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Mechanisms and Frequency of Resistance to Premafloxacin in *Staphylococcus aureus*: Novel Mutations Suggest Novel Drug-Target Interactions

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Premafloxacin is a novel 8-methoxy fluoroquinolone with enhanced activity against *Staphylococcus aureus*. We found premafloxacin to be 32-fold more active than ciprofloxacin against wild-type *S. aureus*. Single mutations in either subunit of topoisomerase IV caused a four- to eightfold increase in the MICs of both quinolones. A double mutation (*gyrA* and either *grlA* or *grlB*) caused a 32-fold increase in the MIC of premafloxacin, while the MIC of ciprofloxacin increased 128-fold. Premafloxacin appeared to be a poor substrate for NorA, with NorA overexpression causing an increase of twofold or less in the MIC of premafloxacin in comparison to a fourfold increase in the MIC of ciprofloxacin. The frequency of selection of resistant mutants was 6.4×10^{-10} to 4.0×10^{-7} at twofold the MIC of premafloxacin, 2 to 4 $\log_{10}$ less than that with ciprofloxacin. Single-step mutants could not be selected at higher concentrations of premafloxacin. In five single-step mutants, only one previously described uncommon mutation (Ala116Glu), and four novel mutations (Arg43Cys, Asp69Tyr, Ala176Thr, and Pro157Leu), three of which were outside the quinolone resistance-determining region (QRDR) were found. Genetic linkage studies, in which incross of $grlA^+$ and outcross of mutations were performed, showed a high correlation between the mutations and the resistance phenotypes, and allelic exchange experiments confirmed the role of the novel mutations in *grlA* in resistance. Our results suggest that although topoisomerase IV is the primary target of premafloxacin, premafloxacin appears to interact with topoisomerase IV in a manner different from that of other quinolones and that the range of the QRDR of *grlA* should be expanded.

Infections due to highly resistant *Staphylococcus aureus* are widespread, and studies to develop novel antibiotics, including fluoroquinolones, against these organisms are continuing. Premafloxacin is a novel fluoroquinolone initially developed for veterinary use (29, 32). Differing from ciprofloxacin, premafloxacin has a pyrrolidinyl substitute at position 7, which has been reported to increase activity against gram-positive bacteria (4), and an 8-methoxy group, which has been associated with increased bactericidal activity in some bacteria (19, 33, 34). Its activity against gram-negative bacteria has been reported to be equivalent to that of other quinolones, but its activity against staphylococci has been shown to be 16- to 32-fold more than that of ciprofloxacin (32). The interaction of premafloxacin with its target enzymes and the mechanisms of resistance of this potent fluoroquinolone, however, have not yet been determined.

Fluoroquinolones exert their bactericidal activity by interacting with two type 2 topoisomerases, DNA gyrase (subunits encoded by the *gyrA* and *gyrB* genes) and topoisomerase IV (subunits encoded by the *grlA* and *grlB* genes for *S. aureus*, and termed *parC* and *parE* for other organisms). These drugs act as topoisomerase poisons, trapping the enzyme reaction intermediate at a step in which the enzyme is bound to DNA and can be converted to a form in which both strands of DNA are cleaved (5, 7, 12). Resistance to quinolones arises from mutations in the primary enzyme target, with mutations in the secondary target enzyme only contributing to resistance in the presence of mutations in the primary target (13). Mutations reported to date in genetically defined resistant mutants for all quinolones cluster in a domain termed the quinolone resistance-determining region (QRDR), which is thought to be the quinolone-binding domain. For *S. aureus*, the primary target of most quinolones is topoisomerase IV. In *Streptococcus pneumoniae*, however, the primary target of sparfloxacin (22) and gatifloxacin (11) has been shown to be DNA gyrase, and clinafloxacin has been shown to target both enzymes similarly (23). A quinolone with dual targets and low frequency of resistant mutant selection in vitro is predicted to carry a lower risk of selection of resistance in clinical settings.

In this study we evaluated the mechanism of action of and resistance to premafloxacin. The effect of mutations in topoisomerase IV and DNA gyrase in genetically defined mutants of *S. aureus* was studied, and mutants selected with premafloxacin were characterized. Although topoisomerase IV was found to be the primary drug target, surprisingly, four novel *grlA* mutations, three of which were outside the QRDR were identified, suggesting a novel mechanism of interaction of premafloxacin with the DNA-enzyme complex.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are shown in Table 1. *Escherichia coli* strains were grown in Luria-Bertani medium, and *S. aureus* strains were grown in brain heart infusion medium. All strains were grown at 37°C except the *S. aureus* strains carrying the thermosensitive plasmids derived from pCL52.1, which were grown at 30°C. Spectinomycin was used at a concentration of 50 μg/ml, and tetracycline was used at a concentration of 5 μg/ml except during integration of the plasmid into the chromosome, when it was used at a concentration of 3 μg/ml.

Drug susceptibility determinations. Premafloxacin was kindly provided by Pharmacia & Upjohn Pharmaceuticals, and ciprofloxacin was provided by Bayer Corporation. Nalidixic acid, novobiocin, and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, Mo.). MICs were determined on Trypticase soy agar containing serial twofold dilutions of antibiotics. Incubations were done at 37°C, and growth was scored at 24 and 48 h. MICs of nalidixic acid were used

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype[a]	Source, reference, or comment
S. aureus		
ISP794	8325 *pig-131*	25
MT5	8325 *nov* (*gyrB142*) *hisG15 pig-131*	10, 26
SS1	8325 *pig-131 nov* (*gyrB142*) *gyrA* (Ser84Leu)	1
MT5224c2	8325 *nov* (*gyrB142*) *hisG15 pig-131 flqA552*	21
MT5224c3	8325 *nov* (*gyrB142*) *hisG15 pig-131 flqA552*	26
MT111	8325 *pig-131 grlA548* (Ala116Glu)	26
MT5224c4	8325 *nov* (*gyrB142*) *hisG15 pig-131 grlA542* (Ser80Phe)	26
MT5224c9	8325 *nov* (*gyrB142*) *hisG15 pig-131 grlB543* (Arg470Asp)	10, 26
MT23142	8325 *pig-131 flqB* Ω(chr::Tn*916*)*1108*	20
EN1252a	8325 *nov* (*gyrB142*) *hisG15 pig-131 grlA542* (Ser80Phe) *gyrA* (Ser84Leu) Ω*1051* (Erm) Nov^+	1
MT1222	8325 *pig-131 grlA553* (Ala116Glu) *flqB gyrA* (Ser84Leu)	26
RN4220	8325-4, r^-	15
EN6	RN4220 *nov-142 gyrA*	21
EN14	RN4220 *grlB543* Ω(chr::Tn*917lac*)*2 nov* (*gyrB142*) *gyrA* (Ser84Leu)	21
EN20	RN4220 *grlA542* Ω(chr::Tn*917lac*)*2*	21
EN9	RN4220 *grlA543* Ω(chr::Tn*917lac*)*2 nov* (*gyrB142*) *gyrA*	21
P4	ISP794 *grlA552* (Arg43Cys)	This study; spontaneous PRFX-R[b]
P5	ISP794 *grlA553* (Ala116Glu)	This study; spontaneous PRFX-R
P6	ISP794 *grlA554* (Pro157Leu)	This study; spontaneous PRFX-R
P10	ISP794 *grlA555* (Ala176Thr)	This study; spontaneous PRFX-R
P21	ISP794 *grlA556* (Asp69Tyr)	This study; spontaneous PRFX-R
DIP4	ISP794 *grlA552*	This study, allelic exchange mutant
DIP6	ISP794 *grlA554*	This study; allelic exchange mutant
DIP10	ISP794 *grlA555*	This study; allelic exchange mutant
DIP21	ISP794 *grlA556*	This study; allelic exchange mutant
ISP2133	8325 *pig-131 trp-489* Ω(chr::Tn*917lac*)*2*	26
ISP2134	8325 *pig-131 trp-489* Ω(chr::Tn*917lac*)*1*	26
ISP225	Ps55	26
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		
pGEM3-zf(+)	3,199-bp cloning vector, Ap^r	Promega
pCL52.1	8,100-bp plasmid containing the replicon of pGB2, Sp^r (*E. coli*) and temperature-sensitive replicon of pE194, Tc^r	16

[a] Abbreviations: Ap, ampicillin; Sp, spectinomycin; Tc, tetracycline.
[b] PRFX-R, premafloxacin resistance.

to screen for *gyrA* mutations, MICs of novobiocin were used to screen for certain *grlB* mutations (10), and MICs of ethidium bromide were used to screen for NorA overexpression (14).

Frequency of selection of mutants. Mutants were selected by plating *S. aureus* ISP794 on brain heart infusion agar containing ciprofloxacin or premafloxacin at two-, four-, and eightfold above the MIC of each drug. Selection plates were incubated at 37°C. Mutation frequencies were calculated as the ratio of the number of resistant colonies at 48 h to the number of cells inoculated.

Limit levels of resistance with serial passage. *S. aureus* ISP794 was serially passaged on brain heart infusion agar containing twofold-increasing concentrations of premafloxacin to define the highest level of resistance achievable.

Sequence analysis. Chromosomal DNA from various single-step and multiple-step mutants of *S. aureus* ISP794 was used as a template. PCR for the QRDRs of *grlA*, *grlB*, *gyrA*, *gyrB*, and the promoter region of *norA* was performed using Vent DNA polymerase (New England Biolabs) (annealing temperature of 53°C for *grlA* and *grlB* and *gyrA* and *gyrB* and 55°C for the *norA* promoter) using primers and PCR conditions previously reported (10). DNA sequencing of the PCR products was performed with the ABI fluorescent system and *Taq* dye terminators (Qiagen). For strains in which no mutation was found in these regions, the entire *grlA*, *grlB*, *gyrA*, and *gyrB* genes were amplified by PCR and sequenced by the same methods.

DNA transformation. For transformation in *S. aureus*, high-molecular-weight DNA was prepared by the method of Stahl and Pattee (25), and cells were made competent for transformation as described by Lindberg et al. (17), except that the original inoculum was grown in Trypticase soy broth at 35°C overnight in a shaking water bath. Bacteriophage Φ55 was maintained on strain ISP225 and was used to render the *S. aureus* strains competent for transformation.

Correlations of resistance phenotype and genotype in genetic crosses. To correlate the resistance phenotype with specific mutations, 10 resistant and 10 susceptible transformants from crosses in which DNA from ISP2133 was used to transform mutants P4, P6, P10, and P21 were selected for PCR amplification and sequencing of the relevant regions of *grlA* or *grlB*. PCR primers used for amplification of *grlA* fragments encompassing the mutations in P4 and P21 were 5′-AGTTGAAGATGAAGTGCGTT-3′ (5′ nucleotide at position 2196 in the sequence published by Yamagishi et al. [31]) and 5′-ACCATTGGTTCGAGTG TCGT-3′ (5′ nucleotide at position 2839). For amplification of fragments containing mutations from strains P6 and P10, the primers were 5′-GCTGAAGA GTTATTACGTGA-3′ (5′ nucleotide at position 2757) and 5′-TGGACGACC ATCACTAATAGCGA-3′ (5′ nucleotide at position 3389). The *grlA* (Ala176Thr) mutation in mutant P10 is marked by loss of the *Bst*UI site, and the *grlA* (Asp69Tyr) mutation in mutant P21 is marked by loss of the *Hph*I site (New England Biolabs) within the PCR fragments. For studies with these two mutants, restriction patterns were examined on agarose gels. For the other mutants, DNA sequencing of PCR products was performed to determine the presence and absence of mutations in resistant and susceptible transformants.

Cloning and allelic exchange. Internal fragments (1,312 bp) of *grlA* and *grlB* from mutants P4, P6, P10, and P21 were amplified by PCR with the upstream primer containing an engineered *Eco*RI site (5′-GAAGAATTCTGGGAAACG ACG-3′ [position 2140]) and the downstream primer containing an engineered *Bam*HI site (5′-GCAGGATCCTCAATTTGGTGATT-3′ [position 3451]), using an annealing temperature of 58°C and an extension time of 1 min 25 s. PCR products were ligated into the *Eco*RI and *Bam*HI sites of pGEM3-zf(+), and the recombinant plasmids were electroporated into *E. coli* DH5α. The insert for each mutant was sequenced from the plasmid to verify that no new mutation was introduced during PCR amplification. After restriction and purification of the inserts from the pGEM3-zf(+) by gel extraction, they were ligated to the *Eco*RI and *Bam*HI site of pCL52.1, a thermosensitive shuttle plasmid, and introduced into *E. coli* DH5α again by electroporation. The resultant plasmids were first

TABLE 2. Activities of ciprofloxacin and premafloxacin against genetically defined mutants of *S. aureus*

Strain	Mutation(s)	MIC (μg/ml) of:	
		Ciprofloxacin	Premafloxacin
ISP794	Wild-type (parent)	0.125–0.25 (0.14)[a]	0.004–0.008 (0.006)
MT5	*gyrB142*	0.25	0.008
SS1	*gyrA* (Ser84Leu)	0.25 (0.14)	0.008–0.016 (0.011)
MT5224c2	*grlA* (Ala116Pro)	1.0	0.032
MT5224c3	*grlA* (Ala116Glu)	2.0	0.032
MT111	*grlA* (Ala116Glu)	1.0	0.032
MT5224c4	*grlA* (Ser80Phe)	1.0	0.032
MT5224c9	*grlB* (Arg470Asp)	2.0	0.032
MT23142	*flqB*	0.5–1.0	0.008–0.016
EN1252a	*grlA* (Ser80Phe) *gyrA* (Ser84Leu)	32	0.25
MT1222	*grlA* (Ala116Glu) *gyrA* (Ser84Leu) *flqB*	32	0.25
RN4220	8325-4, r^-, parent	0.5	0.016
EN6	*gyrA* (Ser84Leu)	0.5	0.016–0.032
EN14	*grlB* (Arg470Asn) *gyrA* (Ser84Leu)	4.0	0.125
EN20	*grlA* (Ser80Phe)	4.0	0.064
EN9	*grlA* (Ser80Phe) *gyrA* (Ser84Leu)	32	0.25

[a] Values in parentheses are from experiments using an arithmetic series of drug concentrations.

electroporated into *S. aureus* RN4220 and then to ISP794, as described previously (2). One colony from the resultant strains was incubated at 42°C for 24 h in the presence of 3 μg of tetracycline per ml of brain heart infusion broth to allow for integration of plasmid into the chromosome via homologous recombination and then plated on brain heart infusion agar containing the same concentration of tetracycline and incubated at 42°C for 36 to 48 h to isolate single colonies with the integrated plasmid (16). A single colony was then incubated in brain heart infusion medium without antibiotic selection for 48 h at 30°C to allow excision of the integrated plasmid from the chromosome. Appropriate dilutions of the 48-h culture were plated on medium without antibiotics to isolate single colonies, and these colonies were screened for tetracycline susceptibility and quinolone resistance. MICs of ciprofloxacin and premafloxacin for tetracycline-susceptible, quinolone-resistant colonies were determined, and the presence of the expected mutation was confirmed by sequencing of PCR products for the appropriate region amplified from chromosomal DNA.

RESULTS

Quinolone activities against genetically defined mutants. Table 2 shows the activities of premafloxacin and ciprofloxacin against previously selected, genetically defined mutants of *S. aureus*. Premafloxacin was 32-fold more active than ciprofloxacin against wild-type *S. aureus* ISP794 and a restriction-deficient mutant, RN4220. MICs of both premafloxacin and ciprofloxacin increased four- to eightfold in various *grlA* and *grlB* mutants of these two strains with altered subunits of topoisomerase IV. A single mutation in *gyrA* of DNA gyrase had no effect on the MIC of ciprofloxacin and about a twofold increase in the MIC of premafloxacin that was reproducible when an arithmetic series of drug concentrations was tested. In double mutants with *gyrA* and either *grlA* or *grlB* mutations, the MIC of premafloxacin (4.0 μg/ml) increased 32-fold, while the MIC of ciprofloxacin increased 128-fold. Overexpression of the NorA efflux pump in an *flqB* mutant caused an increase of twofold or less in the MIC of premafloxacin and a fourfold increase in the MIC of ciprofloxacin.

Frequency of selection of mutants. Table 3 shows the frequencies of selection of single-step resistant mutants of wild-type strain ISP794 with premafloxacin and ciprofloxacin. At twofold the MIC, the selection frequency was 2 to 4 $\log_{10}$ lower with premafloxacin, and at fourfold the MIC, mutants could not be selected with premafloxacin. In contrast, mutants were readily selected at four- and eightfold the MIC of ciprofloxacin.

Limit resistance. By stepwise selection on increasing concentrations of ciprofloxacin, the limit level of resistance was 32 μg/ml, 256-fold higher than the MIC of ciprofloxacin for the wild-type strain. In contrast, the limit level of resistance for premafloxacin was 1.0 μg/ml, 128-fold higher than the MIC of premafloxacin for the parent wild-type strain.

Characterization of mutants selected with premafloxacin. In single-step mutants, MICs of ciprofloxacin and premafloxacin increased four- to eightfold. MICs of novobiocin, nalidixic acid, and ethidium bromide did not change or showed a twofold change. In the serial-passage mutants, MICs of novobiocin decreased fourfold and MICs of nalidixic acid and ethidium bromide did not change or decreased twofold. Only one of the single-step mutants had a previously reported, albeit uncommon, mutation (Ala116Glu). In four other single-step mutants, novel mutations, three of which were outside the QRDR, were found (Table 4). These mutations included Arg43Cys, Asp69Tyr, Pro157Leu, and Ala176Thr. In two serial passage mutants, each from a different experiment, commonly occurring mutations in *grlA* (Ser80Phe) and *gyrA* (Ser84Leu) were

TABLE 3. Frequency of selection of resistant mutants

MIC (μg/ml) for ISP794	Selecting drug concn (Factor of MIC)		Frequency of mutation	
	Ciprofloxacin	Premafloxacin	Ciprofloxacin	Premafloxacin
0.016		2		6.4×10^{-10}–4.0×10^{-7}
0.032		4		9.1×10^{-10}–$<1.0 \times 10^{-9}$
0.064		8		$<9.1 \times 10^{-10}$–$<1.0 \times 10^{-9}$
0.5	2		2.8×10^{-6}–1.5×10^{-5}	
1.0	4		3.0×10^{-8}–6.1×10^{-8}	
2.0	8		$<4.5 \times 10^{-11}$–2.8×10^{-9}	

TABLE 4. Characteristics of mutants of *S. aureus* ISP794 selected with premafloxacin

Strain or mutant	MIC (μg/ml) of[d]:					Mutation in designated gene				
	PRFX	CPFX	NOV	NA	EB	*grlA*	*gyrA*	*grlB*	*gyrB*	*norA*[b]
ISP794	0.0004–0.008	0.125–0.25	0.064–0.128	64–128	2.0–4.0					
Single-step mutants										
P4	0.032	1.0	0.064	128–256	4.0	Arg43Cys	None	None	None	None
P5	0.064	2.0	0.064	256	2.0	Ala116Glu	None	None	None	None
P6	0.032	1.0	0.064	256	2.0	Pro157Leu	Met538Ile	None	None	None
P10	0.032	1.0	0.032–0.064	128	2.0	Ala176Thr	None	None	None	None
P21	0.032	1.0	0.064	128	4.0	Asp69Tyr	None	None	None	None
Serial-passage mutants										
PS1	0.5	16.0	0.032	128	2.0	Ser80Phe[a]	Ser84Leu[a]			
PS2	0.5	16.0	0.032	128	2.0	Ser80Phe[a]	Ser84Leu[a]			
PS3	0.5	8.0–6.0	0.064	256	4.0–8.0	Ala176Thr	Ser84Leu[a]	None[a]		AAAG insertion[c]

[a] QRDR.
[b] Promoter region of *norA*.
[c] Insertion is between the 95th and 96th nucleotide upstream of the ATG start codon of *norA*.
[d] Abbreviations: PRFX, premafloxacin; CPFX, ciprofloxacin; NOV, novobiocin; NA, nalidixic acid; EB, ethidium bromide.

found, and in a third one, one of the novel mutations in *grlA* (Ala176Thr) was found together with two other mutations, a common *gyrA* mutation (Ser84Leu) and a novel mutation in the upstream region of the *norA* gene (insertion of five nucleotides), which may account for the increase in the MIC of ethidium bromide in this mutant.

Genetic linkage of quinolone resistance loci. To define the relationship of these novel mutations to the quinolone resistance phenotype of the mutants, we performed genetic crosses using transformation with high-molecular-weight DNA. Tn*917* transposon insertions, Ω(chr::Tn*917lac*)*2* and Ω(chr::Tn*917lac*)*1*, encoding erythromycin resistance (Erm^r) have been previously shown to be linked to *grlA* and *grlB* (21). For an incross experiment, high-molecular-weight chromosomal DNA from strain ISP2133 Ω(chr::Tn*917lac*)*2* $grlA^+$ $grlB^+$ was used to transform premafloxacin-selected mutants, selecting for the erythromycin resistance of Tn*917*. Linkages similar to those previously reported for established resistance mutations in *grlA* (26) were found and were also shown to be similar to the linkage to *grlA* (Ser80Phe) in parallel experiments (Table 5). To demonstrate that these mutant loci were themselves sufficient to cause resistance, we performed outcross experiments in which ISP794 ($grlA^+$ $grlB^+$) was transformed with high-molecular-weight chromosomal DNA prepared from a resistant transformant obtained in the incross experiments for each mutant. Table 6 shows the linkage results for each premafloxacin-resistant mutant. In the outcross experiments, as in the incross experiments, the linkages of the resistance loci to Ω(chr::Tn*917lac*)*2* and Ω(chr::Tn*917lac*)*1* were similar to those reported for known *grlBA* resistance mutations (21).

Correlation of the presence of specific mutations in outcross transformants. To correlate specific mutations with the resistance phenotype in the transformants obtained in genetic crosses, we determined the presence or absence of mutation-specific restriction fragment length polymorphism patterns or the DNA sequences of the relevant regions of *grlA* of selected transformants. The mutation encoding Ala176Thr (TAC**A**CG) in P10 eliminates the *Bst*UI recognition site, CG ↓ CG, and the mutation encoding Asp69Tyr (GGT**T**AT) in P21 eliminates the *Hph*I recognition site, $GGTGA(N)_8$ ↓ (mutations are shown in boldface type, and vertical arrows show restriction enzyme cutting site). PCR products of nucleotides 2757 to 3389 of *grlA* of 10 resistant outcross transformants of ISP794 transformed with high-molecular-weight DNA from P10, and the P10 mutant all yielded only one band of 633 bp following *Bst*UI digestion, whereas 10 susceptible transformants all yielded two bands of 488 and 145 bp. Similarly, PCR products of nucleotides 2196 to 2839 of *grlA* of 10 resistant outcross transformants

TABLE 5. Genetic linkage of *grlA* mutations and resistance by transformation: incross of $grlA^+$

Donor		Recipient		MIC (μg/ml) for[a]:				% Susceptible (no. of susceptible strains/total no. of transformants)
				Recipient		Susceptible transformants		
Strain	Genotype	Strain	Genotype	PRFX	CPFX	PRFX	CPFX	
ISP2133	*trp-489* Ω(chr::Tn*917lac*)*2*	P4	*grlA* (Arg43Cys)	0.016	1.0	0.008	0.25	21 (15/70)
		P6	*grlA* (Pro157Leu) *gyrA* (Met538Ile)	0.032	1.0	0.008	0.25	39 (37/95)
		P10	*grlA* (Ala176Thr)	0.032	1.0	0.008	0.125	30 (31/104)
		P21	*grlA* (Asp69Tyr)	0.032	1.0	0.008	0.25	24 (24/100)
		MT5224c4	*grlA* (Ser80Phe)	0.032	1.0	0.008	0.25	21 (8/38)
ISP2134	*thrB494* Ω(chr::Tn*917lac*)*1*	P4	*grlA* (Arg43Cys)	0.016	1.0	0.008	0.25	7 (7/100)
		P6	*grlA* (Pro157Leu) *gyrA* (Met538Ile)	0.032	1.0	0.008	0.125	10 (12/115)
		P10	*grlA* (Ala176Thr)	0.032	1.0	0.008	0.125	9 (13/144)
		P21	*grlA* (Asp69Tyr)	0.032	1.0	0.008	0.25	9 (9/100)
		MT5224c4	*grlA* (Ser80Phe)	0.032	1.0	0.008	0.25	5 (8/171)

[a] Abbreviations: PRFX, premafloxacin; CPFX, ciprofloxacin.

TABLE 6. Genetic linkage of *grlA* mutations and resistance by transformation: outcross of mutations

Donor strain[a]	Genotype	MIC (μg/ml) for resistant transformants[b]		% Resistant (no. of resistant strains/total no. of transformants)
		PRFX	CPFX	
P43355	*grlA* (Arg43Cys) Ω(chr::Tn*917lac*)*2*	0.016–0.032	1.0	19 (23/120)
P6332	*grlA* (Pro157Leu) *gyrA* (Met538Ile) Ω(chr::Tn*917lac*)*2*	0.016–0.032	0.5–1.0	16 (17/104)
P1033232	*grlA* (Ala176Thr) Ω(chr::Tn*917lac*)*2*	0.016–0.032	1.0	28 (54/192)
P213360	*grlA* (Asp69Tyr) Ω(chr::Tn*917lac*)*2*	0.016–0.032	1.0	20 (14/70)

[a] Recipient ISP794 *grlA*$^+$ *grlB*$^+$
[b] Abbreviations: PRFX, premafloxacin; CPFX, ciprofloxacin.

of ISP794 transformed with high-molecular-weight DNA from P21 and the P21 mutant all yielded only three bands of 416, 176, and 52 bp, in contrast to the four bands (257, 176, 159, and 52 bp) found with the 10 susceptible transformants. Direct DNA sequencing of the PCR products of *grlA* from P4 and P6 revealed the respective presence and absence of the mutations in 10 resistant and 10 susceptible transformants from each outcross experiment.

Confirmation of the role of novel mutations in resistance by allelic exchange. Following integration of donor DNA containing the mutant sequences of *grlBA* into the chromosome of ISP794 using a thermosensitive shuttle plasmid in cells grown at nonpermissive temperature in the presence of tetracycline, cells were grown at permissive temperature in the absence of tetracycline to allow for plasmid excision. Tetracycline-susceptible cells (lacking the plasmid) were then screened for ciprofloxacin resistance. Tetracycline-susceptible, ciprofloxacin-resistant colonies in which allelic exchange is predicted were then tested for the presence of the expected mutation and for the MICs of ciprofloxacin and premafloxacin in relation to the original mutants. The MICs of ciprofloxacin (1.0 μg/ml) and premafloxacin (0.032 μg/ml) for the original and allele exchange mutants containing the GrlA Arg43Cys (P4 and DIP4), Ala176Thr (P10 and DIP10), and Asp69Tyr (P21 and DIP21) mutations were the same. Comparison of the original mutant P6, which had double mutations [GrlA (Pro157Leu), GyrA (Met538Ile)], with the allele exchange mutant DIP6, which had only the GrlA (Pro157Leu) mutation, revealed the same MIC of ciprofloxacin (1.0 μg/ml) and a slightly lower MIC of premafloxacin (twofold lower) for DIP6 (0.032 versus 0.064 μg/ml). Allelic exchange mutants DIP10, DIP21, DIP4, and DIP6 all contained the expected *grlA* mutations as shown by direct DNA sequencing of the PCR products of chromosomal DNA.

DISCUSSION

We found that premafloxacin was highly potent against *S. aureus*, being 32-fold more active than ciprofloxacin against both wild-type and *grlA* mutant strains of *S. aureus*. A four- to eightfold increase in the MIC of premafloxacin was seen in defined *grlA* and *grlB* mutants, but a *gyrA* mutation had a slight but reproducible effect (approximately twofold) on the activity of premafloxacin in the absence of a *grlA* mutation. This phenomenon has been reported before with quinolone-resistant mutants of *S. pneumoniae* (27). These data, together with the identification of *grlA* mutations in premafloxacin-selected, first-step mutants indicate that topoisomerase IV is the primary target of premafloxacin in *S. aureus*. In the presence of a mutation in the primary target enzyme, the intrinsic sensitivity of the secondary enzyme may determine the susceptibility of the cell to the quinolone. The increase in the MIC of premafloxacin for strains with *grlA gyrA* dual mutations in comparison to a strain with a *grlA* mutation alone was 8-fold, in contrast to a 32-fold increase in the MIC of ciprofloxacin. These findings further suggested that the intrinsic activity of premafloxacin against gyrase was greater than that of ciprofloxacin. This higher intrinsic activity might contribute to the lower frequency of selection of resistant mutants. For other quinolones that, like premafloxacin, have a C-8 methoxy group, the frequency of selection of resistant mutants is also low (6). Thus, the presence of the 8-methoxy group in premafloxacin may contribute to its low frequency of mutant selection.

In addition, the overexpression of the NorA multidrug resistance efflux pump in one mutant (20) had little or no effect on the activity of premafloxacin. Thus, premafloxacin appears to be a poor substrate for NorA and possibly for related pumps, thereby limiting the range of the types of mutations that can contribute to resistance to premafloxacin. This presumption is also supported by our findings that no premafloxacin-selected mutants exhibited a substantial increase in the MIC of ethidium bromide, a common substrate of multidrug resistance pumps.

Clustering of mutations near the 5′ ends of *grlA* and *gyrA* in *S. aureus* has led to the QRDRs being defined between residues 68 and 107 in GrlA and between residues 64 and 103 in GyrA (8). In genetic studies, mutations in three codons have been shown to cause quinolone resistance, Ser80Phe or -Tyr, Glu84Lys or -Leu, and Ala116Glu or -Pro (8, 21, 31). Mutations at codon 80 and 84 are more common than codon 116 mutations. Although a range of other mutations has been found in resistant clinical isolates, genetic studies to link the mutations to the resistance phenotype were often not done (13).

Strikingly, we did not select any of the common codon 80 and 84 mutations in *grlA* in our single-step mutants, and only one mutant had a previously described, but uncommon, Ala116Glu mutation. We have shown by genetic mapping that these novel mutations are indeed highly correlated with the resistance phenotype and by allelic exchange that all four mutations were definitively responsible for the resistance phenotype.

The X-ray crystallographic structure of the breakage-reunion domain of gyrase in *E. coli* localizes the QRDR to a positively charged surface that is predicted to bind the negatively charged phosphate backbone of DNA and that bridges the two GyrA subunits (3). The commonly occurring mutations map on α-helix 4 of GyrA, and one mutation (Ser83Trp) has been shown to cause reduced quinolone binding to the gyrase-DNA complex (30).

The novel resistance mutation in codon 69 in mutant P21 maps to the α3 helix, the first helix in the helix-turn-helix motif, which dominates the head dimer by packing side by side with the α3 helix of the other subunit (3). Although no other mutation in this domain has been reported in genetically defined

mutants, the same mutation (Asp69Tyr) has been reported in a resistant clinical isolate of *Mycoplasma hominis* (13), and we have shown here by allelic exchange experiments that this mutation is responsible for resistance.

Mutant P6 carried a particularly unusual double mutation, Pro157Leu in GrlA and Met538Ile in GyrA. The mutation in GrlA is localized to the region between the β5 strand and the α9 helix, which is not highly conserved and seems to lie on a plane below that of the active site Tyr. The role of the Met538Ile mutation in GyrA in the resistance phenotype of this strain was initially uncertain. We were able to outcross the quinolone resistance phenotype with the *grlA* mutation from P6. Also allelic exchange experiments showed that substitution of only the Pro157Leu mutation caused resistance. Thus, the *gyrA* mutation does not itself appear to contribute to resistance, nor does it appear to serve as a compensatory mutation necessary for the presence of the *grlA* mutation.

Another novel mutation at position 176, aligns with the β-strand 7 of the *grlA* subunit. The crystal structure of the breakage-reunion domain of DNA gyrase in *E. coli* has revealed that isoleucine 174 (β-strand 6 in *E. coli* and *Saccharomyces cerevisiae* topoisomerase) and glycine 177 (β-strand 7 in yeast topoisomerase II) are also highly conserved and protein-DNA contacts are also made with the β-strand 6 and 7 hairpin. Although alanine 176 is not a conserved amino acid in *E. coli* and yeast topoisomerase, by genetic crosses and allelic exchange we have shown that it is both necessary and sufficient for quinolone resistance. Interestingly we have seen a similar mutation at codon 176, Ala176Gly, in a mutant selected with gatifloxacin, a quinolone which also has an 8-methoxy substitutent (D. Ince and D. C. Hooper, unpublished data).

We also report for the first time a novel resistance mutation at codon 43, which encodes a highly conserved arginine in *E. coli* gyrase and yeast topoisomerase II, mapping to the α2 helix in the helix-turn-helix motif of the catabolite activator protein-like domain (3). In yeast topoisomerase II, Arg704, the counterpart of Arg43 of *S. aureus* GrlA has been modified to alanine by site-directed mutagenesis and shown to decrease substantially the relaxation and cleavage activity of the enzyme (18). In addition, in *E. coli*, Arg46, the counterpart of Arg43, has been suggested to serve a role in anchoring the 3′ end of the cleaved DNA together with Arg47, which corresponds to Arg44 in *S. aureus* (3). Thus, the Arg43Cys mutation might be predicted to have an effect on enzymatic function.

The molecular mechanisms of quinolone resistance for these novel mutations remain to be determined. These mutants did, however, exhibit low levels of coumarin hypersusceptibility (two- to fourfold for mutant P10 but twofold or less for other mutants), a phenotype reported with GrlB (Asn470Asp) and GrlA (Ala116Glu) mutants that had suggested reduced catalytic efficiency as contributing to resistance (9, 10, 28). In the case of topoisomerase IV reconstituted with the purified GrlB (Asp470) subunit, quinolone resistance and coumarin hypersusceptiblity as well as reduced catalytic efficiency have been demonstrated (X. Zhang and D. C. Hooper, unpublished observations). Thus, it is possible that these novel mutations in the *grlA* structural gene affect the catalytic activity and structure of topoisomerase IV such that the mutant enzymes form unstable or reduced levels of drug-enzyme-DNA complexes. This concept is supported by previous work in which a mutant yeast topoisomerase II that causes resistance to a quinolone derivative in vivo exhibited reduced stability and cellular levels despite the purified enzyme's having hypersensitivity to the same quinolone derivatives in vitro (24).

Although common mutations in *grlA* and *gyrA* (together with *grlA*) had an effect on the activity of premafloxacin, none of these mutations was selected directly by single-step plating on premafloxacin-containing agar. In contrast, premafloxacin-selected mutants were commonly found outside the conventional QRDR region of GrlA. These findings suggest that premafloxacin interacts with topoisomerase IV-DNA complexes in a manner different from that of many other quinolones, possibly generating unique contact points between the drug and the enzyme-DNA complex. Interestingly, two of three mutants serially passaged on premafloxacin contained common *grlA* and *gyrA* mutations, and one contained the novel *grlA* (Ala176Thr) mutation in addition to a common *gyrA* mutation. The reasons for the differences in mutational patterns seen with the single-step-selected and the serially passaged mutants is not clear but might be explained by more stringent requirements for fitness in order for mutants to persist under conditions of serial versus single platings on drug-containing agar. Thus, if, as we have suggested above, the novel mutants may have a fitness cost due to catalytic inefficiency of topoisomerase IV or otherwise relative to more commonly identified mutants, then the common mutants will be enriched in serial-passage experiments, which may mimic drug exposures in clinical settings. It is also noteworthy that serially passaged mutants are likely to accumulate multiple mutations (as illustrated by mutant PS3), some in as yet undefined genetic loci, contributing to resistance above that attributable to the identified mutations and some of which might compensate for fitness defects, as could be the case for the *grlA* (Ala176Thr) mutant identified after serial passage.

In summary, premafloxacin is a highly potent fluoroquinolone that is especially active against even multiply mutant strains of *S. aureus*. The primary target of premafloxacin in *S. aureus* is also topoisomerase IV, as is the case with many other quinolones. In five single-step mutants selected with premafloxacin, however, four had novel mutations, three of which were outside the QRDR region of GrlA. Thus, as further development of novel quinolones continues, studies of resistance will need to expand the range of the QRDR region of *grlA* and possibly *gyrA* as well. Identification of novel mutations may also further our understanding of drug target interaction and broaden the range of the molecular mechanisms of quinolone resistance due to target alteration.

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