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Mutations in Topoisomerase IV and DNA Gyrase of *Staphylococcus aureus*: Novel Pleiotropic Effects on Quinolone and Coumarin Activity

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Previous studies have shown that topoisomerase IV and DNA gyrase interact with quinolones and coumarins in different ways. The MICs of coumarins (novobiocin and coumermycin) for MT5, a *Staphylococcus aureus nov* mutant, are higher than those for wild-type strains. Sequencing the *gyrB* gene encoding one subunit of the DNA gyrase revealed the presence of a double mutation likely to be responsible for this resistance: at codon 102 (Ile to Ser) and at codon 144 (Arg to Ile). For single-step *flqA* mutant MT5224c9, previously selected on ciprofloxacin, the fluoroquinolone MIC was higher and the coumarin MIC was lower than those for its parent, MT5. Sequencing the *grlB* and *grlA* genes of topoisomerase IV of MT5224c9 showed a single Asn-470-to-Asp mutation in GrlB. Genetic outcrosses by transformation with chromosomal DNA and introduction of plasmids carrying either the wild-type or the mutated *grlB* gene indicated that this mutation causes both increased MICs of fluoroquinolones and decreased MICs of coumarins and that the mutant *grlB* allele is codominant for both phenotypes with multicopy alleles. Integration of these plasmids into the chromosome confirmed the codominance of fluoroquinolone resistance, but *grlB*$^+$ appeared dominant over *grlB* (Asp-470) for coumarin resistance. Finally, the *gyrA* (Leu-84) mutation previously described as silent for fluoroquinolone resistance increased the MIC of nalidixic acid, a nonfluorinated quinolone. Combining the *grlA* (Phe-80) and *grlB* (Asp-470) mutations with this *gyrA* mutation also had differing effects. The findings indicate that alterations in topoisomerases may have pleiotropic effects on different classes of inhibitors as well as on inhibitors within the same class. A full understanding of drug action and resistance at the molecular level must take into account both inhibitor structure-activity relationships and the effects of different classes of topoisomerase mutants.

Staphylococcus aureus is a major pathogen, many strains of which are now susceptible to only a few antibiotics. Resistance has begun to compromise the utility of fluoroquinolones as antistaphylococcal agents.

Four genes related to fluoroquinolone resistance in *S. aureus* have been reported so far: *norA* (24), *gyrA* (15, 32), *gyrB* (15), and *grlA* (8, 25, 41). Resistance involving the *norA* gene is due to increased expression of the NorA efflux pump. The other resistance genes encode the structural proteins of DNA topoisomerases: *gyrA* and *gyrB* encode the structural proteins of DNA gyrase, and *grlA* (referred to as *parC* in other species) encodes one of the two subunits of DNA topoisomerase IV. The *grlB* gene encoding the second subunit of topoisomerase IV has not been previously implicated in fluoroquinolone resistance in *S. aureus*, but the mutation of its homolog in *Escherichia coli* and *Streptococcus pneumoniae*, *parE*, has been shown to cause resistance (4, 31).

The fluoroquinolones act on type 2 topoisomerases by trapping or stabilizing an enzyme reaction intermediate in which both DNA strands are cleaved and covalently linked to the breakage-reunion subunits (GyrA in the case of gyrase and GrlA in the case of topoisomerase IV) (14). The stabilization of this cleavage complex initiates a series of events that result in cell death. In contrast to the findings for *E. coli*, in staphylococci and other gram-positive bacteria that have been studied, it appears that DNA gyrase is only a secondary target of many fluoroquinolones and that topoisomerase IV is the primary target (8, 25, 27). Mutations in *gyrA* are found only in more highly resistant clinical strains of *S. aureus* that also have mutations in *grlA*, and *grlA* mutations precede *gyrA* mutations in single-step resistance (9). In addition, *gyrA* mutations producing fluoroquinolone resistance in *S. aureus* are silent in the absence of *grlA* mutations (25). Mutations in three codons were described for *grlA*: codon 80 (Ser→Phe or Tyr), 84 (Glu→Lys), and 116 (Ala→Glu or Pro) (9, 25, 41).

A quinolone resistance locus referred to as *flqA* was localized to the A fragment of chromosomal DNA of first-step ciprofloxacin- and ofloxacin-selected resistant mutants digested with *Sma*I between the *thr* and *trp* loci (37). This region has been shown to contain the *grlB* and *grlA* genes (25). In a previous work (25), some *flqA* mutants were shown to have *grlA* mutations but one single-step *flqA* mutant selected on ciprofloxacin (MT5224c9) (37) showed no nucleotide change in the quinolone resistance-determining region of *grlA*, suggesting that additional resistance mutations may occur either in *grlA* outside the region sequenced, or in the adjacent *grlB* gene, or in another as yet undefined but linked locus. In addition to quinolone resistance, mutant MT5224c9, which was selected from novobiocin-resistant parent strain MT5 (*nov-142*), showed a substantial decrease in novobiocin resistance (37).

Coumarins such as novobiocin and coumermycin act differently from quinolones: they act by competitive inhibition of ATP hydrolysis by the B subunit of DNA gyrase (10, 12). The gyrase B protein consists of two domains: a 43-kDa N-terminal domain containing the ATPase site as well as the coumarin-binding site and a 47-kDa C-terminal domain responsible for interaction with the A protein and DNA (21). The binding of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristic[a]	Source or reference
Strains		
S. aureus		
ISP794	8325 *pig-131*	33
ISP2133	8325 *pig-131 trp-489* Ω(chr::Tn*917lac*)*2*	37
MT5	8325 *nov* (*gyrB142*) *hisG15 pig-131*	37
MT5224c9	8325 *nov* (*gyrB142*) *hisG15 pig131 flqA* (*grlB543*)	37
EN3	8325 *nov* (*gyrB142*) *hisG15 pig-131 flqA* (*grlB543*) Ω(chr::Tn*917lac*)*2*	25
RN4220	8325-4 r^-	17
EN5	RN4220 *gyrB142 flqC* (*gyrA*)	25
EN8	RN4220 *flqA* (*grlA542*) Ω(chr::Tn*917lac*)*2 nov* (*gyrB142*) *flqC* (*gyrA*)	25
EN14	RN4220 *flqA* (*grlB543*) Ω(chr::Tn*917lac*)*2 nov* (*gyrB142*) *flqC* (*gyrA*)	25
EN20	RN4220 *flqA* (*grlA542*) Ω(chr::Tn*917lac*)*2*	25
EN22	RN4220 *flqA* (*grlA543*) Ω(chr::Tn*917lac*)*2*	25
BF2	RN4220 *nov* (*gyrB142*)	This study[b]
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
Plasmids		
pSK950	10.5-kb plasmid carrying the *att* site of phage L54a; replicon of pSC101, Sp^r (*E. coli*) and temperature-sensitive replicon of pE194, Tc^r (*S. aureus*)	G. L. Archer
pGEM3-zf(+)	2.9-kb cloning vector, Ap^r	Promega
pYL112Δ19	7-kb plasmid carrying the integrase gene of phage L54a; Ap^r (*E. coli*); Cm^r (*S. aureus*)	18
pBFISB	2.2-kb *Bam*HI fragment of PCR product containing the *grlB* gene from ISP794 cloned into pSK950	This study
pBFC9B	2.2-kb *Bam*HI fragment of PCR product containing the *grlB* gene from MT5224c9 cloned into pSK950	This study

[a] Ap, ampicillin; Cm, chloramphenicol; Sp, spectionmycin; Tc, tetracycline.
[b] Cross between DNA of MT5 and RN4220.

coumarin and ATP is competitive because of overlap in their binding sites (20). All reported mutations that result in coumarin resistance lie at the periphery of the ATP-binding site of GyrB. In contrast, quinolone resistance mutations in GyrB have been localized to a different subunit domain in a position midway between the amino and carboxy termini.

The aim of this study was to determine the origin of the modification of the quinolone and coumarin resistance in the mutant MT5224c9 and to determine if the *nov-142* locus was a mutant allele of *gyrB*. We have found that a novel mutation in the *grlB* gene resulting in the replacement of an asparagine by an aspartate at position 470 is responsible for the phenotype of MT5224c9. In addition, we found that the *nov-142* locus is a novel doubly mutant allele of *gyrB*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani medium, and *S. aureus* strains were grown in Trypticase soy (TS) medium. All strains were grown at 37°C except the *S. aureus* strains carrying the thermosensitive plasmids derived from pSK950, which were grown at 30°C.

Determination of susceptibility patterns. Antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.), except sparfloxacin and DU-6859a, which were kindly provided by Rhone-Poulenc Rorer and Daiichi Pharmaceuticals, respectively. MICs were determined with Mueller-Hinton agar supplemented with serial twofold-increasing concentrations of antibiotics. The cells were grown at 30°C when thermosensitive plasmids were used (free or integrated). For other determinations of MICs, cells were grown at 37°C.

Gene amplification and cloning. The *grlB* genes from strains ISP794 and MT5224c9 were amplified by PCR with two primers, both of which contain an engineered *Bam*HI site (5′-AAC GTA CGG ATC CAG GAG GCG AAA T-3′; 5′ nucleotide at position 352 in the oligonucleotide coordinates used by Yamagishi et al. [41]; and 5′-GGC AAT GGA TCC TCT TGA ATA-3′; position 2470). PCR included annealing at 53°C with Vent DNA polymerase (New England Biolabs). PCR products were digested with *Bam*HI and ligated into the *Bam*HI site of pGEM3-zf(+). Recombinant plasmids were introduced into *E. coli* DH5α. In a similar manner, the *grlA* genes of ISP794 and MT5224c9 were amplified by PCR with two primers containing *Bam*HI sites (5′-AGT GGA TCC TGA AGT GCG TT-3′; position 2196; and 5′-TAA GGA TCC GTA CTT GGT CA-3′; position 4881) and were cloned into pGEM3-zf(+).

The cloning into pSK950 was performed by using a PCR product amplified with the following primers: 5′-GAT TAA TAT ATG GGA TCC AGC TAT GAA AGT (position 259) and 5′-ACT GGA TCC TCC AAA GCG AT-3′ (position 2441). The PCR products were digested with *Bam*HI, ligated into the *Bam*HI site of pSK950, and introduced into *E. coli* DH5α

The 5′ termini of the *gyrB* genes of ISP794, MT5, and MT5224c9 were amplified by PCR with primers 5′-AAA GCG ATG GTG ACT GCA TTG-3′ (position 275) and 5′-GTG GCA TAT CCT GAG TTA TAT-3′ (position 1102 in the sequence published by Brockbank and Barth [5]), and PCR products were sequenced directly.

Sequencing. The sequences were determined with the ABI Fluorescent System and *Taq* Dye terminators (Qiagen). In order to verify that sequenced mutations were not introduced by Vent polymerase, each mutation was verified by direct sequencing on PCR products.

DNA transformation. Transformation of plasmids in *S. aureus* was performed by electroporation as previously described (4).

Chromosomal DNA was obtained by the procedure of Stahl and Pattee (33). Transformation with high-molecular-weight chromosomal DNA was performed as previously described (33).

Integration of plasmids derived from pSK950 into the chromosome. Plasmids derived from pSK950 were first introduced into *S. aureus* strains and grown at 30°C. pSK950 is derived from plasmid pCL84, which contains the *attP* site of staphylococcal phage L54a (18) and which is capable of integrating specifically into the chromosomal *attB* site located just 3′ of the *geh* gene, which encodes staphylococcal lipase. Integration is facilitated by the presence of plasmid pYL112Δ19, which carries the L54a *int* gene encoding integrase. pYL112Δ19 was introduced into the strains carrying the derivatives of pSK950 and selected at 30°C for 48 h on tetracycline (5 μg/ml) and chloramphenicol (10 μg/ml). A first shift to 42°C was done in TS broth with chloramphenicol (10 μg/ml) for 24 h, and another incubation was performed at 42°C on TS agar for 24 h. One colony was isolated on TS agar containing 2 μg of tetracycline per ml at 37°C.

The site-specific integration of the plasmid into the *S. aureus* chromosome was confirmed by scoring for loss of lipase activity resulting from the disruption of the lipase gene. The lipase assay was performed as previously described by plating overnight cultures on J_1 medium containing egg yolk (19). In order to determine that plasmids derived from pSK950 did not remain in the cells, the absence of these plasmids was verified by minipreps (Promega).

RESULTS

Sequencing of *grlB* and *grlA*. The entire *grlA* genes from ISP794 and MT5224c9 were amplified by PCR, generating a 2.7-kb fragment that was cloned into the *Bam*HI site of

TABLE 2. Sites associated with quinolone resistance in the quinolone-resistance-determining regions of GrlB and GyrB

Protein	Species	Type	Sequence and sites associated with quinolone resistance[a]	Mutation	Source or reference
GrlB (ParE)	*S. aureus*	Wild type	VEGDSAGGSA KLGRDRKFQA ILPLRGKVIN TEKARLEDIF KNEEINTIIT		
	S. aureus	Mutant	*	Asn470Asp	This study
	S. pneumoniae	Mutant	*	Asp435Asn	31
	E. coli	Mutant	*	Leu445His	4
GyrB	*S. aureus*	Wild type	VEGDSAGGST KSGRDSRTQA ILPLRGKILN VEKARLDRIL NNNEIRQMIT		
	S. aureus	Mutant	*	Asp437Asn	15
	S. pneumoniae	Mutant	*	Asp435Asn	27
	E. coli	Mutant	*	Asp426Asn	42
	S. aureus	Mutant	*	Arg458Gln	15
	E. coli	Mutant	*	Lys447Glu	42
	S. typhimurium	Mutant	*	Ser463Tyr	11

[a] Sites associated with quinolone resistance are indicated by asterisks.

pGEM3-zf(+). The entire *grlA* sequence of MT5224c9 was identical to that of ISP794 (data not shown).

The 2.2-kb PCR products containing the entire *grlB* gene were cloned into pGEM3-zf(+). The sequence of *grlB* from strain MT5224c9 revealed a single mutation in comparison to that of *grlB* from ISP794, an A-to-G change resulting in the replacement of an asparagine (codon AAT) by an aspartate (codon GAT) at position 470 (Table 2).

Sequencing of *gyrB*. The 5′ regions of *gyrB* genes (830 bp) from strains ISP794, MT5, and MT5224c9 were amplified by PCR, and the region of each gene from nucleotide 342 (codon 21) to 983 (codon 243) in the sequence published by Brockbank and Barth (5) was directly sequenced after PCR. Two mutations producing the substitution of two amino acids were observed in MT5 and MT5224c9 in comparison to the sequence of *gyrB* from strain ISP794: a T→G substitution at nucleotide 586 modifying codon 102 (ATT [Ile] to AGT [Ser]) and a G→T substitution at position 702 modifying codon 144 (AGA [Arg] to ATA [Ile]) (Fig. 1). These two mutations characterize the *gyrB142* allele. Two other silent mutations were observed in the DNA from MT5 and MT5224c9 in comparison to that from strain ISP794: a C-to-T change at nucleotide 617 and an A-to-G change at position 872. The *nov-142* marker originated in strain 655 (30) and was introduced into strain 8325 by several DNA outcrosses to produce MT5 (30, 37). As ISP794 was derived from strain 8325, the two silent mutations are likely due to strain-to-strain variabilities in the sequence of *gyrB*.

Genetic linkage of quinolone resistance and novobiocin hypersusceptibility phenotypes. To determine the relationship of the quinolone resistance and novobiocin susceptibility of MT5224c9, we performed both incross and outcross transformation experiments to define the linkage of these phenotypes to Ω(chr::Tn*917lac*)*2*, a Tn*917* transposon inser-

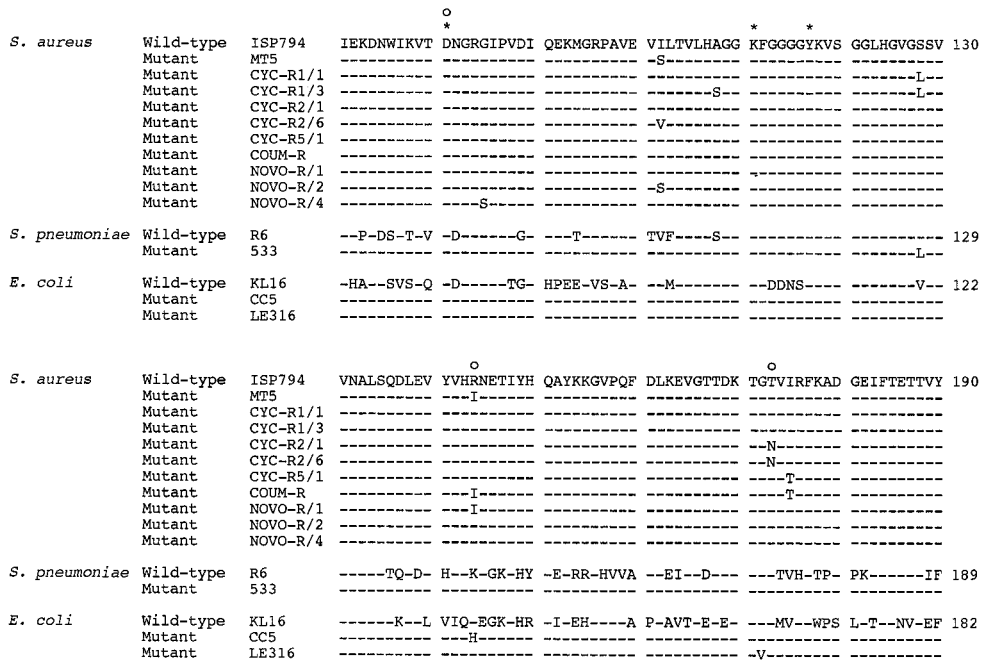

FIG. 1. Alignment of the amino acid sequences of GyrB involved in coumarin resistance (positions 71 to 190) from *S. aureus* (5), *S. pneumoniae* (22), and *E. coli* (1) and identification of positions of the mutations responsible for coumarin resistance. The asterisks and the circles indicate the sites involved in ATP (39) and novobiocin binding (20), respectively. The mutants of *S. aureus* (except MT5), *S. pneumoniae*, and *E. coli* GyrB were previously described (references 34, 22, and 6, respectively). Numbers at the right refer to the positions of the last amino acids.

TABLE 3. Outcross and incross of *flqA* (*grlB543*) locus by transformation with chromosomal DNA

Donor		Recipient		MIC (μg/ml) for recipient:		Phenotype of transformant[a]		No. of transformants
Strain	Genotype	Strain	Genotype	Ciprofloxacin	Novobiocin	Ciprofloxacin	Novobiocin	
ISP2133	*trp-489* Ω(chr::Tn*917lac*)*2*	MT5224c9	*nov* (*gyrB142*) *grlB543*	1.0[b]	5.0[c]	R	S	137
						S	R	109
EN3	*grlB543* Ω(chr::Tn*917lac*)*2*	MT5	*nov* (*gyrB142*)	0.25[c]	40.0[b]	S	R	92
						R	S	57

[a] S, susceptible; R, resistant. The phenotype was determined by plating each colony on ciprofloxacin at 0.125, 0.5, and 1.0 μg/ml and on novobiocin at 1.25 and 20 μg/ml. For scoring transformants for ciprofloxacin susceptibility, R was defined as growth at 0.125 and 0.5 μg/ml and no growth at 1.0 μg/ml and S was defined as growth at 0.125 μg/ml and no growth at 0.5 and 1.0 μg/ml. For scoring transformants for novobiocin susceptibility, R was defined as growth at 1.25 and 20 μg/ml and S was defined as growth at 1.25 μg/ml and no growth at 20 μg/ml.
[b] Value indicates resistance.
[c] Value indicates susceptibility.

tion encoding erythromycin resistance (Erm^r) and previously shown to be linked to *grlA* and *grlB* (25). For the incross experiment, high-molecular-weight chromosomal DNA from strain ISP2133 Ω(chr::Tn*917lac*)*2 grlA*$^+$ *grlB*$^+$ was used to transform MT5224c9, selecting for Erm^r. Forty-four percent of 246 Erm^r transformants showed both loss of quinolone resistance and an increase in novobiocin resistance (Table 3). The remaining Erm^r transformants had no change in these resistances from those of MT5224c9.

For the outcross experiment, chromosomal DNA was prepared from strain EN3 *grlB543* Ω(chr::Tn*917lac*)*2* and used to transform strain MT5 (*nov-142*), selecting for Erm^r. Thirty-eight percent of 149 Erm^r transformants exhibited increases in quinolone resistance and decreases in novobiocin resistance, with the remaining transformants showing no change relative to the recipient (Table 3). Thus, both the quinolone resistance and novobiocin susceptibility phenotypes of the *grlB543* mutants are 38 to 44% linked to Ω(chr::Tn*917lac*)*2*, and no dissociation of phenotypes was seen. These data indicate that the quinolone resistance and novobiocin susceptibility of *grlB543* are tightly linked and likely due to the single *grlB543* mutation.

Dominance characteristics of *grlB543* in multicopy and single copy. To determine the dominance characteristics of *grlB543*, the *grlB* genes of strains ISP794 (*grlB*$^+$) and MT5224c9 (*grlB543*) were cloned into hybrid shuttle plasmid pSK950. This plasmid carries the thermosensitive replicon of staphylococcal plasmid pE194, which is stable at 30°C but unstable at 42°C, and the replicon of *E. coli* plasmid pSC101. *grlB* was cloned directly from PCR products into pSK950, which has a low copy number in *E. coli* (10 copies per cell). We were able to clone *grlB* without its promoter in *E. coli* plasmid pGEM3-zf(+), a high-copy-number plasmid, but were unable to clone *grlB* with its promoter in this plasmid. The resulting plasmids, derived from pSK950, pBFISB (*grlB*$^+$), and pBFC9B (*grlB543*), were introduced into strains RN4220 and EN22, the latter being a strain derived from RN4220 in which the Ω(Tn*917lac*)*2* marker is linked to the *grlB543* chromosomal mutation. The effects of the introduction of these plasmids on drug susceptibility are shown in Table 4. RN4220 (*grlB*$^+$) carrying pBFC9B (*grlB543*) increased its resistance to norfloxacin (eightfold) relative to RN4220 containing pSK950. The effect of the mutated gene on resistance to quinolones ciprofloxacin, ofloxacin, and sparfloxacin was less (twofold). A surprising result was the absence of an effect of pBFC9B on the MICs of nalidixic acid and oxolinic acid. In addition to the effects of *grlB543* on quinolone resistance, novobiocin and coumermycin MICs were decreased fourfold. These data definitively demonstrated that the *grlB543* allele itself causes the quinolone resistance and novobiocin hypersusceptibility phenotypes. For the merodiploid of EN22 (*grlB543*) containing pBFISB (*grlB*$^+$) there were decreases in the MICs of the tested fluoroquinolones relative to those of the merodiploid containing plasmid-encoded *grlB543* (in strain RN4220); the coumarin MICs were similar. Thus, *grlB543* is codominant to *grlB*$^+$, with the phenotype determined by the allele present on the multicopy plasmid.

To determine further the dominance characteristics of *grlB543* in the presence of the novobiocin resistance mutations carried by *gyrB142*, plasmids were extracted from RN4220 and introduced by electroporation into derivatives of strain ISP794 (*gyrB*$^+$ *grlB*$^+$), MT5 (*gyrB142 grlB*$^+$), or MT5224c9 (*gyrB142 grlB543*) (Table 5). A pattern similar to that for RN4220 and EN22 was seen, confirming the codominance of fluoroquinolone resistance in *grlB* merodiploids. The modifications of the MICs of ciprofloxacin, norfloxacin, and ofloxacin in the presence of the different plasmids were, however, somewhat greater in these strains than in RN4220: 8-, 8- to 32-, and 4-fold, respectively. In the MT5 (*gyrB142*) background, the *grlB543* mutation decreased by four- to eightfold the MICs of coumarins. Conversely, *grlB*$^+$ increased by four- to eightfold the MICs of coumarins.

To determine the dominance characteristics of single copies of *grlB543* and *grlB*$^+$, pBFISB and pBFC9B were integrated

TABLE 4. Susceptibility patterns of *S. aureus* RN4220 strains diploid for *grlB*$^+$ and mutant *grlB*

Strain/plasmid	Type of *grlB*[a] in:		MIC (μg/ml)[b] of:							
	Chromosome	Plasmid	CPFX	NFLX	OFLX	SPFX	NA	OA	NOV	COU
RN4220/pSK950	Wild	None	0.5	1.0	0.5	0.06	64.0	2.0	0.16	0.01
RN4220/pBFC9B	Wild	N470D	1.0	8.0	1.0	0.12	32.0	2.0	0.04	0.0025
EN22/pSK950	N470D	None	1.0	8.0	1.0	0.12	64.0	2.0	0.04	0.0025
EN22/pBFISB	N470D	Wild	0.25	0.5	0.25	0.06	64.0	2.0	0.08	0.01

[a] Wild, wild-type gene; none, plasmid vector only; N470D, mutant gene (*grlB543*) encoding Asn470Asp mutation.
[b] CPFX, ciprofloxacin; NFLX; norfloxacin; OFLX, ofloxacin; SPFX, sparfloxacin; NA, nalidixic acid; OA, oxolinic acid; NOV, novobiocin; COU, coumermycin. The MICs are the means of three different transformants.

TABLE 5. Susceptibility patterns of *S. aureus* MT5 *gyrB142* strains diploid for *grlB*$^+$ and mutant *grlB*[a]

Strain/plasmid	Type of *grlB*[b] in:		Plasmid position[c]	MIC (μg/ml)[d]							
	Chromosome	Plasmid		CPFX	NFLX	OFLX	SPFX	NA	OA	NOV	COU
MT5/pSK950	Wild	None	Plasmid	0.125	0.5	0.25	0.06	128.0	4.0	40.0	5.0
MT5/pBFC9B	Wild	N470D	Plasmid	1.0	4.0	1.0	0.12	128.0	2.0	10.0	1.25
MT5224c9/pSK950	N470D	None	Plasmid	1.0	8.0	1.0	0.12	128.0	4.0	5.0	1.25
MT5224c9/pBFISB	N470D	Wild	Plasmid	0.125	0.25	0.25	0.06	128.0	4.0	40.0	5.0
MT5/pSK950	Wild	None	Chromo.	0.125	0.25	0.25	ND[e]	ND	ND	40.0	5.0
MT5/pBFC9B	Wild	N470D	Chromo.	0.5	1.0	0.5	ND	ND	ND	20.0	5.0
MT5224c9/pSK950	N470D	None	Chromo.	1.0	4.0	1.0	ND	ND	ND	2.5	0.62
MT5224c9/pBFISB	N470D	Wild	Chromo.	0.25	1.0	0.5	ND	ND	ND	40.0	5.0

[a] MT5 and MT5224c9 carry a double mutation of *gyrB* (Ile102Ser and Arg144Ile).
[b] Wild, wild-type gene; none, plasmid vector only; N470D, mutant gene (*grlB543*) encoding Asn470Asp mutation.
[c] Position of the plasmid: free in the cell (plasmid) or integrated in the chromosome (chromo.).
[d] CPFX, ciprofloxacin; NFLX; norfloxacin; OFLX, ofloxacin; SPFX, sparfloxacin; NA, nalidixic acid; OA, oxolinic acid; NOV, novobiocin; COU, coumermycin. The MICs are the means of three different transformants.
[e] ND, not determined.

into the chromosomal lipase gene. MICs for the strains carrying both *grlB*$^+$ and *grlB543* on the chromosome were identical for both merodiploids and corresponded to an average of the MICs of quinolones for the wild-type and resistant strains, indicating codominance for quinolone resistance (Table 5). In contrast, in MT5 (*gyrB142*) with chromosomally integrated merodiploids, *grlB*$^+$ appeared dominant over *grlB543* for coumarin resistance (Table 5).

Effects of mutations in *grlA*, *grlB*, and *gyrA* alone and in combination on susceptibility to quinolones and novobiocin. In a previous work, we showed that a mutation in *gyrA* encoding GyrA (Ser-84 to Leu [Ser84Leu]) caused no fluoroquinolone resistance in the absence of a *grlA* mutation (25). To expand these observations, we determined the MICs of additional quinolones for strains with single, dual, and triple mutations (Table 6). In the absence of mutations in the *grlB* and *grlA* genes, the MICs of the fluoroquinolones tested were unchanged for the strain carrying the *gyrA* mutation. However, the MIC of nalidixic acid was increased fourfold. When the *grlA* mutation was combined with the *gyrA* mutation, a major increase in the MIC of sparfloxacin in comparison to that produced by the *grlA* mutation alone was observed: 133-fold. Increases of 16-, 8-, and 4-fold were observed for the MICs of ciprofloxacin, DU6859a, and norfloxacin, respectively. In contrast, for the combination of the *grlB* and *gyrA* mutations, MICs for most of the quinolones were unchanged or increased only twofold in comparison with MICs associated with *grlB* alone. The only substantial effect of this combination was observed with sparfloxacin: a 16-fold increase. For nalidixic acid, neither the *grlA* nor *grlB* mutation conferred resistance when present alone, nor did they increase resistance in combination with the *gyrA* mutation.

DISCUSSION

Spontaneous mutant MT5224c9 was previously obtained in one step by plating MT5 *gyrB142* on ciprofloxacin (37). This mutant showed an increase in the MIC of ciprofloxacin (about fourfold) and a decrease in the MIC of novobiocin (about eightfold) in comparison to its parent, MT5. In a previous study, the mutation in MT5224c9 was localized to *Sma*I chromosomal fragment A by its linkage to a Tn*551* insertion in this fragment (37), which also carries the *grlB* and *grlA* genes (25). This previous observation suggested that the resistance to quinolones was due to a mutation in topoisomerase IV. However, no mutation was found in the region of *grlA* in which several mutations responsible for quinolone resistance in other mutants had been found (8, 25, 41). These mutations in *grlA* did not affect novobiocin resistance (25). We have shown here that MT5224c9 contains a novel *grlB543* mutation encoding Asp-470 of GrlB, which is responsible for both quinolone resistance and coumarin hypersusceptibility.

The *nov-142* locus of *S. aureus* has been used for genetic mapping and has been presumed to be *gyrB*, as are novobiocin resistance loci in other species (6, 13, 21, 22, 34–36), based on its close linkage to *gyrA*, which is adjacent to *gyrB* in *S. aureus* (25). Sequencing the *gyrB* gene of MT5, a *nov-142* mutant (37), revealed two different mutations, Ile102Ser and Arg144Ile. These two mutations were previously described individually, but not together, as being in the *gyrB* genes of coumarin-

TABLE 6. Effects of *grlA*, *grlB*, and *gyrA* mutations on the activities of selected quinolones

Strain	Genotype	MIC (μg/ml) of:						
		Ciprofloxacin	Norfloxacin	DU-6859a	Sparfloxacin	Nalidixic acid	Oxolinic acid	Novobiocin
RN4220		0.5	1.0	0.0312	0.12	64.0	2.0	0.16
BF2	*nov* (*gyrB142*)	0.5	1.0	ND[a]	ND	64.0	2.0	40.0
EN5	*nov* (*gyrB142*) *gyrA*	0.5	1.0	0.0625	0.12	256.0	4.0	40.0
EN22	*grlB543*	2	16.0	0.0625	0.12	64.0	4.0	0.08
EN20	*grlA542*	2	16.0	0.0625	0.12	64.0	4.0	0.16
EN14	*nov* (*gyrB142*) *grlB543 gyrA*	4	8.0	0.125	2.0	128.0	8.0	10.0
EN8	*nov* (*gyrB142*) *grlA542 gyrA*	32	64.0	0.5	16.0	256.0	128.0	40.0

[a] ND, not determined.

resistant strains of *S. aureus* and had not been linked specifically to the *nov* genetic locus (34) (Fig. 1). The second mutation at position 144 has also been described for *E. coli gyrB* (7). As the novobiocin and coumermycin MICs for MT5 are particularly high, it is not surprising that two mutations may be involved in this level of coumarin resistance. MICs of coumarins for strains carrying the individual Ile102Ser and the Arg144Ile mutations are 8 and 16 μg of novobiocin/ml and 0.03 and 1 μg of coumermycin/ml, respectively (34). The MICs for MT5 are 40 μg of novobiocin/ml and 5 μg of coumermycin/ml, values compatible with the incremental effects of the two mutations.

grlB has similarities to *gyrB*, including conservation of the domains involved in ATPase activity, resistance to novobiocin, and resistance to quinolones (Fig. 1; Table 2) (8). A single GrlB mutation (Asn470Asp) was found in MT5224c9, in comparison to strain ISP794. Since this mutation was associated with two phenotypes, increased quinolone resistance and decreased resistance to coumarins, it seemed possible that an additional undetected mutation might be responsible for one of the phenotypes and that the *grlB* mutation might be responsible for the other. Several findings indicated, however, that the *grlB* mutation alone is responsible for both effects on quinolone and coumarin susceptibility. First, the mutant was obtained in a single step with a frequency of 5×10^{-9} (37), a frequency consistent with a single mutational event. Second, no other mutation was found in the entire extent of the *grlB* and *grlA* genes. Third, the genetic incross and outcross experiments showed no segregation of the quinolone and novobiocin phenotypes. Finally, the introduction of the plasmid carrying the mutant *grlB* gene into a quinolone-susceptible strain increased quinolone resistance and decreased novobiocin resistance.

In *E. coli* a region (PLKGKILN; amino acids 451 to 458, numbered as in *S. aureus* GrlB) is highly conserved between ParE (homologous to GrlB) and GyrB (4). This region is also conserved between GrlB (ParE) subunits of *S. aureus* (8) and *S. pneumoniae* (28). The Asn470Asp mutation encoded by the *grlB543* allele is located slightly outside this conserved region. A quinolone resistance mutation also in this region in *Salmonella typhimurium gyrB* (position 463) has been described (11) (Table 2). By alignment with the sequence of the yeast topoisomerase II, position Asn-470 was found to be homologous to Asn-493 (data not shown). The crystal structure of yeast (*Saccharomyces cerevisiae*) topoisomerase II indicated that Asn-493 is on the B′α3 helix, which is distant from the active-site tyrosine directly involved in DNA strand breakage (2). The mechanism by which a mutation distant from the active site may affect quinolone activity is unknown. GrlA and GyrA mutations involved in quinolone resistance are, in contrast, close to the active-site tyrosine. It has been postulated that the active-site region of DNA gyrase, and by homology of topoisomerase IV, may constitute the region of quinolone binding, since GyrA mutations result in decreased drug binding (40). No structural data on topoisomerase-DNA-quinolone complexes have, however, been reported. Therefore, the exact mode of binding of quinolones to the enzyme-DNA complex remains unknown. The pattern of resistance due to mutations in *E. coli gyrB* had led to the hypothesis that direct electrostatic interactions of quinolones with GyrB amino acid residues might be involved in quinolone binding (42). This possibility seems unlikely for the GrlB (Asn470Asp) mutation, however, because the negative charge of the mutant amino acid is predicted by this hypothesis to increase the affinity of GrlB for quinolones with a positively charged piperazinyl group (ciprofloxacin or norfloxacin), thereby increasing susceptibility, rather than causing resistance, to these drugs (42). Because the phenotype of GrlB (Asp-470) is resistance to amphoteric quinolones, our findings are not consistent with such a model.

Another hypothesis is that the *grlB* mutation attenuates the intrinsic catalytic efficiency of the enzyme. Two mutants of yeast topoisomerase II carrying mutations in the PLRGKMLN region (Leu480Pro and Arg476Gly, each in combination with Leu475Ala, corresponding to positions 457, 453, and 452 in GrlB of *S. aureus*, respectively) were selected for resistance to amsacrine, a DNA topoisomerase-targeting drug that acts analogously to quinolones in forming drug-enzyme-DNA complexes. The mutant enzymes also exhibited decreased relaxation of supercoiled DNA with or without amsacrine (38). Thus, in such a model, if quinolone binding to the enzyme-DNA complex occurs only at certain steps in the catalytic cycle, then enzyme conformational changes that slow the catalytic cycle might reduce the steady-state level of enzyme-DNA complexes in the proper conformation to bind quinolones. This reduction is postulated to be sufficient to reduce the number of drug-enzyme-DNA complexes that initiate events leading to cell death and thereby cause drug resistance but to be insufficient to impair cell growth in the absence of a drug. Such conformational changes might act by reducing DNA binding, by altering subunit association, by altering enzyme affinity for ATP, or by impairing other steps in the catalytic cycle. We currently have no information about the catalytic efficiency of purified topoisomerase IV containing GrlB (Asp-470). Although MT5224c9 grows identically to its parent strain, MT5 (data not shown), this does not exclude a possible catalytic defect in topoisomerase IV containing GrlB543, since the *gyrB* (Arg-136) coumarin resistance mutation has been shown to impair enzyme catalytic function but not cell growth (6).

The codominance of fluoroquinolone resistance seen with multicopy and single-copy mutant alleles of *grlB* is similar to that seen with multicopy alleles of either $parE^+$ or the resistance mutant *parE* gene (4) and $parC^+$ or the resistance mutant *parC* gene (equivalent to *grlA*) in *E. coli* (16). In contrast, the dominance of $grlA^+$ by gene dosage in *S. aureus* was reported to require the additional presence of plasmid-encoded *grlB* (41). This discrepancy might be explained if *grlA* alone was not expressed from the complementary plasmid. Our preliminary results indicated that the *grlA* gene cloned alone with its putative promoter is not expressed (or is only poorly expressed) and may need the *grlB* promoter to be expressed (data not shown).

The *grlB543* mutation also increases coumarin susceptibility in both *nov* and nov^+ cells (Table 4). Coumarins act by competitively inhibiting ATP binding to the B subunit of DNA gyrase (10, 12). All of the mutations that result in coumarin resistance lie at the periphery of the ATP-binding site of the B subunit of gyrase (6, 7, 13, 22, 34, 36) (Fig. 1). Recently, the crystal structure of a complex between the 24-kDa N-terminal fragment of the *E. coli* DNA gyrase B protein and novobiocin was described (20). Novobiocin and ATP, which are dissimilar in chemical structure, bind to the protein in different ways, but their binding is competitive because of the overlap of their binding sites (Fig. 1) (20). Hypersusceptibility to coumarins might be envisioned to occur if topoisomerase IV containing GrlB543 had reduced affinity for ATP. In such a circumstance coumarins become more effective as competitors of ATP binding. Unfortunately, no crystal structure that includes both the region of amino acid 470 of GrlB (or its equivalent) and the coumarin-binding site has yet been reported.

The 50% inhibitory concentration (IC_{50}) of novobiocin for inhibition of decatenation of wild-type *S. aureus* topoisomerase

IV (30 μg/ml) (3) is 54- to 167-fold higher than the IC_{50}s of novobiocin for inhibition of DNA supercoiling by wild-type *S. aureus* DNA gyrase (0.18 to 0.56 μg/ml) (23, 26). Thus, in *S. aureus*, topoisomerase IV is considerably less sensitive to novobiocin than DNA gyrase. In the *gyrB142* background, topoisomerase IV may, however, become the actual target of novobiocin when gyrase is first protected from inhibition by mutation, since *grlB543* is epistatic to *gyrB142* for coumarin susceptibility.

Codominance for coumarin resistance was observed when the $grlB^+$ or *grlB543* alleles were on multicopy plasmids. In contrast, when plasmids were integrated into the chromosome, the wild-type gene appeared dominant over the mutant gene (Table 5). The codominance of fluoroquinolone resistance in these chromosomal merodiploids makes it unlikely that the dominance of $grlB^+$ for coumarin susceptibility is due to a modification of the expression of the *grlB* gene integrated in the lipase gene. One possible explanation for this finding is that GrlB543 subunits form complexes with GrlA subunits poorly. When *grlB543* is hyperexpressed on plasmids, the GrlB543 subunits are in excess and form mutant enzymes in sufficient numbers to affect the cell phenotype, but when GrlB543 and $GrlB^+$ subunits are present in similar numbers, holoenzyme complexes form dominantly with $GrlB^+$ subunits.

The *gyrA* (Leu-84) mutation is silent for fluoroquinolone resistance in the absence of *grlA* and *grlB* mutations, but unexpectedly GyrA (Leu-84) causes resistance to nalidixic acid, a nonfluorinated member of the quinolone group. The opposite effect is observed with *grlA* and *grlB* mutations: increased MICs of fluoroquinolones (ciprofloxacin and norfloxacin) but no modification of the MIC of nalidixic acid (Table 6). Although chromosomal $grlA^+$ or $grlB^+$ is epistatic to chromosomal mutant *gyrA* in determining fluoroquinolone susceptibility (25), this is not the case for nalidixic acid susceptibility. This pattern suggests that the fluorine substituent at position 6 on the quinolone nucleus may specifically enhance activity against topoisomerase IV.

When the *gyrA* mutation was combined with the *grlA* mutation, MICs of all tested fluoroquinolones were substantially increased in comparison to those associated with the individual mutations. In contrast, the combination of a *gyrA* mutation with a *grlB* mutation only increased the MIC of sparfloxacin. The consequences of a *grlA* mutation in the presence of a *gyrA* mutation differ from those of a *grlB* mutation, indicating that the combined effects of these two mutations are different. Also noteworthy is the observation that for sparfloxacin and DU6859a either a *grlA* or a *gyrA* mutation alone had little or no effect on activity. The combination of these same two mutations, however, produced substantial increments in resistance (16-fold for DU6859a and 128-fold for sparfloxacin). This pattern is consistent with the hypothesis that these drugs have similar activities in vivo against both DNA gyrase and topoisomerase IV (4). The findings with nalidixic acid, which appears to target gyrase, and those with other fluoroquinolones (ciprofloxacin and norfloxacin), which appear to target topoisomerase IV, amplify previous observations for *S. pneumoniae* (29), indicating that either or both enzymes may be targets of the quinolone drugs depending on drug structure. A study of a larger series of compounds should allow better definition of structural features that determine the selectivity of drug targeting. Our findings also indicate that alterations in topoisomerases may have pleiotropic effects not only on different classes of inhibitors such as fluoroquinolones and coumarins but also on inhibitors within the same class such as ciprofloxacin and sparfloxacin for *grlB* and *grlA* mutations or nalidixic acid and oxolinic acid for the *gyrA* mutation. Full understanding of drug action and resistance at the molecular level must take into account both inhibitor structure-activity relationships and the effects of different classes of topoisomerase mutants.

In summary, the *nov-142* locus in MT5 has a double mutation in the GyrB protein: Ile102Ser and Arg144Ile, which likely explains the high level of resistance of the strain to coumarins. The *grlB* mutation encoding the Asn470Asp substitution in the GrlB protein of MT5224c9 causes an increase in quinolone resistance and a decrease in coumarin resistance. The combination of *grlA* (Phe-80) and *grlB* (Asp-470) mutations with the *gyrA* (Leu-84) mutation had differing effects on drug susceptibility. For sparfloxacin and DU6859a the pattern of minimal to no resistance caused by single *grlA*, *grlB*, and *gyrA* mutations and substantial resistance caused by the same mutations together suggests these drugs have dual primary targets.

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