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Quinolone Resistance Mutations in Topoisomerase IV: Relationship to the *flqA* Locus and Genetic Evidence that Topoisomerase IV Is the Primary Target and DNA Gyrase Is the Secondary Target of Fluoroquinolones in *Staphylococcus aureus*

EVA Y. NG[1], MICHELE TRUCKSIS,[1,2]† AND DAVID C. HOOPER[1,2]*

Infectious Disease Unit and Medical Services,[1] Massachusetts General Hospital, Harvard Medical School,[2] Boston, Massachusetts 02114-2696

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Mutations in the *flqA* (formerly *ofx*/*cfx*) resistance locus of *Staphylococcus aureus* were previously shown to be common after first-step selections for resistance to ciprofloxacin and ofloxacin and to map on the *S. aureus* chromosome distinctly from *gyrA*, *gyrB*, and *norA*. *grlA* and *grlB*, the genes for the topoisomerase IV of *S. aureus*, were identified from a genomic λ library on a common *Kpn*I fragment, and *grlB* hybridized specifically with the chromosomal *Sma*I A fragment, which contains the *flqA* locus. Amplification of *grlA* sequences (codons 1 to 251) by PCRs from nine independent single-step *flqA* mutants, one multistep mutant, and the parent strain identified mutations encoding a change from Ser to Phe at position 80 in four mutants, a novel change from Ala to either Glu or Pro at position 116 in three mutants, and no change in three mutants. In the multistep mutant, another resistance locus, *flqC*, was mapped by transformation to the chromosomal *Sma*I G fragment by linkage to Ω(*chr*::Tn*551*)*1051* (58%) and *nov* (97.9%), which encodes resistance to novobiocin. This fragment contains the *gyrA* gene, and *flqC* mutants had a mutation in *gyrA* encoding a change from Ser to Leu at position 84, a change previously found in resistant clinical isolates. In genetic outcrosses, the *flqC* (*gyrA*) mutation expressed resistance only in *flqA* mutants, including those with both types of *grlA* mutations. The silent mutant allele of *gyrA* was present in an *flqA* background and expressed resistance only upon introduction of a *grlA* mutation. At fourfold the MIC of ciprofloxacin, the bactericidal activity of ciprofloxacin was reduced in a *grlA* mutant and was abolished in *gyrA grlA* double mutants. These findings provide direct genetic evidence that topoisomerase IV is the primary target of current fluoroquinolones in *S. aureus* and that this effect may result from the greater sensitivity of topoisomerase IV relative to that of DNA gyrase to these agents. Furthermore, resistance from an altered DNA gyrase requires resistant topoisomerase IV for its expression.

Fluoroquinolones such as norfloxacin, ciprofloxacin, and ofloxacin are broad-spectrum, synthetic antimicrobial agents that have been used widely for the treatment of a broad range of infections (14). The current members of this group have the greatest potency against aerobic gram-negative pathogens and less activity against gram-positive pathogens. Within a relatively brief period after the introduction of the fluoroquinolones into clinical practice, rising levels of resistance to these antimicrobial agents were noted in some organisms, particularly in *Staphylococcus aureus* (50). The genetics and mechanisms of bacterial fluoroquinolone resistance have been most studied in *Escherichia coli* and *Pseudomonas aeruginosa* isolates, but more recently, the mechanisms of resistance in *S. aureus* have been evaluated in both laboratory and clinical isolates (7, 8, 15, 16, 32, 33, 43, 44, 51).

The fluoroquinolones are known to act on DNA gyrase, an essential topoisomerase composed of two A (GyrA) and two B (GyrB) subunits, by trapping or stabilizing an enzyme reaction intermediate in which a DNA strand is cleaved and covalently linked to each of the two A subunits (9, 54). The stabilization of this cleavage complex initiates a series of as yet poorly defined events that results in cell death. Alterations in both the *gyrA* and *gyrB* genes (encoding GyrA and GyrB, respectively) have been shown to cause fluoroquinolone resistance in *E. coli*, identifying DNA gyrase as a principal drug target in this organism. All such resistance mutations have been localized to specific domains within the respective genes (5, 13, 15, 20, 34, 42, 43, 57, 58). In *S. aureus*, alterations in the homologous domain of *gyrA* have been associated with resistance in clinical isolates, particularly those isolates with higher levels of resistance and multiple resistance mutations (15, 20, 43). In contrast to *E. coli* and other gram-negative bacteria, in *S. aureus* such *gyrA* mutations have not been found in any mutants selected for first single-step resistance to fluoroquinolones, and genetic studies have not definitively linked quinolone resistance with *gyrA* mutations. These findings suggested that in *S. aureus*, *gyrA*-determined resistance differs from that in *E. coli* and other gram-negative bacteria.

Resistance mechanisms other than alterations in DNA gyrase have been identified in both gram-negative and gram-positive bacteria. Altered fluoroquinolone permeation may cause resistance as a consequence of the interaction of inner membrane efflux pumps and reduced outer membrane permeability in gram-negative bacteria (27) and as a consequence of increased levels of expression of efflux pumps in gram-positive bacteria, including specifically enhanced expression of the *S. aureus* NorA efflux transporter associated with the *flqB* resistance locus (31, 32, 59). The relative occurrences of these types of resistance mechanisms are as yet unclear, but the pleiotropic

* Corresponding author. Mailing address: Infectious Disease Unit, Massachusetts General Hospital, 14 Fruit St., Boston, MA 02114-2696. Phone: (617) 726-3812. Fax: (617) 726-7416. Electronic mail address: hooperd@a1.mgh.harvard.edu.

† Present address: Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, MD 21201.

TABLE 1. Bacterial strains used in the study

Strains	Genotype	Origin or reference
S. aureus		
ISP86	8325[a] *uraA141 purA102 pig-131 nov-142 hisG15*	51
ISP794	8325 *pig-131*	45
ISP2133	8325 *pig-131 trp-489* Ω(chr::Tn*917lac*)2	51
ISP2462	8325 *pig-131* Ω(*chr*::Tn*551*)*1051* Ω(*chr*::Tn*916*)*1105*	P. A. Pattee (35)
MT1222	8325 *pig-131 flqA* (*grlA553*) *flqB flqC* (*gyrA*)	51
MT5224c9	8325 *pig-131 nov-142 hisG15 flqA543*[b]	51
MT5224c4	8325 *pig-131 nov-142 hisG15 flqA* (*grlA542*)	51
MT5224c2	8325 *pig-131 nov-142 hisG15 flqA552*	This study; selection on ciprofloxacin agar
MT52222	8325 *pig-131 nov-142 hisG15 flqA* (*grlA541*)	51
MT52184	8325 *pig-131 nov-142 hisG15 flqA545*	51
MT201	8325 *pig-131 flqA549*	51
MT111	8325 *pig-131 flqA* (*grlA548*)	51
MT211	8325 *pig-131 flqA* (*grlA550*)	51
MT221	8325 *pig-131 flqA* (*grlA551*)	51
MT23142	8325 *pig-131 flqB* Ω(*chr*::Tn*916*)*1108*	32
MT1293	8325 *pig-131 flqA* (*grlA553*) *flqB flqC* (*gyrA*) Ω(chr::Tn*551*)*1051*	This study; ISP2462 DNA × MT1222
MT3229	8325 *pig-131 flqA* (*grlA553*) *flqB flqC* (*gyrA*) *nov-142*	This study; ISP86 DNA × MT1222
JC5233	8325 *pig-131 nov-142 hisG15 flqA* (*grlA541*) Ω(*chr*::Tn*917lac*)*2*	This study; ISP2133 DNA × MT5222
RN4220	8325-4 r^-	25
EN1	RN4220 *flqA* (*grlA542*) Ω(*chr*::Tn*917lac*)*2*	This study; ISP2133 DNA × RN4220
EN3	RN4220 *flqA543* Ω(*chr*::Tn*917lac*)*2*	This study; ISP2133 DNA × RN4220
EN5	RN4220 *nov-142 flqC* (*gyrA*)	This study; MT3229 DNA × RN4220
EN6	RN4220 *nov-142* flqC (*gyrA*)	This study; MT3229 DNA × RN4220
EN8	RN4220 *flqA* (*grlA542*) Ω(*chr*::Tn*917lac*)*2 nov-142 flqC* (*gyrA*)	This study; EN1 DNA × EN5
EN9	RN4220 *flqA543* Ω(*chr*::Tn*917lac*)*2 nov-142 flqC* (*gyrA*)	This study; EN1 DNA × EN6
EN12	RN4220 $flqA^+$ Ω (*chr*::Tn*917lac*)*2 nov-142 flqC* (*gyrA*)	This study; EN1 DNA × EN6
EN18	RN4220 $flqA^+$ Ω(*chr*::Tn*917lac*)*2 nov-142 flqC* (*gyrA*)	This study; EN3 DNA × EN6
EN20	RN4220 *flqA* (*grlA542*) Ω(*chr*::Tn*917lac*)*2*	This study; EN1 DNA × RN4220
EN22	RN4220 *flqA543* Ω(*chr*::Tn*917lac*)*2*	This study; EN3 DNA × RN4220
E. coli		
DH5α	F^- ϕ80dlacZΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 phoA hsdR17* (r_k^-, m_k^+) *supE44* λ^- *thi-1 gyrA96 relA1*	GIBCO-BRL
SRB	$e14^-$ ($McrA^-$) Δ(*mcrCB-hsdSMR-mrr*)*171 sbcC recJ uvrC umuC*::Tn*5 supE44 lac gyrA96 relA1 thi-1 endA1* [F′ *proAB* $lacI^qZ\Delta M15$][a]	Stratagene

[a] *S. aureus* phage group III strain NCTC 8325 (35).
[b] The *flqA* locus was formerly designated the *cfx*/*ofx* locus (51).

resistance phenotype of many reduced permeation mechanisms and the broad substrate profile of many efflux pumps suggest that the occurrence of this type of resistance might be augmented by bacterial exposure to other classes of antimicrobial agents in addition to fluoroquinolones.

More recently, another essential topoisomerase related to DNA gyrase, topoisomerase IV, was identified in *E. coli* (21, 22, 37). This enzyme, like DNA gyrase, is a heterodimer composed of two subunits, ParC (homologous to GyrA) and ParE (homologous to GyrB). The conservation in *parC* and *parE* of respective domains similar to those in *gyrA* and *gyrB* that are involved in fluoroquinolone resistance and the observation that purified topoisomerase IV was inhibited by fluoroquinolones in vitro (18, 37) suggested that this enzyme may also be a drug target in vivo and that altered topoisomerase IV may be another mechanism of fluoroquinolone resistance. In *S. aureus*, a resistance locus, *flqA* (previously designated *cfx*/*ofx*), which is distinct from *norA*, *gyrA*, and *gyrB*, was found in all single-step mutants isolated after various independent selections with ciprofloxacin and ofloxacin (51). Most *flqA* mutations affected the expression of novobiocin resistance encoded by the *nov* locus, which in other species is *gyrB*. These findings suggested that *flqA* might encode a topoisomerase or other gene product that interacts with DNA gyrase. Subsequently, mutations in the *S. aureus* homolog of *parC*, termed *grlA*, have been associated in clinical isolates with low levels of fluoroquinolone resistance, a phenotype similar to that of *flqA* mutants (8). Clinical isolates with higher levels of quinolone resistance had both *grlA* and *gyrA* mutations, suggesting that *grlA* mutations precede *gyrA* mutations, a presumption that was confirmed in subsequent studies of first-step and second-step laboratory mutants (7). *grlA* and *grlB*, which is the *S. aureus* homolog of *parE*, were also found to be contiguous genes, and cloned *grlA* and *grlB* complemented *Salmonella typhimurium parC* and *parE* mutants (8).

We report here the localization of *grlA* on the *S. aureus* chromosome and the identification of established and novel *grlA* mutations in genetically defined and mapped *flqA* mutants. We report further the mapping of the *flqC* resistance locus associated with a *gyrA* mutation and define the dominance characteristics between chromosomal *grlA* and *gyrA* fluoroquinolone resistance mutations. The bactericidal activity of ciprofloxacin against *grlA* and *gyrA* single mutants and *grlA gyrA* double mutants was also characterized.

MATERIALS AND METHODS

Bacterial strains, plasmids, and conditions of growth. The bacterial strains used in the study are listed in Table 1. Plasmids pGEM5zf(+) and pGEM7zf(+) (Promega, Madison, Wis.) were used for subcloning. *E. coli* strains were grown in Luria-Bertani medium at 37°C, and *S. aureus* strains were grown in brain heart infusion medium at 35°C. When required, the following antibiotics were added to

the growth medium at the indicated concentrations: ampicillin (100 μg/ml), erythromycin (20 μg/ml), and novobiocin (10 μg/ml) (all purchased from Sigma Chemical Co., St. Louis, Mo.). MICs were determined with Mueller-Hinton agar supplemented with serial twofold increasing concentrations of ciprofloxacin or penicillin. The MBCs of penicillin were determined by broth macrodilution.

Construction of a bacteriophage λ library. An *S. aureus* genomic library was constructed by using λ Dash II (Stratagene, La Jolla, Calif.). The 9- to 20-kb insert DNAs were prepared from *S. aureus* MT5224c9 (*flqA543*) that had been partially digested with *Sau3A*, size fractionated in a sucrose gradient, and treated with calf intestinal phosphatase. The packaging, amplification, and propagation of the λ lysates were performed according to the protocols of the manufacturer.

***S. aureus* transformation.** Chromosomal DNA was obtained by the procedure of Stahl and Pattee (45). The resultant DNA was resuspended in 0.1× Tris-EDTA (TE) buffer. Transformation with high-molecular-weight chromosomal DNA was performed as described previously (45).

Recombinant DNA techniques, nucleotide sequencing, and computer analysis. Recombinant DNA techniques were performed by standard protocols (40). ^{32}P-labeled DNA probes for Southern blot analyses were made by using the random prime kit from Promega. Southern blot analyses and genomic library screenings were performed with GeneScreen membranes (DuPont) according to the manufacturer's directions. Nucleotide sequences were determined by the dideoxy chain termination method (41) by using Sequenase, version 2.0, with double-stranded DNA templates following the manufacturer's instructions. A combination of universal and internal primers was used to determine the sequences of both strands from single and multiple clones of the desired regions of *grlA* and *gyrA*. Nucleotide sequences were analyzed by using the Genetics Computer Group software package, version 7.0.

Amplification of *grlB* and its mapping on the *S. aureus* chromosome. Degenerate primers were made to two conserved regions of *gyrB* (19), taking into account the codon usage pattern of *S. aureus*. These regions correspond to amino acids 106 to 112 (LHAGGKF) and amino acids 323 to 329 (EGDSAGG) of *S. aureus* GyrB (3). These primers were used to amplify *gyrB* and *grlB* sequences from *S. aureus* MT5224c9 chromosomal DNA by using Vent DNA polymerase (New England Biolabs) at an annealing temperature of 50°C. The resulting fragments were predominantly 0.8 and 1.0 kb in size and were cloned into the *Sma*I site of pGEM5zf(+) (Promega). Several resulting clones were sequenced. The sequences of two clones were found to be identical to that of *S. aureus gyrB*, and the sequence of one clone was found to have 58% amino acid identity with that of *S. aureus gyrB* and to be identical to that of *S. aureus grlB* (8). This *grlB* fragment was used to probe the *S. aureus* λ library and a Southern blot containing *Sma*I-digested *S. aureus* chromosomal DNA separated by pulsed-field gel electrophoresis as described previously (51).

Amplification of the quinolone resistance-determining regions of *gyrA* and *grlA*. Primers and amplification conditions were those described previously (8). Chromosomal DNAs from various single-step- and multiple-step-derived quinolone-resistant *S. aureus* mutants were used as templates. The amplified fragments were cut with *Eco*RI or *Hin*dIII and were cloned into the corresponding sites of pGEM7zf(+).

Confirmation of the mutant genotypes in *S. aureus* transformants. DNA fragments containing the quinolone resistance-determining region of *gyrA* and *grlA* were amplified from the *S. aureus* transformants of various genetic crosses as described above. In the case of *gyrA*, the mutation encoding a change from Ser to Leu at position 84 (Ser84Leu) is marked by the loss of a *Hin*fI site within this fragment. In the case of *grlA*, the Ser80Phe mutation is marked by the loss of one *Mnl*I site. The PCR products of the strains of interest were digested with the respective enzymes, and the restriction patterns were examined on agarose gels.

Quinolone bactericidal activity in mutant and wild-type strains. To control growth rates precisely, measurements of quinolone killing were done in MOPSO medium supplemented with 1% Casamino Acids, 1% yeast extract, and 0.5% glucose (30). Cells were grown to the mid-logarithmic phase (optical density at 600 nm, 0.3 to 0.5) at 35°C with vigorous shaking. Ciprofloxacin or penicillin was added, and the incubation was continued. To assess the number of surviving bacteria, samples were taken immediately prior to drug exposure and at hourly intervals up to 5 h thereafter. Samples were then diluted in saline and were plated onto Mueller-Hinton agar.

RESULTS

Localization of *grlB* and *grlA* on the *S. aureus* chromosome. Using degenerate oligonucleotide primer pairs based on regions of amino acids conserved among *S. aureus* GyrB, *E. coli* GyrB, and *E. coli* ParE, we amplified by PCR DNA fragments of the expected sizes of 0.8 and 1.0 kb from the chromosomal DNAs of *S. aureus* MT5224c4 *flqA* and ISP794 *flqA*$^+$. The 1-kb fragment was then cloned into the *Eco*RV site of pGEM5zf(−). Sequencing of the 1-kb fragment from 13 clones identified two that contained sequences identical to that of *S. aureus gyrB* and one that had sequences identical to that of *S. aureus grlB* (8) (data not shown). This 1-kb *grlB* fragment was gel purified, labeled with ^{32}P, and used to probe a Southern blot of the *Sma*I fragments of chromosomal DNA from ISP794 *flqA*$^+$. Under conditions of high stringency, only the *Sma*I A fragment hybridized with the probe. The A fragment contains the *flqA* locus but does not contain the *gyrB* and *gyrA* genes, which are located on the *Sma*I G fragment (51). Thus, *grlB*, like the *flqA* locus (51), is located on the *Sma*I A fragment of the *S. aureus* 8325 chromosome.

Using similar approach, we were unsuccessful at identifying *grlA* sequences by PCR amplification with degenerate primers based on regions conserved among *S. aureus* GyrA, *E. coli* GyrA, and *E. coli* ParC. While this work was ongoing, however, candidate *grlA* sequences from *S. aureus* were identified by degenerate primer amplification (19a) and were subsequently verified to be identical to that of *grlA* found in clinical isolates (8). We used these sequences to design oligonucleotide primers that were used to amplify by PCR a 240-bp internal fragment of *grlA*. This fragment was cloned and used as a *grlA* probe.

The *grlB* probe was also used to screen a λ library constructed from genomic DNA of strain MT5224c9 *flqA*. Four clones were identified under conditions of high stringency and were secondarily amplified, and two were mapped in greater detail. Restriction digests of clones λ3d1 and λ3a1 were probed with both *grlB* and *grlA* probes. Both *grlA* and *grlB* sequences were located on a common *Kpn*I fragment, indicating that *grlA* and *grlB* are closely linked in the *S. aureus* 8325 background, as is the case for clinical isolates (8). Thus, *grlA*, because of its proximity to *grlB*, is also located on the *Sma*I A fragment of the *S. aureus* chromosome.

Amplification and sequencing of *grlA* in *flqA* mutants. Using *grlA* primers, we amplified by PCR, subcloned into pGEM7zf (+), and sequenced codons 9 through 251 of *grlA* from *S. aureus* ISP794 (wild-type parent), MT1222 (*flqA*, *flqB*, *flqC*) selected by serial passage on increasing concentrations of norfloxacin, and eight previously mapped *flqA* mutants selected independently by plating strains ISP794 or MT5 (ISP794 *nov*) on plates containing either ciprofloxacin or ofloxacin (51). The nucleotide sequence of *grlA* of ISP794 (wild type) was identical to that of the *grlA* of the susceptible strain published previously (8), with the exception of a single, silent C-to-T change in the third position of codon 47.

Four single-step *flqA* mutants (mutants MT5224c4, MT52222, MT211, and MT221) had single nucleotide differences (TCC→TTC) at codon 80 encoding a change from Ser to Phe (Table 2), as was found in genetically undefined clinical isolates with low-level quinolone resistance (8). In addition, we found previously unreported mutations in *grlA* in MT1222 (the *flqA flqB flqC* multiply mutated strain) and two other single-step *flqA* mutants (mutants MT52184 and MT111). Single nucleotide changes encoded a change in Ala at position 116 (homologous to Ala119 of GyrA) to either glutamic acid (GCA→GAA; n = 2) or proline (GCA→CCA; n = 1). These changes (set in boldface and underscored) occur in a region (motif: A**A**MRYTE) that is completely conserved between *grlA* and *gyrA* (19) and that includes the tyrosine at position 122 of GyrA, which is in the active site of DNA gyrase (17, 52). Changes in Ala116 of GrlA (or Ala120 of GyrA) have not been previously reported to be associated with quinolone resistance. No nucleotide changes were detected between codons 9 and 251 in the three other single-step *flqA* mutants (mutants MT5224c9, MT5224c2, and MT201), suggesting that additional resistance mutations may occur either in *grlA* outside the region sequenced, in *grlB* (possibly homologous to those reported in *gyrB*), or in another linked locus. These possibilities are under study.

Mapping of the *flqC* resistance locus and identification of a *gyrA* mutation in an flqC mutant. Highly resistant strain

TABLE 2. Deduced amino acid changes in *grlA* in *flqA* mutants of *S. aureus*

Mutant strain	Parent strain	Selecting quinolone	Amino acid sequence[a]						
			80	81	115	116	117	118	119
	ISP794	None	S	S	A	A	M	R	Y
MT1222[c]	ISP794	Norfloxacin	S	S	A	**E**	M	R	Y
MT5224c4	MT5	Ciprofloxacin	**F**	S	A	A	M	R	Y
MT5224c9	MT5	Ciprofloxacin	S	S	A	A	M	R	Y
MT52222	MT5	Ofloxacin	**F**	S	A	A	M	R	Y
MT5224c2	MT5	Ciprofloxacin	S	S	A	A	M	R	Y
MT52184	MT5	Ofloxacin	S	S	A	**P**	M	R	Y
MT201	ISP794	Ciprofloxacin	S	S	A	A	M	R	Y
MT111	ISP794	Ofloxacin	S	S	A	**E**	M	R	Y
MT211	ISP794	Ciprofloxacin	**F**	S	A	A	M	R	Y
MT221	ISP794	Ofloxacin	**F**	S	A	A	M	R	Y

[a] Numbers refer to amino acids of GrIA (codons of *grlA*). The sequences between *grlA* codons 9 and 251 for all strains except strains MT111 (codons 9 to 230) and MT211 (codons 9 to 151) were determined. For all mutants relative to ISP794, no other nucleotide changes were found in the regions sequenced. Boldface and underscoring indicate nucleotide changes. Abbreviations: S, serine; A, alanine; M, methionine; R, arginine; Y, tyrosine; E, glutamic acid; F, phenylalanine; C, cysteine; P, proline.

[b] Strain selected by serial passage on norfloxacin; it contains *flqA*, *flqC*, and *flqB* mutations.

MT1222 had been selected by serial passage on increasing concentrations of norfloxacin to generate multiple mutations. This strain contains an *flqA* (*grlA* Glu116) mutation (Table 2) and has previously been shown to contain a *flqB* resistance locus on the *Sma*I D fragment that is associated with an increased level of expression of the *norA* gene, which encodes the NorA quinolone efflux protein (32). Because of its high level of resistance, we expected that MT1222 would also contain a *gyrA* mutation. We have previously shown that *S. aureus gyrA* is located on the *Sma*I G fragment, a fragment that also contains the *nov* locus, which encodes resistance to novobiocin (51). Novobiocin and coumermycin resistance is caused by mutations in *gyrB* in other species (11, 46, 47), and in *S. aureus*, *gyrB* and *gyrA* are contiguous genes (15, 28). Thus, we postulated that *nov*, which is a selectable marker in *S. aureus*, would be tightly linked to *gyrA*. To determine if MT1222 contained a quinolone resistance locus (which we designated *flqC*) that is tightly linked to *nov*, we prepared high-molecular-weight chromosomal DNA from strain ISP86 (*hisG nov*) and used this DNA to transform MT1222, selecting for novobiocin resistance. For 187 of 192 transformants (97.9%), the ciprofloxacin MIC was reduced four- to eightfold, from 64 to 8 to 16 μg/ml, and 67 (35%) became His^-. The linkage of *hisG* and *nov* was similar to that reported previously (36). Thus, *flqC* is tightly linked to *nov* and is a candidate for mutant *gyrA*.

To determine the presence of a *gyrA* mutation(s) in the *flqC* mutant, we amplified by PCR and sequenced the region between nucleotides 2299 and 2738 (8, 28) of *gyrA*, which encodes amino acids 6 to 152 that encompass the region of *S. aureus* GyrA that is homologous to the quinolone resistance-determining region of *E. coli* GyrA and in which previous *gyrA* mutations associated with high-level quinolone resistance have been found (8, 43, 44). We found a single nucleotide change (TCA→TTA) in codon 84 that encoded Leu in strain MT1222 instead of the Ser found in the ISP794, the wild-type susceptible strain. The Ser84Leu mutation has previously been associated with high-level quinolone resistance in clinical isolates of *S. aureus* (8, 43, 44). Thus, the *flqC* resistance locus encodes mutant GyrA.

***gyrA* mutations contribute to resistance in the presence of mutant but not wild-type *grlA*.** Because *flqC* (*gyrA*) was found in a multiply mutated resistant strain, we attempted to outcross this locus to determine its contribution to quinolone resistance (Table 3). We first constructed strain MT1293 by introducing Ω*1051* (a chromosomal Tn*551* insertion on the *Sma*I G fragment) into strain MT1222 to provide a selectable erythromycin resistance marker linked to *flqC* (*gyrA*). High-molecular-weight chromosomal DNA from MT1293 was used to transform strains ISP794 (wild type) and MT23142 *flqB*, which exhibits increased levels of expression of *norA*, selecting for resistance to erythromycin. Surprisingly, none of 94 erythromycin-resistant transformants of ISP794 and none of 83 transformants of MT23142 had an alteration in their resistance to ciprofloxacin. In contrast, when the same preparation of high-molecular-weight DNA was used to transform *flqA* strains M5224c9, MT5224c4, and MT52222, a 16-fold increase in ciprofloxacin resistance was found in 58 to 69% of erythromycin-resistant transformants (Table 3). Thus, Ω*1051* is linked to *flqC* (*gyrA*), but the *gyrA* resistance phenotype can be outcrossed only into *flqA* mutants.

These findings suggested at least two possibilities. Either the *flqC* (*gyrA*) mutation was lethal in the absence of *flqA* (*grlA*) mutations or, alternatively, *flqC* (*gyrA*) $flqA^+$ strains were viable but the resistance phenotype of *flqC* (*gyrA*) was silent in the absence of *flqA* mutations encoding altered topoisomerase IV. To distinguish between these possibilities, we constructed a *nov flqC* (*gyrA*) double mutant to allow for the outcross of *flqC* (*gyrA*) using the tightly (>95%) linked *nov* marker. If *flqC* (*gyrA*) was present but silent in the $flqA^+$ background, its presence should then be detected upon introduction of a mutant *flqA* allele.

In the first transformation, donor DNA from MT3229 (*nov gyrA*) was used to transform wild-type strain RN4220, selecting for resistance to novobiocin (Table 4). As expected, for all *nov* transformants there was no change in the MIC of ciprofloxacin relative to that for strain RN4220. Three candidate *nov gyrA* transformants were then used as recipients for transformation with DNA prepared from strain EN1 [Ω(Tn*917lac*)*2 grlA542*] in which the Ω(Tn*917lac*)*2* (erythromycin resistance) marker is linked to *grlA*. As expected, increases in the ciprofloxacin MIC were found for from 31 to 55% of the transformants tested for each cross. For the transformed *nov* $gyrA^+$ recipients, the increase was from 0.5 to 1 μg/ml (the wild-type level for RN4220) to 2 to 4 μg/ml, a fourfold increment consistent with the effect of the *grlA* mutation alone (51). In contrast, for all three of the transformed candidate *nov gyrA* recipients, the increase in MIC was from 1 to ≥32 μg/ml, an increment greater than that which can be attributed to the mutant *grlA*

TABLE 3. Conditional outcross of *flqC* (*gyrA*) by transformation with chromosomal DNA from strain MT1293[a]

Recipient	Recipient genotype	Ciprofloxacin MIC (μg/ml) for recipient	Transformants (no./total no. [%]) in class for which change in the ciprofloxacin MIC (fold) was	
			No change	≥8
M55224c4	*flqA* (*grlA542*)	4	74/187 (40)	113/187 (60)
MT52222	*flqA* (*grlA541*)	4	85/203 (42)	118/203 (58)
MT5224c9	*flqA543*	4	56/179 (31)	123/179 (69)
ISP794	Wild type	0.5	94/94 (100)	0/94 (0)
MT23142	*flqB*	2	83/83 (100)	0/83 (0)

[a] MT1293 [8325 *pig-131 flqA* (*grlA553*) *flqB flqC* (*gyrA*) Ω(*chr*::Tn*551*)*1051*] donor DNA was used; the same preparation of high-molecular-weight chromosomal DNA was used for all of the crosses shown; transformants were selected on erythromycin agar.

TABLE 4. Demonstration of the presence of *gyrA* silent resistance alleles in *nov gyrA* transformants by introduction of *flqA* (*grlA542*) linked to Ω(*chr*::Tn*917lac*)*2*

Donor DNA	Recipient	MIC (μg/ml) for recipient	Transformants (no./total no. [%]) in class for which ciprofloxacin MIC (μg/ml) is as follows:		
			0.5–1	4–8	≥32
MT3229 *gyrA nov*[a]	RN4220	0.5–1	9/9 (100)	0/9 (0)	0/9 (0)
EN1 *grlA542* Ω(*chr*::Tn*917lac*)*2*[b]	RN4220	0.5–1	49/101 (49)	52/101 (52)	0/101 (0)
EN1 *grlA542* Ω(*chr*::Tn*917lac*)*2*[b]	EN5 *nov gyrA*	0.5–1	40/58 (69)	0/58 (0)	18/58 (31)
EN1[b]	EN6 *nov gyrA*	0.5–1	13/24 (55)	0/58 (0)	11/24 (45)
EN1[b]	EN7 *nov gyrA*	0.5–1	38/56 (68)	0/58 (0)	18/56 (32)

[a] Selection for *nov* on novobiocin agar.
[b] Selection for Tn*917* (Erm) on erythromycin agar.

allele alone. The absence of the Ser84Leu *gyrA* mutation in the RN4220 recipient and its presence in the three candidate *nov gyrA* recipients was confirmed by the *Hin*fI restriction site polymorphism (see below). Thus, the *flqC* (*gyrA*) mutation does not cause lethality in the absence of a *grlA* mutation, but a *grlA* mutation is required for the resistance of mutant *gyrA* to be expressed. These findings were also confirmed for the *grlA541* allele and in the genetic background of strain ISP794 (data not shown).

Restriction fragment length polymorphisms confirming *grlA* and *gyrA* mutations. To confirm the presence of the *grlA* and *gyrA* mutations in the strains used in these genetic crosses, fragments of these genes from selected transformants were amplified and were cut with restriction enzymes: *Mnl*I in the case of *grlA* and *Hin*fI in the case of *gyrA*. *Mnl*I, with the recognition site CCTC(N)$_7$, cuts *grlA*$^+$ at codons 80 and 81 (TCC TCA), and its recognition site is destroyed by the mutation (T**T**C TCA) encoding Ser80Phe. *grlA*$^+$ between nucleotides 1 and 810 contains four *Mnl*I sites which are predicted to generate fragments of 539, 243, 17, and 11 bp, respectively, whereas the mutant *grlA* (Phe80) should generate fragments of 782, 17, and 11 bp, with the two smallest bands being undetectable by conventional agarose gel electrophoresis. The presence of the *grlA* mutation was confirmed by this analysis in strains MT224c4 (Table 2), EN8, and EN9 (Table 4), which exhibited single visible bands of the appropriate size. In contrast, as expected, strains MT1222, MT5224c9, ISP794 (Table 2), EN5, and EN6 (Table 4) exhibited the two expected visible bands, consistent with the absence of a *grlA* (Phe80) mutation.

*Hin*fI (recognition site GANTC) cuts *gyrA*$^+$ at codons 83 and 84 (GAC TCA), and its recognition site is destroyed by the mutation (GAC T**T**A) encoding Ser84Leu. *gyrA*$^+$ between nucleotides 1 and 474 contains three *Hin*fI sites which are predicted to generate fragments of 231, 189, and 54 bp, respectively, whereas the mutant *gyrA* (Leu84) should generate fragments of 420 and 54 bp (3). Strains MT1222 (Table 2), EN9, EN8, EN6, and EN5 (Table 4) all exhibited two bands with sizes consistent with those expected for *gyrA* (Leu84). In contrast, strains ISP794 and RN4220 exhibited the wild-type pattern.

Bacterial killing by fluoroquinolones is determined by both topoisomerase IV and DNA gyrase. In order to determine the effect of mutations in *grlA* and *gyrA* on the bactericidal activity of ciprofloxacin, strains RN4220 and its mutant derivatives EN20 (*grlA* [Ser80Phe]), EN6 (*gyrA* [Ser84Leu]), and EN9 (*grlA* [Ser80Phe] *gyrA* [Ser84Leu]) were grown to the log phase under conditions in which each strain was doubling at the same rate. Ciprofloxacin was then added to the culture at a concentration of fourfold the MIC for that strain. The numbers of CFU were determined immediately prior to the addition of ciprofloxacin and at intervals thereafter. Strain EN20 (*grlA*) was consistently killed more slowly than strains RN4220 and EN6 (*gyrA*) (Fig. 1). Only in EN9, the *grlA gyrA* double mutant, however, was killing completely abolished at fourfold the MIC.

Because quinolones may exhibit paradoxical reductions in bactericidal activity at high drug concentrations, strain RN4220 was exposed to the same concentration of ciprofloxacin used for EN9, the *grlA gyrA* double mutant. The reductions in the viable counts of RN4220 at this drug concentration (128 μg/ml) were comparable to those seen at fourfold the MIC (4 μg/ml) (data not shown). Thus, the absence of killing seen in the double mutant cannot be attributed to a paradoxical effect of the higher drug concentration.

To determine whether the reduced rate of killing seen for the double mutant was specific for fluoroquinolones, we tested the effect of penicillin on strains RN4220, EN9, EN6, and EN20. The MICs and MBCs of penicillin for all strains were 0.05 μg/ml. In addition, for RN4220 and EN9, similar rates of killing were seen after exposure to 0.2 μg of penicillin per ml (data not shown). Thus, the reduced rate of killing found for

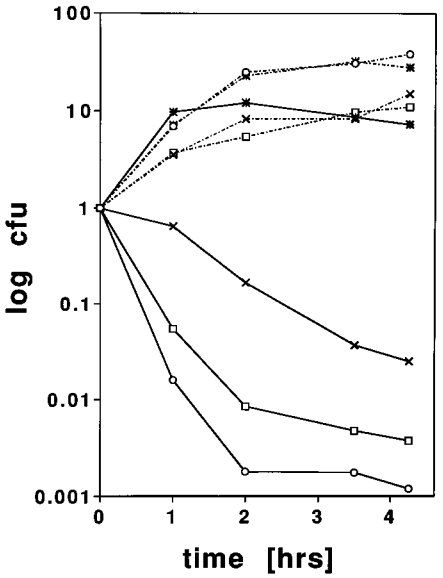

FIG. 1. Viable counts of *S. aureus* strains in the presence (solid lines) and absence (dashed lines) of ciprofloxacin at 4× the MIC for each strain. Results are of a representative experiment with three repetitions. Strains RN4220 (□); 4× the MIC = 4 μg/ml), EN6 *gyrA* (○); 4× the MIC = 4 μg/ml), EN9 *grlA gyrA* (*; 4× the MIC = 128 μg/ml), and EN20 *grlA* (×; 4× the MIC = 32 μg/ml) were tested.

the *grlA gyrA* double mutant was specific for ciprofloxacin and likely for other fluoroquinolones.

DISCUSSION

We have localized the *grlA* and *grlB* genes to the *S. aureus* chromosomal *Sma*I A fragment on which the *flqA* quinolone resistance locus was previously mapped between *thrB* and *trp*. *flqA* mutations were genetically distinct from *gyrA*, *gyrB*, and *flqB* mutations, and the ability of some mutations to affect the expression of novobiocin resistance from the unlinked *nov* locus had suggested that *flqA* mutations might involve another topoisomerase (51). We have now found mutations in *grlA* in six of nine independently selected single-step *flqA* mutants and one highly resistant mutant strain with multiple mutations. Three lines of evidence strongly suggest that the *grlA* mutations found are responsible for the resistance phenotypes of these mutants. First, Ser80 of *grlA* is homologous to Ser83 of *E. coli gyrA*, changes of which are known to cause quinolone resistance. Second, individual *flqA* mutants selected by single-step plating occurred at frequencies (6×10^{-6} to 7×10^{-9}) consistent with those for single spontaneous mutants, and their mutations mapped genetically in a manner consistent with a single locus (51). Thus, Ser80Phe is likely to be a single mutation in these mutants. Third, *grlA* mutations at codon 80 have also been associated with low-level quinolone resistance in clinical isolates (8) and, more recently, laboratory mutants (7). Thus, most genetically defined *flqA* mutants have *grlA* mutations predicted to cause resistance based on homology with *gyrA*.

The Phe80 mutation was the principal mutation found in GrlA, accounting for four of seven independent mutations in our strains. This finding is consistent with those reported for clinical isolates in which changes at position 80 were the only GrlA mutations seen (8). The common occurrence of resistance mutations at both Ser80 of GrlA (7, 8) and Ser84 of GyrA of *S. aureus* (7, 12, 39, 43, 44) and the homology between DNA gyrase and topoisomerase IV (21) suggest that fluoroquinolones may interact with similar structural motifs in the two enzymes.

Mutations at codon 116 of *grlA* or codon 120 of *gyrA* of *S. aureus* have not previously been associated with quinolone resistance. That these changes occurred in 3 of 10 mutants makes it unlikely that they occurred because of random polymerase errors in the cloned PCR fragment. Codon 120 is part of a highly conserved motif, AAMRYTE, in which tyrosine is known to be at the active site of GyrA (9, 52, 53). Thus, the tyrosine at position 119 of GrlA is likely the active site of topoisomerase IV. Because of proximity to the active site and the conservation of the amino acid sequence, it is possible that mutations in this region may affect enzyme function. The existence of viable mutants containing these mutations, however, suggests three possibilities: that enzyme function is not severely affected by the mutation, that *S. aureus* topoisomerase IV (in contrast to *E. coli* topoisomerase IV [21]) is not an essential enzyme, or that mechanisms for complementing a defective enzyme also exist in these mutants.

The absence of mutations in three of the nine single-step *flqA* mutants sequenced indicates that additional mechanisms of resistance may occur, either in the regions of *grlA* not yet sequenced, in the adjacent *grlB* gene (perhaps in regions homologous to those in *gyrB* that cause quinolone resistance), or in another as yet undefined but linked mutant gene.

We had previously found that some *flqA* mutants reduced the level of novobiocin resistance in both wild-type strains and *nov* mutants (51). Of particular note, all four *flqA* mutants identified with *grlA* (Ser80Phe) mutations (strains MT52222, MT5224c4, MT211, and MT221) in both *nov* and *nov*$^+$ genetic backgrounds did not affect novobiocin resistance. In contrast, for the two *flqA* mutants that had no mutation in the region of *grlA* sequenced and for which there was information on novobiocin resistance, both mutations caused reductions in novobiocin MICs: for MT5224c9 (*nov*), the MIC was 2.5 μg of novobiocin per ml, whereas it was 80 μg/ml for its parent strain, strain MT5; for MT201 (*nov*$^+$), the MIC was 0.04 μg of novobiocin per ml, whereas it was 0.16 μg/ml for its parent strain, strain ISP794 (51). For two strains with mutations in codon 116 of *grlA*, there was either no detectable alteration in the novobiocin MIC (for strain MT111 (*nov*$^+$ *grlA* [Ala116Glu]), the MIC was 0.16 μg/ml) or a lesser reduction in the MIC than that for the *nov* strain without an identified mutation in *grlA* (for MT52184 [*nov grlA* (Ala116Pro)], the MIC was 20 μg/ml, whereas it was 80 μg/ml for MT5).

The *nov* locus of *S. aureus* is likely *gyrB*. Novobiocin and coumermycin resistance in other species are caused by *gyrB* mutations (1, 4, 11, 26, 29, 34, 46), and in *S. aureus*, *gyrA* and *gyrB* are contiguous genes (3, 28). Thus, our findings of the tight linkage of *flqC* (*gyrA*) and *nov* provide additional support for the likelihood that the *nov* mutation is in *gyrB*. In this context, it is interesting to speculate about the mechanism of the effects of certain *flqA* mutants on the levels of novobiocin susceptibility in both *nov* and *nov*$^+$ backgrounds. One intriguing possibility is that the mutations that have the greatest effect on novobiocin resistance are in *grlB*, which has similarities to *gyrB*, including conservation of the domains involved in ATPase activity and resistance to novobiocin (8). The activity of purified *E. coli* topoisomerase IV is also known to be inhibited by novobiocin (22, 37, 38). Thus, alterations in GrlB that cause fluoroquinolone resistance might also enhance the affinity of novobiocin for *S. aureus* topoisomerase IV.

Mutations in *gyrA* have commonly been found in quinolone-resistant clinical isolates (12, 39, 43, 44) and in second- but not first-step laboratory mutants of *S. aureus* (7, 16, 20). We originally found no *gyrA* mutants among 14 independently selected single-step resistant mutants of *S. aureus* 8325 plated onto ciprofloxacin or ofloxacin (51). It was only in the multiply resistant strain that had been serially passed on increasing concentrations of norfloxacin that we found other mutations, including the *flqB* locus associated with increased levels of expression of chromosomal *norA* (32) and the *flqC* locus reported here. These findings are in contrast to those for *E. coli* and other gram-negative bacteria (10, 13, 48, 55–57, 60), in which mutations in *gyrA* are found in first single-step mutants.

In *S. aureus*, the *gyrA* (Leu84) mutation, which is homologous to those causing resistance in *E. coli* and associated with higher-level resistance in clinical and laboratory isolates, does not cause resistance by itself. *gyrA* (Leu84) is, however, capable of causing incremental resistance in the presence of *grlA* and *flqA* mutations but not other fluoroquinolone resistance loci such as *flqB*. These findings indicate that chromosomal wild-type *grlA* is dominant over chromosomal mutant *gyrA* in determining quinolone susceptibility. Thus, a sensitive wild-type topoisomerase IV determines the fluoroquinolone MIC for *S. aureus* strains regardless of the presence of mutant DNA gyrase. Our findings thus provide direct genetic evidence that in *S. aureus*, in contrast to in *E. coli*, topoisomerase IV is the principal fluoroquinolone target enzyme.

The explanation for the relative roles of topoisomerase IV and DNA gyrase in sensitivity and resistance to fluoroquinolones might be envisioned at two levels. The simplest explana-

tion is perhaps that the affinities of the current fluoroquinolones for *S. aureus* topoisomerase IV are higher than their affinities for *S. aureus* DNA gyrase and that the interaction of fluoroquinolones with either enzyme may result in cell death. Thus, cell susceptibility is determined directly by the most sensitive enzyme. An alternative explanation is that in *S. aureus* the affinities of fluoroquinolones for the two enzymes are similar but that the consequences of the interactions of fluoroquinolones with the two enzymes differ in vivo. In this scheme, the interaction of fluoroquinolones with *S. aureus* topoisomerase IV but not *S. aureus* DNA gyrase initiates the sequence of events that result in cell death. In an attempt to distinguish these possibilities, we determined the bactericidal activity of ciprofloxacin against *grlA* and *gyrA* single mutants and a *grlA gyrA* double mutant, adjusting for the effect of the mutations on the MICs. At ciprofloxacin concentrations of fourfold the MIC, rates of bacterial killing were greatest for the wild-type strain and *gyrA* single mutant and intermediate for the *grlA* mutant (Fig. 1). Thus, the *grlA* mutation alone did not completely abolish killing, suggesting that at higher drug concentrations, fluoroquinolones may interact with sensitive DNA gyrase to produce bacterial killing. Only when both *grlA* and *gyrA* were mutated were the bactericidal activities of the fluoroquinolones completely abolished. These findings suggest that at suitable drug concentrations, fluoroquinolone interaction with either topoisomerase IV or DNA gyrase may result in bacterial killing and that drug potency is determined by the relative sensitivities of the two enzymes. This interpretation awaits further support from the comparisons of the activities of fluoroquinolones against purified *S. aureus* topoisomerase IV and *S. aureus* DNA gyrase.

The relative sensitivities of these two enzymes to fluoroquinolones appear to differ among species. In *E. coli* the relationships are the reverse of those in *S. aureus. E. coli parC* resistance mutations are expressed only in the presence of *gyrA* mutations (23), and purified *E. coli* topoisomerase IV is less sensitive to fluoroquinolones than *E. coli* DNA gyrase (18, 23). The relationship of *parC* and *gyrA* mutations in resistant clinical isolates of *Neisseria gonorrhoeae* is also the reverse of what is found in clinical isolates of *S. aureus* (2). *N. gonorrhoeae* strains with low-level resistance contained *gyrA* mutations, strains with higher levels of resistance contained both *gyrA* and *parC* mutations, and *parC* mutations were not found alone in resistant isolates. For enterococci, the absence of *gyrA* mutations in first-step fluoroquinolone-resistant mutants and their presence in second-step mutants (24) suggests the possibility that first-step mutants may contain *grlA* mutations, as in *S. aureus* (8). Whether these differences will be consistent between gram-negative and gram-positive species remains to be determined.

The differences among fluoroquinolones in their activities against topoisomerase IV and DNA gyrase in vitro and in vivo have been determined with current drug congeners. The structure-activity relationships of the fluoroquinolones have been studied largely for the DNA gyrase of *E. coli* and much less so for the topoisomerase IV of any species (6, 18, 49). Thus, it is possible that some quinolones or related compounds will have different relative potencies against these enzymes. It might be predicted that potent compounds with equivalent activities against both enzymes would be particularly advantageous, not only because they act on dual targets but also because alteration in the drug targets would require concomitant rather than sequential mutations in the genes for both enzymes for high-level resistance to occur.

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