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Characterization of *grlA*, *grlB*, *gyrA*, and *gyrB* Mutations in 116 Unrelated Isolates of *Staphylococcus aureus* and Effects of Mutations on Ciprofloxacin MIC

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One hundred sixteen unrelated clinical isolates of *Staphylococcus aureus* (70 ciprofloxacin resistant and 46 ciprofloxacin susceptible) from eight countries were studied for the presence of mutations in the *grlA*, *grlB*, *gyrA*, and *gyrB* gene loci. Two mutations within *grlA* (located at codons 80 and 84) and two mutations within *gyrA* (located at codons 84 and 88) were clearly associated with ciprofloxacin resistance, although other mutations detected within the four genes studied may also contribute to decreased susceptibility.

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are a therapeutic challenge due to multiple antibiotic resistance (10). Fluoroquinolones (FQs), of which ciprofloxacin is the most widely used, are broad-spectrum antibiotics with good activity against gram-positive organisms, including both methicillin-sensitive *S. aureus* and MRSA. The widespread use of FQs has led to the emergence of FQ-resistant *S. aureus*, especially among MRSA strains (5). Mutations within *norA* (20), *gyrA*, *gyrB* (6, 8, 18), and *grlA* (3, 13, 19) have been shown to be associated with FQ resistance in *S. aureus*. *norA* encodes a membrane protein which acts as a efflux pump (9, 14). *gyrA* and *gyrB* encode subunits of DNA gyrase (2, 11). Strains with mutations in *gyrA* and *gyrB*, but without *grlA* mutations, which confer high-level FQ resistance, can be isolated by single-step selection with FQs for *Escherichia coli* but not for *S. aureus* (3), although *gyrA* mutations have been detected in FQ-resistant clinical isolates of *S. aureus* (6, 8, 18). *grlA* and *grlB* encode the structural proteins of DNA topoisomerase IV (4), and mutations in the *grlA* gene, with or without *gyrA* mutations, have been described for FQ-resistant *S. aureus* strains (3, 17). Genetic and biochemical evidence suggests that the primary target site of ciprofloxacin, and probably of other FQs, in *S. aureus* is DNA topoisomerase IV (1, 7, 13) and not DNA gyrase, as in *E. coli* and *Neisseria gonorrhoeae.*

This study aimed to characterize mutations in *grlA*, *grlB*, *gyrA*, and *gyrB* of 116 unrelated *S. aureus* isolates derived from eight countries and to correlate the effects of mutations or combinations of mutations within these genes with ciprofloxacin MICs.

Ninety-three MRSA isolates (67 ciprofloxacin resistant and 26 ciprofloxacin susceptible) and 23 methicillin susceptible *S. aureus* (MSSA) isolates (3 ciprofloxacin resistant and 20 ciprofloxacin susceptible) were included in this study. Eighty *S. aureus* isolates from patients residing in Germany, collected between 1990 and 1995, and 36 *S. aureus* isolates from seven other countries (8 from Japan, 8 from Brazil, 6 from Switzerland, 4 from Sri Lanka, 4 from Spain, 3 from the United Kingdom, and 3 from Hungary), collected between 1983 and 1989, were tested. All 116 clinical isolates from different patients were screened for the presence of the *mecA* and *coa* genes by multiplex PCR (16). All isolates were selected on the basis of belonging to different pulsed-field gel electrophoresis types (15).

Ciprofloxacin MICs were derived by using a broth microdilution method according to guidelines recommended by the National Committee for Clinical Laboratory Standards (12).

Based on published sequences for *grlA* and *grlB* (19) and *gyrA* and *gyrB* (8), the appropriate oligonucleotide primers were selected as follows: for *grlA*, the 5′ primer 2402-ACTTGAAG ATGTTTTAGGTGAT-2423 and the 3′ primer 2942-TTAGG AAATCTTGATGGCAA-2961; for *grlB*, the 5′ primer 1520-C GATTAAAGCACAACAAGCAAG-1541 and the 3′ primer 1874-CATCAGTCATAATAATTACTC-1894; for *gyrA*, the 5′ primer 2311-AATGAACAAGGTATGACACC-2330 and the 3′ primer 2514-TACGCGCTTCAGTATAACGC-2533; and for *gyrB*, the 5′ primer 1400-CAGCGTTAGATGTAGCAAGC-1419 and the 3′ primer 1631-CCGATTCCTGTACCAAATG C-1650.

Four independent PCR amplifications were carried out with a GeneAmp PCR System 2400 (Perkin-Elmer, Weiterstadt, Germany), and all reagents (GeneAmp deoxynucleoside triphosphates, high-fidelity *Taq* DNA polymerase, and 10 × PCR buffer) were purchased from Perkin-Elmer or Boehringer Mannheim (Mannheim, Germany). To prepare cell lysates for use as template DNA in PCR, approximately 1/10 of a single bacterial colony was picked with a pipette tip and mixed in the PCR amplification mixture, consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 100 μM deoxynucleoside triphosphates, 3 U of high-fidelity *Taq* DNA polymerase, and 0.4 μM primers in a final volume of 50 μl. Samples were denatured at 94°C for 10 min, followed by 25 amplification cycles with the following parameters: 94°C for 20 s, 55°C for 20 s, and 72°C for 50 s. A final cycle of 72°C for 5 min was used to fully extend amplicons.

PCR products were purified with a PCR purification kit (Qiagen, Hilden, Germany). PCR-amplified DNA was sequenced by the dye terminator method in both the forward and reverse

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TABLE 1. Mutations within the *grlA*, *grlB*, *gyrA*, and *gyrB* genes in 116 clonally unrelated clinical isolates of *S. aureus* from eight countries

Gene and base position(s) (change[s])[a]	Amino acid change	No. of strains			
		Ciprofloxacin susceptible	Ciprofloxacin resistant	MSSA	MRSA
grlA					
2495 (A→C)*	None	2	0	2	0
2614 (C→T)	Ser-80→Phe	1	60	1	60
2651 (C→T)*	None	3	0	3	0
2756 (A→G)*	None	1	0	0	1
2614 (A→C); 2626 (A→T)*	Ser-80→Phe; Glu-84→Val	0	4	0	4
2614 (A→C); 2751 (T→C)*	Ser-80→Phe; none	0	1	0	1
2517 (G→A); 2614 (C→T)*	Ala-48→Thr; Ser-80→Phe	0	4	1	3
2510 (T→G); 2516 (T→C); 2606 (T→C)*	Ile-45→Met; none; none	2	0	2	0
2714 (G→A); 2798 (T→C)*; 2805 (C→T); 2825 (A→G)*	None; none; Pro-144→Ser; none	4	0	2	2
2510 (T→G); 2516 (T→C)*; 2606 (T→C); 2614 (C→T)*	Ile-45→Met; none; none; Ser-80→Phe	1	0	1	0
2497 (T→G); 2510 (T→G); 2516 (T→C)*; 2606 (T→C); 2614 (C→T)*	Val-41→Gly; Ile-45→Met; none; none; Ser-80→Phe	0	1	1	0
No mutations		32	0	10	22
Total		46	70	23	93
grlB					
1644 (C→T)*	None	1	0	1	0
1650 (A→T)*	Glu-422→Asp	1	0	1	0
1677 (T→C)*	None	1	6	0	7
1679 (A→G)*	Asp-432→Gly	0	1	0	1
1735 (C→T)*	Pro-451→Ser	0	1	0	1
1866 (T→C)*	None	1	0	0	1
1650 (A→T); 1776 (A→G); 1866 (T→C)*	Glu-422→Asp; none; none	1	0	1	0
1644 (C→T); 1650 (A→T); 1710 (A→T)*; 1770 (A→T); 1866 (T→C)*	None; Glu-422→Asp; none; none; none	3	1	4	0
1644 (C→T); 1647 (T→A); 1650 (A→T)*; 1776 (A→G); 1866 (T→C)*	None; none; Glu-422→Asp; none; none	3	0	2	1
No mutations		35	61	14	82
Total		46	70	23	93
gyrA					
2402 (C→T)	Ser-84→Leu	0	43	1	42
2409 (T→C)	None	16	0	3	13
2413 (G→A)	Glu-88→Lys	1	0	1	0
2481 (T→C)	None	2	1	3	0
2487 (A→G)	Ser-112→Arg	1	0	1	0
2402 (C→T); 2409 (T→C)	Ser-84→Leu; none	0	8	1	7
2402 (C→T); 2481 (T→C)*	Ser-84→Leu; none	0	1	1	0
2409 (T→C); 2413 (G→A)*	None; Glu-88→Lys	0	16	0	16
2402 (C→T); 2409 (T→C); 2468 (G→A)*	Ser-84→Leu; none; Gly-106→Asp	0	1	0	1
No mutations		26	0	12	14
Total		46	70	23	93
gyrB					
1534 (G→A)	None	2	0	2	0
1579 (A→G)*	None	16	26	7	35
1525 (C→T); 1579 (A→G)*	None; none	1	0	0	1
1474 (T→A); 1498 (C→T)*	None; none	1	0	1	0
No mutations		26	44	13	57
Total		46	70	23	93

[a] *, new single point mutations or combinations of single point mutations.

directions. The reaction was carried out with 50 ng of DNA and 0.1 μmol of primers, by using a Ready Reaction Dye Terminator Cycle Sequencing Kit (Perkin-Elmer) according to the manufacturer's instructions. The products were resolved and automatically analyzed with a 310 DNA sequencer (Perkin-Elmer).

Sequence data from codon 16 to codon 189 of the *grlA* gene, from codon 386 to codon 497 of the *grlB* gene, from codon 70 to codon 121 of the *gyrA* gene, and from codon 413 to codon 483 of the *gyrB* gene were obtained for further analysis. Wild-type sequences with no mutations were identified on the basis of being identical to the published sequences of *grlA* and *grlB* (19) and *gyrA* and *gyrB* (8). Mutations in these genes were identified by comparison.

The mutations identified are summarized in Table 1. Within the *grlA* gene, 11 single or combination mutations were found in 84 isolates; within the *grlB* gene, 9 single or combination mutations were located in 20 isolates; within the *gyrA* gene, 9 single or combination mutations were found in 90 isolates; and within the *gyrB* gene, 4 single or combination mutations were found in 46 isolates.

The effect of the three amino acid changes within *grlB* (Table 2) is unclear, as the MICs of ciprofloxacin for several isolates without these mutations are lower (Table 2).

TABLE 2. Amino acid changes encoded by mutations in the *grlA*, *grlB*, and *gyrA* gene loci[a] and corresponding ciprofloxacin MICs

Amino acid change(s)			No. of strains with the following ciprofloxacin MIC (μg/ml):													Total no. of strains
GrlA	GrlB	GyrA	≤0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	
—[b]	—	—		11	15	6	3	1								36
	—	Ser-112→Arg			1											1
	Glu-422→Asp	Glu-88→Lys					1									1
Ile-45→Met	Glu-422→Asp	—				2										2
Pro-144→Ser	—	—			1											1
	Glu-422→Asp	—			1	1		1								3
Ser-80→Phe	—	—						1								1
	—	Ser-84→Leu								4	22	13	7	1		47
	—	Glu-88→Lys								1	9	2				12
	—	Ser-84→Leu; Gly-106→Asp									1					1
	Asp-432→Gly	Ser-84→Leu									1					1
Ser-80→Phe; Glu-84→Val	—	Glu-88→Lys											1	2	1	4
Ser-80→Phe; Ala-48→Thr	—	Ser-84→Leu											2		1	3
	Pro-451→Ser	Ser-84→Leu													1	1
Ser-80→Phe; Ile-45→Met	Glu-422→Asp	—						1								1
Ser-80→Phe; Val-41→Gly; Ile-45→Met	Glu-422→Asp	Ser-84→Leu									1					1

[a] No amino acid changes encoded by mutations in the *gyrB* gene have been observed.
[b] —, no amino acid change.

From the correlation of the characterized mutations with the resulting MICs of ciprofloxacin (Table 2), it is clear that all the isolates studied that do not have the *grlA* mutation Ser-80→Phe are ciprofloxacin susceptible.

All ciprofloxacin-resistant isolates for which MICs were ≥4 μg/ml had the *grlA* mutation Ser-80→Phe in combination with either a Ser-84→Leu mutation or a Glu-88→Lys mutation within the *gyrA* gene.

In two isolates a Ser-80→Phe mutation was combined with no mutations in the *gyrA* gene, resulting in a ciprofloxacin MIC of 2 μg/ml, which, while elevated from a wild-type level, is still below the breakpoint for resistance. These data support the finding that in *S. aureus*, *grlA* mutations precede *gyrA* mutations in the development of resistance to ciprofloxacin (3). However, in contrast, two isolates, each of which had a single mutation in the *gyrA* gene without a corresponding Ser-80→Phe mutation in *grlA*, were associated with MICs of ciprofloxacin of 0.25 and 1 μg/ml.

Combinations of single point mutations within the *gyrA* gene have been shown to be associated with higher ciprofloxacin MICs than single point mutations (18). Similarly, two combinations of single point mutations within *grlA*, a Glu-84→Val or an Ala-48→Thr mutation in combination with a Ser-80→Phe mutation, were associated with relatively higher ciprofloxacin MICs (range, 64 to 256 μg/ml) than only a single Ser-80→Phe mutation (range, 8 to 128 μg/ml) (Table 2).

However, other factors have some effect on ciprofloxacin resistance, as evidenced, for example, by the fact that in the 47 isolates with a single *grlA* mutation (Ser-80→Phe) in combination with the Ser-84→Leu mutation in *gyrA*, 16-fold differences in ciprofloxacin MICs occurred. This implicates additional resistance mechanisms associated with elevated MICs of ciprofloxacin (1, 9, 14).

In summary, our data support previous findings and provide evidence that two mutations within the *grlA* gene (located at codons 80 and 84), as well as two mutations within the *gyrA* gene (located at codons 84 and 88), are clearly associated with the development of ciprofloxacin resistance. From 116 unrelated isolates we have found 9 combinations of amino acid changes within in GrlA, GrlB, and GyrA associated with resistance to ciprofloxacin. However, some mutations reported by previous workers (3, 6, 8, 17–19) as associated with ciprofloxacin resistance were not found among these isolates, suggesting that other, unknown mutations are likely to exist. The association of mutations within *grlA* at codon 48, as well as that of polymorphisms in *grlB* and *gyrB*, with increased ciprofloxacin MICs is not known. Sequence data from unrelated clones of *S. aureus* isolated from different countries show that some *grlA* and *gyrA* mutations are conserved in both MRSA and MSSA.

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