	88	35	CPFX LVFX DU-6859a	12.5–100 3.13–12.5 0.2–0.78	25 6.25 0.39	100 12.5 0.78
80		13	CPFX LVFX DU-6859a	3.13–12.5 0.39–6.25 0.05–0.39	12.5 3.13 0.2	12.5 3.13 0.39
80	73 + 84	2	CPFX LVFX DU-6859a	50 12.5 0.78		
80	84 + 86	8	CPFX LVFX DU-6859a	50–100 12.5–25 0.78–1.56		
80	84 + 88	3	CPFX LVFX DU-6859a	50–800 12.5–50 0.78–3.13		
84	84	4	CPFX LVFX DU-6859a	100–800 25–100 1.56–6.25		
84	84 + 88	1	CPFX LVFX DU-6859a	800 400 50		
80 + 84	84	41	CPFX LVFX DU-6859a	100–>800 25–100 0.78–6.25	800 50 6.25	800 100 6.25
80 + 84	73 + 84	1	CPFX LVFX DU-6859a	200 50 1.56		
80 + 84	84 + 85	6	CPFX LVFX DU-6859a	\cong 800 \cong 800 25		
80 + 84	84 + 86	1	CPFX LVFX DU-6859a	200 50 3.13		
80 + 81	88	1	CPFX LVFX DU-6859a	100 12.5 0.78		
81 + 84	84	1	CPFX LVFX DU-6859a	200 50 3.13		
77 (silent)		7	CPFX LVFX DU-6859a	0.2–1.56 0.2–0.39 0.025–0.05		
100 (silent)		4	CPFX LVFX DU-6859a	0.39 0.2 0.025–0.05		
	86 (silent)	8	CPFX LVFX DU-6859a	0.39–3.13 0.2–0.78 0.025–0.1		
		132	CPFX LVFX DU-6859a	0.2–3.13 0.1–0.78 0.013–0.1	0.78 0.39 0.05	1.56 0.39 0.05

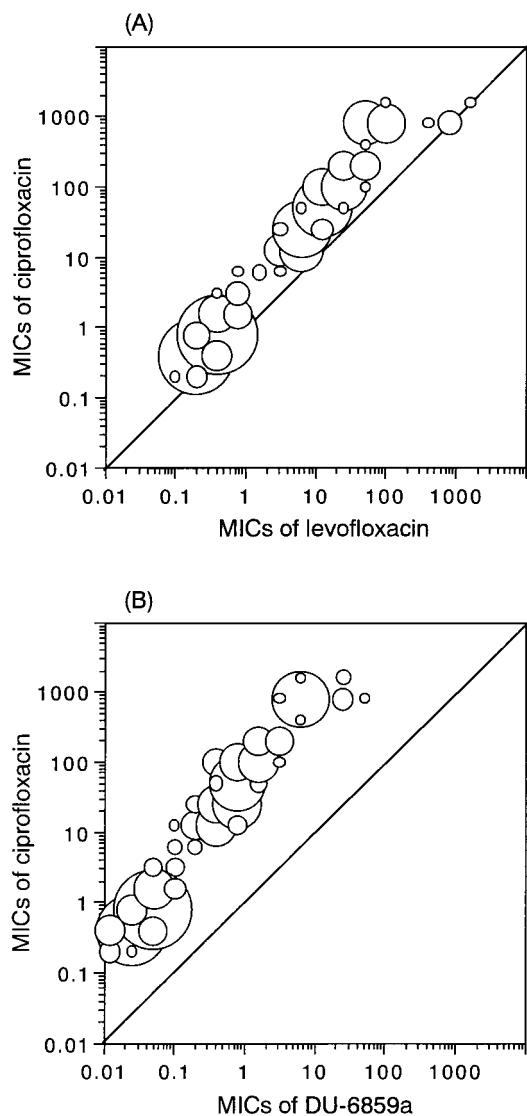


FIG. 2. Distribution of the MICs of LVFX and DU-6859a versus the MICs of ciprofloxacin. The sizes of the circles reflect the numbers of strains.

gyrA gene. By SSCP analysis and direct sequencing, four types of single-point mutations and four types of double mutations were detected at codons 73, 84, 86 (silent), 88, 84 and 73, 84 and 85, 84 and 86, and 84 and 88. The single-point mutations of TCA→TTA (Ser-84→Leu) and GAA→AAA (Glu-88→Lys) were principal; they were found in 121 (35.0%) and 36 (10.5%) isolates, respectively. The GAC→GGC (Asp-73→Gly) change was a novel point mutation in the *gyrA* gene. The eight types of *gyrA* mutants and the wild type had distinct SSCP patterns (Fig. 1B); however, the SSCP patterns in lanes 6 (alteration at codon 73) and 7 (alterations at codons 73 and 84) of Fig. 1B were similar, so the mutations of four strains were confirmed by sequencing. There was no strain which possessed amino acid changes in GyrA in the absence of a *gla* mutation.

Susceptibilities of mutants to quinolones. A total of 19 mutation combinations were found in the *gla* and *gyrA* genes of the 344 *S. aureus* strains (Table 3). Isolates with *gla-gyrA* mutation combinations of codon 77–none, codon 100–none, and none–codon 86 were found to have quinolone susceptibilities similar to that of the wild type, because these silent mu-

tations are not responsible for quinolone resistance. The other 15 types observed in 193 mutants (56.1%) were related to CPFV resistance (MIC \geq 3.13 μ g/ml). Overall, those strains mutated in both genes showed higher-level CPFV resistance. The MIC at which 50% of the isolates are inhibited (MIC₅₀) and MIC₉₀ were 50 and 800 μ g/ml, respectively. Among such strains, those with combined mutations at codons 84–84, 84–84 plus 86, 80 plus 84–84, 80 plus 84–73 plus 84, 80 plus 84–84 plus 86, 80 plus 84–84 plus 85, and 81 plus 84–84 were highly resistant to CPFV (MIC \geq 100 μ g/ml). The combination of the Ser-80→Phe or Tyr alteration in *gla* but wild-type *gyrA* was present in strains with lower-level resistance to CPFV. The MIC range, MIC₅₀, and MIC₉₀ for these strains were 3.13 to 12.5, 12.5, and 12.5 μ g/ml, respectively.

With respect to LVFX and DU-6859a, the distribution of susceptibilities of mutants had a tendency similar to that of susceptibility to CPFV (Fig. 2). LVFX was active against 68 of 193 mutants (35.2%) which contained alterations codon as codon 80–73, 80–84, 80–88, or 80–none (MIC of LVFX \leq 6.25 μ g/ml). The other mutants were moderately or highly resistant to LVFX. DU-6859a had good activity against 120 of the mutants (62.2%; MIC of DU-6859a \leq 0.78 μ g/ml). The MICs of DU-6859a for 66 of the mutants (34.2%) were from 1.56 to 6.25 μ g/ml. Six mutants with the codon 80 plus 84–84 plus 85 alterations and one with the codon 84–84 plus 88 alterations were highly resistant to DU-6859a (MIC \geq 25 μ g/ml).

DISCUSSION

In clinical *S. aureus* strains, five types of *gla* mutations (causing Ser-80→Phe, Ser-80→Tyr, Glu-84→Lys, Glu-84→Gln, and Ser-80→Phe plus Glu-84→Lys changes) were demonstrated to be responsible for quinolone resistance (2–4, 10, 15, 18). In *gyrA*, 10 types of mutations (causing Ser-84→Leu, Ser-84→Ala, Ser-84→Val, Ser-85→Pro, Glu-88→Lys, Glu-88→Gly, Ser-84→Leu plus Ser-85→Pro, Ser-84→Leu plus Ile-86 [silent], Ser-84→Leu plus Glu-88→Lys, and Ser-84→Leu plus Glu-88→Gly changes) were found in strains with high-level quinolone resistance (5, 7, 13–17).

In this study, we examined 344 clinical isolates of *S. aureus* by a combination of SSCP, RFLP analysis, and direct sequencing and found nine types of mutations in *gla* and eight types in *gyrA*. Among them, five types in *gla* and two types in *gyrA* were novel. The *gla* mutation at codon 80 and the *gyrA* mutation at codon 84 were principal, being found in 137 of 204 *Gla* mutants (67.2%) and 121 of 188 *GyrA* mutants (64.4%), respectively. This result is consistent with findings reported for clinical isolates (4, 14). *gla* mutations at codons 80 and 84 and *gyrA* mutations at codons 84, 85, and 88 were detected in the CPFV-resistant strains (MIC \geq 3.13 μ g/ml), which is in agreement with other reports that these point mutations are responsible for quinolone resistance (5, 18). Further study is necessary to determine whether the Ser-81→Pro alteration encoded by *gla* and the Asp-73→Gly alteration encoded by *gyrA* directly contributed to quinolone resistance.

Fifteen types of mutation combinations of both genes observed in this investigation were related to CPFV resistance. Among them, 13 mutants with lower-level CPFV resistance contained a *gla* mutation at codon 80 but no mutation in *gyrA*. This supports the notion that in *S. aureus* topoisomerase IV is a primary target of fluoroquinolones. The finding that *gla-gyrA* double mutants exhibited higher-level CPFV resistance (MIC₅₀ = 50 μ g/ml) confirmed previous genetic studies (10, 18). The combinations of mutations including the Glu-84→Lys alteration encoded by *gla* and the Ser-84→Leu alteration

encoded by *gyrA* conferred high-level quinolone resistance (MIC of CPF_X \geq 100 μ g/ml).

Diversity in the MICs of fluoroquinolones for strains with same mutation combination suggests that the strains may possess other mechanisms of resistance. Some strains with no mutations in both genes or with a silent mutation in either gene showed low-level CPF_X resistance (MIC, 1.56 to 3.13 μ g/ml), which suggests that other resistance mechanisms are present. In this study, the alteration at codon 116 in the sequence encoding GrlA, which considered a DNA binding site (10), was not checked because the length of the DNA fragment was limited for detection by SSCP analysis. The amino acid change in the remaining region of GrlA and GyrA, alteration in GrlB and GyrB, and the association of the quinolone efflux system were possibly the cause of the diversity in the MICs. The numerous and complicated mutations seen may explain the rapid and widespread development of quinolone resistance described for *S. aureus*. DU-6859a showed good activity against CPF_X- or LVFX-resistant mutants because of its high inhibitory activity against both topoisomerase IV and DNA gyrase as demonstrated by Tanaka et al. (15).

In this study, SSCP analysis was found to be a rapid, simple, and effective method for detection of point mutations in both the *grlA* gene and the *gyrA* gene of *S. aureus* strains. The phenomenon that one strand could be separated into two bands due to different stable conformations (6) was observed as well under our SSCP conditions.

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