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Analysis of *gyrA* and *grlA* Mutations in Stepwise-Selected Ciprofloxacin-Resistant Mutants of *Staphylococcus aureus*

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Fluoroquinolone-resistant mutants were obtained in vitro from *Staphylococcus aureus* RN4220 by stepwise selection on increasing concentrations of ciprofloxacin. Results from sequence analysis of the quinolone resistance-determining region of GyrA and of the corresponding region of GrlA, the DNA topoisomerase IV subunit, showed an alteration of Ser-80 to Tyr (corresponding to Ser-83 of *Escherichia coli* GyrA) or Glu-84 to Lys in GrlA of both low- and high-level quinolone-resistant mutants. Second-step mutants were found to have, in addition to a mutation in *grlA*, reduced accumulation of norfloxacin or an alteration in GyrA at Ser-84 to Leu or Glu-88 to Lys. Third-step mutants derived from second-step mutants with reduced accumulation were found to have a mutation in *gyrA*. The results from this study demonstrated that mutations in *gyrA* or mutations leading to reduced drug accumulation occur after alteration of GrlA, supporting the previous findings (L. Ferrero, B. Cameron, B. Manse, D. Lagneaux, J. Crouzet, A. Famechon, and F. Blanche, Mol. Microbiol. 13:641–653, 1994) that DNA topoisomerase IV is a primary target of fluoroquinolones in *S. aureus*.

The development of fluoroquinolone resistance in *Staphylococcus aureus* has been widespread in recent years (13, 14, 31, 32). Three mechanisms by which *S. aureus* acquires resistance to fluoroquinolones have been proposed. The first of these, reported in both *S. aureus* and other bacteria (10), involves an alteration of the target DNA gyrase, a type II DNA topoisomerase, composed of two subunits encoded by *gyrA* and *gyrB*. In *Escherichia coli*, DNA gyrase is the primary target of fluoroquinolones in which at least 10 point mutations leading to amino acid substitutions in the GyrA subunit have been described to confer high-level resistance. In both laboratory and clinical isolates, Ser-83 is the site most commonly associated with resistance when substituted (5, 37). Furthermore, these mutations map within a narrow region defined as the quinolone resistance-determining region (QRDR) located between residues 67 and 106 on the *E. coli* GyrA sequence (37). The region in *S. aureus* GyrA and GrlA corresponding to the QRDR of *gyrA* of *E. coli* is located between residues 68 and 107 and between residues 64 and 103 on the GyrA (24) and GrlA (6) sequences, respectively.

In *S. aureus* a second mechanism proposed to be involved in resistance to fluoroquinolones is provided by the membrane protein NorA, encoded by *norA*, which actively transports norfloxacin and other hydrophilic fluoroquinolones out of the bacterial cell, thus effectively decreasing the intracellular concentration of the drugs (16, 38).

The notion that another locus is involved in resistance to fluoroquinolones has been recently alluded to by groups studying resistance mechanisms in other bacteria (20, 39). A third locus proposed to be responsible for resistance in *S. aureus*, termed *flqA* (36), was shown to be distinct from either the structural genes encoding DNA gyrase or NorA. Whether the *flqA* locus described in ciprofloxacin-selected first-step *S. aureus* laboratory isolates corresponds to the recently described DNA topoisomerase IV genes *grlA* and *grlB* (6) remains unknown. The region of *gyrA* and *grlA* corresponding to the QRDR of *E. coli gyrA* was analyzed from clinical fluoroquinolone-resistant isolates of *S. aureus* with various levels of resistance. No mutation was found in the QRDR of GyrA in low-level quinolone-resistant isolates, whereas Ser-80 of GrlA, which corresponds to Ser-83 of the *E. coli* GyrA, is altered to Phe or Tyr in both high- and low-level quinolone-resistant isolates, indicating that a mutation in *grlA* is associated with low-level resistance. This finding led to the proposal that DNA topoisomerase IV is a primary target of fluoroquinolones (6). It has also been recently determined that DNA topoisomerase IV is an in vivo target in *E. coli* by constructing isogenic mutants leading to a Ser-80–to–Leu substitution in the ParC subunit (18). Undisputable evidence that *S. aureus* DNA topoisomerase IV is a target of the quinolone drugs will be provided by biochemical analysis of the purified enzyme, as has been shown for *E. coli* DNA topoisomerase IV (12, 29), and by reverse genetics.

Many in vitro studies involved in the isolation of fluoroquinolone-resistant mutants have shown that high-level resistance is obtained in a minimum of two steps (1, 9, 11, 22, 34). Investigation of fluoroquinolone resistance mechanisms in *S. aureus* by a number of workers (11, 15, 36) demonstrated that mutations in the gyrase genes are never found in first-step mutants. In 1994 Korten et al. (19) studied fluoroquinolone resistance in *Enterococcus faecalis*, in which no detectable changes were found in the QRDR of the putative GyrA subunit in first-step mutants.

Exposure of clinically derived isolates to various concentrations of fluoroquinolones and other antibiotics can lead to multiple mutations in the chromosome, making it difficult to analyze the contribution of each component to resistance. The aim of this study was to characterize first-, second-, and third-step *S. aureus* RN4220 mutants selected in vitro on ciprofloxacin. The contribution of the three known mechanisms of quin-

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olone resistance was determined at each step of the selection procedure.

MATERIALS AND METHODS

Bacterial strains and vectors. *S. aureus* RN4220 (20) was kindly provided by R. P. Novick. *S. aureus* SA-1199 and SA-1199B, used in norfloxacin accumulation experiments, were obtained from G. A. Kaatz. Transformation experiments were carried out with *E. coli* TG1 (7). Phage M13mp18 was purchased from Boehringer. All cultures were grown at 37°C unless otherwise stated.

Antibiotics. Ciprofloxacin, norfloxacin, oxacillin, and novobiocin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Temafloxacin and erythromycin were purchased from Abbott Laboratories (North Chicago, Ill.). Lomefloxacin was purchased from Searle (Augusta, Ga.), sparfloxacin and spiramycin were both obtained from Rhône-Poulenc Rorer (Vitry-sur-Seine, France), and ofloxacin was purchased from Diamant (Paris, France).

Isolation of spontaneous ciprofloxacin-resistant *S. aureus* RN4220 mutants. First-step spontaneous ciprofloxacin-resistant mutants were selected by plating 500 μl of a stationary-phase culture of RN4220 (MIC of ciprofloxacin, 0.5 μg/ml) onto tryptic soy agar (Difco Laboratories) medium supplemented with increasing (twofold) concentrations of the antibiotic from 2 to 128 μg/ml (4 to 256 times the MIC for RN4220). Second-step mutants were obtained by growing the first-step mutants in trypic soy broth in the presence of ciprofloxacin (at the concentration at which the first-step mutant was selected) to exponential phase and then plating 500-μl aliquots onto increasing antibiotic concentrations. Third-step mutants were obtained in a similar manner from a second-step mutant by using ciprofloxacin at the concentration at which the second-step mutant was selected. For each stepwise selection, the frequency of mutation was calculated by plating serial dilutions of the culture onto nonselective plates and plates containing the concentration of ciprofloxacin used for mutant selection. Resulting resistant clones were reisolated on plates containing the appropriate antibiotic concentration to ensure resistance.

Susceptibility testing. The wild-type and mutant isolates were grown in Mueller-Hinton broth (Difco Laboratories) for 18 h, and the cultures were diluted to obtain 10^7 bacteria per ml. A multipoint replicator (Denley) was used to spot 10^4 bacteria onto plates containing serial twofold dilutions of the appropriate antibiotic to be tested. The MICs were determined after incubation at 37°C for 18 h.

DNA methods. All standard DNA manipulations involving *E. coli* were performed according to the methods described by Sambrook et al. (30). Genomic DNA from *S. aureus* was prepared by the procedure of Novick (28).

Amplification of *gyrA* and *grlA* fragments from *S. aureus* spontaneous ciprofloxacin-resistant mutants. Oligonucleotides used in PCRs have been previously described (6). Sense primer 2612 and antisense primer 4345 were used to amplify the N-terminal fragment of *gyrA* corresponding to a region encoding the QRDR from *S. aureus* mutants; for the amplification of the corresponding region of *grlA*, sense primer 3358 and antisense primer 3902 were used. PCR was performed on genomic DNA of each mutant with a Perkin-Elmer Cetus thermal cycler using *Taq* DNA polymerase (Pharmacia) for 30 cycles in which the conditions were 1 min at 90°C for denaturation, 2 min at 42°C for annealing, and 3 min at 72°C for polymerization. Each amplification yielded one band of the expected molecular size. The resulting PCR-amplified products of 473 and 766 bp for *gyrA* and *grlA*, respectively, were purified, digested with *Eco*RI and *Bam*HI (New England Biolabs), and cloned into the corresponding sites of M13mp18. The recombinant phages were transformed into *E. coli* TG1.

DNA sequencing and analysis. The DNA sequence of the PCR-amplified products cloned into M13mp18 was determined for two to three clones from each cloning by using fluorescence-based universal primer and AmpliTaq DNA polymerase (Applied Biosystems Inc.) and then run on an Applied Biosystems sequencer (model 373A). The sequence was considered correct when the same sequence was obtained for duplicate or triplicate clones.

Norfloxacin accumulation. The kinetics of norfloxacin accumulation was studied by the modified method of Chapman and Georgopapadakou (4) as described by Mortimer and Piddock (26). Norfloxacin was used at a final concentration of 10 μg/ml, and the fluorescence of norfloxacin within the cells was measured at excitation and emission wavelengths of 277 and 440 nm, respectively, with a fluorescence spectrophotometer (model F-2000; Hitachi Ltd.).

RESULTS

Selection of spontaneous ciprofloxacin-resistant mutants of *S. aureus* RN4220. Spontaneous ciprofloxacin-resistant mutants were selected by plating *S. aureus* RN4220 onto medium containing ciprofloxacin at concentrations from 2 to 128 μg/ml. Figure 1 is a summary of how first-, second-, and third-step ciprofloxacin-resistant mutants were derived. Table 1 shows the level of ciprofloxacin used and the frequency at which the mutants were obtained in relation to their respective parents. First-step mutants were selected on ciprofloxacin at 4 times the MIC for RN4220; second-step mutants were selected on ciprofloxacin at 64 times the MIC for RN4220 and at 4 times the MIC for their first-step parent. The third-step mutants were selected on ciprofloxacin at 256 times the MIC for RN4220. The results of MIC determinations for the various mutants are shown in Table 2. In general, cross-resistance to all the fluoroquinolones tested was found. MICs of novobiocin for second-step mutant 2C32C and its third-step derivative 2C32C128B were found to be, respectively, four- and eightfold higher than that for RN4220 (Table 2). No difference was observed in MICs for RN4220 and the mutant strains when tested on representatives of other classes of antibiotics (Table 2).

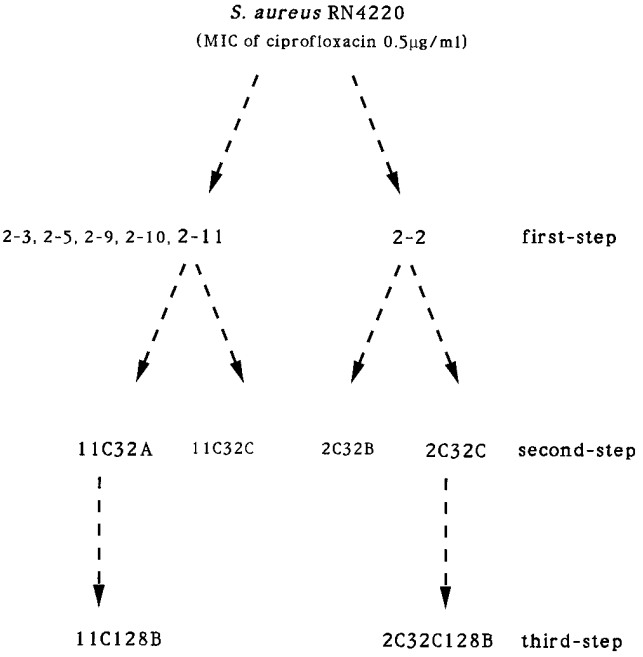

FIG. 1. Schematic representation showing how first-, second-, and third-step mutants were derived from their respective parents.

Analysis of the GyrA and GrlA QRDR from first-, second-, and third-step ciprofloxacin mutants. Genomic DNA from first-, second-, and third-step mutants was used to amplify by PCR the first 473 bases of the *gyrA* gene and the first 766 bases of the *grlA* gene, encoding the region homologous to the *E. coli*

TABLE 1. First-, second- and third-step mutants from *S. aureus* RN4220[a] isolated on ciprofloxacin

Mutant	Parent	Concn of ciprofloxacin (μg/ml)	Frequency of mutation
1st step			
2-2	RN4220	2	9.3×10^{-9}
2-11	RN4220	2	9.3×10^{-9}
2nd step			
2C32B	2-2	32	2.9×10^{-8}
2C32C	2-2	32	2.9×10^{-8}
11C32A	2-11	32	2.4×10^{-6}
11C32C	2-11	32	2.4×10^{-6}
3rd step			
2C32C128B	2C32C	128	1.4×10^{-9}
11C128B	11C32A	128	4.2×10^{-9}

[a] MIC of ciprofloxacin for RN4220 is 0.5 μg/ml.

TABLE 2. Antibiotic susceptibility of first-, second-, and third-step mutants of *S. aureus* RN4220

Strain (step)	MIC (μg/ml) of[a]:										
	CFX	NFX	OFX	SPX	TMX	LMX	FLX	NOV	ERY	SPR	OXA
SA1199	0.5	2	0.25	0.12	0.12	0.5	0.25	0.06	0.25	8	ND
SA1199B	16	128	2	0.25	2	8	4	0.06	0.25	8	ND
RN4220 (parent)	0.5	1	2	0.25	0.25	1	1	0.12	0.25	4	0.12
2-2 (1st)	8	32	2	1	2	8	8	0.12	0.06	2	0.06
2-11 (1st)	8	64	2	1	2	8	8	0.06	0.12	2	0.12
2C32B (2nd)	128	>128	64	32	32	128	64	0.12	0.12	2	0.25
2C32C (2nd)	128	>128	16	2	32	64	32	0.5	0.06	1	ND
11C32A (2nd)	64	>128	16	2	8	32	32	0.12	0.25	2	0.5
11C32C (2nd)	>128	>128	32	2	16	64	32	0.25	0.25	2	ND
2C32C128B (3rd)	>128	>128	>128	64	128	>128	>128	1	0.12	1	ND
11C28B (3rd)	>128	>128	128	64	32	>128	128	0.12	0.12	ND	0.25

[a] CFX, ciprofloxacin; NFX, norfloxacin; OFX, ofloxacin; SPX, sparfloxacin; TMX, temafloxacin; LMX, lomefloxacin; FLX, fleroxacin; NOV, novobiocin; OXA, oxacillin; ERY, erythromycin; SPR, spiramycin; ND, not determined.

gyrA QRDR. The sequence was analyzed over 220 bp containing the QRDR for both genes (Table 3). Sequence analysis of first-step mutants revealed that five of six mutants had a Glu-84 (GAA)–to–Lys (AAA) substitution in *grlA*, whereas the sixth mutant had an alteration of Ser-80 (TCC) to Tyr (TAC). Since five of six isolates (2-3, 2-5, 2-9, 2-10, and 2-11) showed the same sequence over the region analyzed, one representative (2-11) was used for further work. No mutation was found in *gyrA* in the region analyzed for any of the six first-step mutants. No other mutations were found in *grlA* in two second-step mutants, 11C32A and 11C32C, derived from first-step parent 2-11, and no mutation was found in *gyrA* in either mutant. For the two second-step mutants derived from 2-2, no other mutations were found in *grlA*. Sequence analysis of the *gyrA* region, however, showed that no mutation was present in second-step mutant 2C32C, whereas a Glu-88 (GAA)–to–Lys (AAA) substitution was found in mutant 2C32B. No other mutations in *grlA* were found in the third-step mutants 11C128B and 2C32C128B; however, both mutants were found to have a Ser-84 (TCA)–to–Leu (TTA) substitution in *gyrA* (Table 3).

Norfloxacin accumulation. Norfloxacin accumulation by whole cells of *S. aureus* RN4220; the derived first-, second-, third-step mutants; and the control strains SA1199 and SA1199B is shown in Fig. 2. These results represent data obtained from two independent experiments, each performed in duplicate. A reproducible difference was observed in the accumulation levels between SA1199 and SA1199B, which was shown to export hydrophilic quinolones (16). No differences were noted between RN4220 and the first-step mutants 2-2 and 2-11, which all showed accumulation levels two- to threefold higher than that of SA1199B after 5 min. The second-step mutant 2C32B (derived from parent 2-2), which was found to harbor a mutation in the QRDR of both *gyrA* and *grlA*, had drug accumulation levels close to those of SA1199. On the other hand, second-step mutant 2C32C, derived from the same parent but with no mutation in the *gyrA* QRDR, and the third-step mutant 2C32C128B both showed a reproducible reduction in drug accumulation, having levels similar to those of SA1199B. The second-step mutant 11C32A, derived from 2-11, which was shown to have a mutation in *grlA*, and its third-step mutant 11C128B both showed accumulation levels similar to those of SA1199B (data not shown).

DISCUSSION

Ciprofloxacin-selected mutants showed cross-resistance to the other fluoroquinolones tested. No differences were observed between the mutants and RN4220 when tested on erythromycin and spiramycin, both of which target protein synthesis (3). This indicates that the mutations found in the ciprofloxacin-selected isolates are specific to the fluoroquinolones examined. MICs of novobiocin, whose target is GyrB (25), for second-step mutant 2C32C and its third-step derivative 2C32C128B were found to differ from that for RN4220. Since neither the *gyrB* nor *grlB* gene was analyzed in this study, the possibility that 2C32C acquired a mutation(s) in these

TABLE 3. Analysis of the *gyrA* and *grlA* QRDRs from mutants obtained in this study

Mutant	Ciprofloxacin concn (μg/ml)	*gyrA* mutation		*grlA* mutation	
		Nucleic acid position changed	Codon changed	Nucleic acid position changed	Codon changed
Parent (RN4220)	0	None	None	None	None
1st step					
2-2	2	None	None	2270 (C→A)	Ser-80→Tyr
2-11	2	None	None	2281 (G→A)	Glu-84→Lys
2nd step					
2C32B	32	2544 (G→A)	Glu-88→Lys	2270 (C→A)	Ser-80→Tyr
2C32C	32	None	None	2270 (C→A)	Ser-80→Tyr
11C32A	32	None	None	2281 (G→A)	Glu-84→Lys
11C32C	32	None	None	2281 (G→A)	Glu-84→Lys
3rd step					
2-32C128B	128	2533 (C→T)	Ser-84→Leu	2270 (C→A)	Ser-80→Tyr
11C128B	128	2533 (C→T)	Ser-84→Leu	2281 (G→A)	Glu-84→Leu

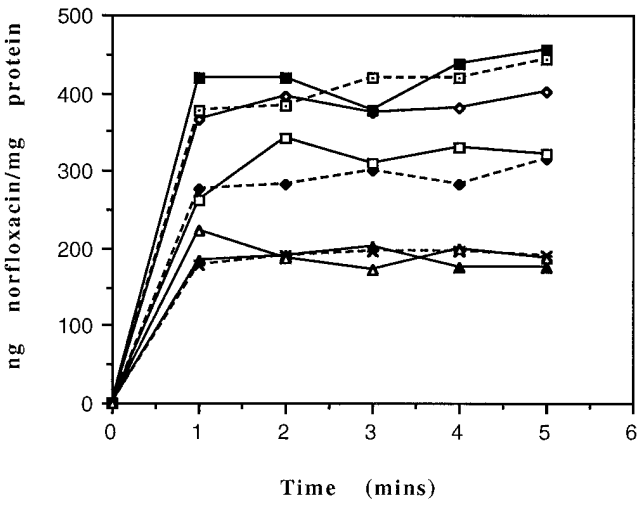

FIG. 2. Norfloxacin accumulation (10 μg/ml) by whole cells of *S. aureus*. ⊡, RN4220; ◆, SA1199; ×, SA1199B; ◇, 2-11; ■, 2-2; □, 2C32B; ▲, 2C32C; △, 2C32C128B.

genes cannot be ruled out. Interestingly, in second-step mutants without an additional mutation in the *gyrA* region examined, there was a lesser increment in resistance for sparfloxacin relative to the other quinolones tested (Table 2), suggesting that the mechanism(s) reducing accumulation of other quinolones affects sparfloxacin less, as has been previously proposed for *norA* mutants (38).

Sequence analysis of the *gyrA* and *grlA* regions homologous to the *E. coli gyrA* QRDR showed that only *grlA* mutations occurred in first-step mutants, supporting the previous finding that in *S. aureus* DNA topoisomerase IV is a primary target of fluoroquinolones (6). This may in part explain why some first-step mutants reported in the literature (19, 21, 39) have no mutation in the QRDR of *gyrA*. In contrast, no mutation has been described in ParC for *E. coli*, even though it has been demonstrated that topoisomerase IV activity was inhibited by the coumarin and quinolone drugs, requiring concentrations 3- to 30-fold higher than those required to inhibit *E. coli* DNA gyrase (12, 29). More recently Belland et al. (2) reported that in stepwise-selected isolates of *Neisseria gonorrhoeae* mutations leading to fluoroquinolone resistance were observed primarily in the QRDR of GyrA, followed by mutation in the QRDR of ParC (the *N. gonorrhoeae* GyrA and ParC being identified on the basis of sequence homology with *E. coli* GyrA and ParC). Does the primary target differ according to bacterial type? Analysis of gyrase and DNA topoisomerase IV genes from a variety of bacteria and their fluoroquinolone-resistant derivatives holds the vital answer to this question.

In *S. aureus* (16, 38), it has been proposed that the active transport of hydrophilic quinolones out of the cell by the membrane protein NorA, chromosomally encoded by *norA*, may provide a mechanism of resistance to quinolones. It has been demonstrated that an *S. aureus* strain containing *norA* on a plasmid accumulated 50% less enoxacin than the plasmid-free strain (38). Similar results were also obtained in *E. coli* (16). Recent findings have shown that in resistant mutants with reduced accumulation, NorA-mediated efflux occurs because of increased *norA* transcripts (17, 27). Decreased norfloxacin accumulation was observed with second-step mutants 2C32C and 11C32C and third-step mutants 2C32C128B and 11C128B, which gave results similar to those with the previously described clinically isolated efflux mutant SA1199B. This may account for the increase in MIC seen with the second-step mutants 2C32C and 11C32A, which were shown to have no mutation in *gyrA*. The nature of reduced norfloxacin accumulation was not characterized in these mutants; therefore, whether reduced accumulation is due to enhanced efflux (*norA*), as previously reported for *S. aureus* (16, 38), is not known. In the second-step mutants in which no decrease in norfloxacin accumulation was observed, the increase in MIC may be attributed to a mutation in *gyrA*. Third-step isolates were found to harbor mutations in *gyrA* and *grlA* and to have reduced norfloxacin accumulation.

From this study, the relative contribution to quinolone resistance made by decreased drug accumulation versus mutation in *gyrA* is difficult to determine. The clinically isolated efflux mutants described in the literature and used in this study as controls in accumulation experiments have been characterized only in terms of efflux (16, 17). Whether these mutants harbor other mutations, and therefore whether drug efflux is a major contributor to quinolone resistance, is not known.

Tankovic and his colleagues (35) studied three sets of clinical and parental resistant isolates obtained during the course of quinolone therapy. In two of three mutants, no mutation was found in the *gyrA* QRDR and no enhanced efflux occurred compared with that in the parents, despite an increase in the MICs of ciprofloxacin and norfloxacin, suggesting the involvement of another gene(s).

Results obtained in this study with first-step mutants demonstrate that a mutation in *gyrA* or a mutation leading to reduced accumulation of quinolones contributes to the acquisition of high-level resistance but occurs after alteration of GrlA (the A subunit of *S. aureus* DNA topoisomerase IV). In first-step mutants with low-level resistance to fluoroquinolones, a mutation was found in *grlA*, whereas high-level fluoroquinolone resistance was associated with additional mutation in *gyrA* and reduced drug accumulation. The roles of *gyrB* and *grlB* were not addressed in this study; therefore, the possibility that mutations in these genes contribute to increased quinolone resistance cannot be dismissed.

It was clearly demonstrated in the work presented that at least three mechanisms of resistance are operative in *S. aureus*, as previously proposed (36). Although *grlA* appears to be a primary target of fluoroquinolones in *S. aureus*, this requires confirmation. The findings from this study support the proposal that an alteration occurs in the QRDR of the GrlA subunit prior to an alteration in the QRDR of GyrA. Whether a mutation leading to reduced drug accumulation precedes a mutation in *gyrA* in the second step remains to be determined. Studies are currently under way to determine how quinolones interact with the GrlA and GrlB subunits of *S. aureus* DNA topoisomerase IV.

The mutants used in this study were obtained in vitro by sequential passage on increasing concentrations of ciprofloxacin, followed by genetic analysis of the mutants at each step. All the mutations encountered in this study, whether in *gyrA* or in *grlA*, have been previously reported for fluoroquinolone-resistant clinical *S. aureus* isolates (6, 8, 23, 33). This indicates that results from studies on fluoroquinolone resistance mechanisms in laboratory *S. aureus* mutants may be applicable to the clinical situation.

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