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Mechanisms of Fluoroquinolone Resistance in Genetically Related Strains of *Staphylococcus aureus*

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Fluoroquinolone resistance in *Staphylococcus aureus* results from amino acid substitutions at particular locations in the DNA gyrase A and B subunits as well as in the topoisomerase IV A subunit and from NorA-mediated efflux. More than one resistance mechanism may be present in a single strain. Fluoroquinolone-resistant derivatives of SA-1199, a methicillin-susceptible *S. aureus* strain, were selected in vivo or in vitro, and their mechanisms of fluoroquinolone resistance were identified. We found that many of the resistance mechanisms described above can develop in derivatives of a single parent strain, either singly or in combination, and can arise in a single step. Variances in MICs for strains with the same apparent resistance mechanisms likely are due to the presence of new or undetected but established means of fluoroquinolone resistance. NorA-mediated resistance can occur in the apparent absence of topoisomerase mutations and in some strains may be the result of a promoter region mutation causing increased expression of *norA*. However, increased expression of *norA* can occur independently of this mutation, suggesting that a regulatory locus for this gene exists elsewhere on the chromosome.

Much work has been done to define the mechanisms of fluoroquinolone resistance in *Staphylococcus aureus*. To date, three means by which such resistance is attained have been described. The first involves mutations in the genes encoding the DNA gyrase and topoisomerase IV A subunits (*gyrA* and *grlA*, respectively), all of which are clustered in a highly homologous region near their 5′ ends (the quinolone resistance-determining region [QRDR]). Amino acid substitutions correlating with fluoroquinolone resistance in GrlA include Ser80→Phe or Tyr, Glu84→Lys, and Ala116→Glu or Pro (5, 6, 16, 24). With respect to GyrA, Ser84→Leu or Ala, Ser85→Pro, and Glu88→Lys mutations are associated with fluoroquinolone resistance (7, 21). The second mechanism involves mutations in *gyrB*, the DNA gyrase B-subunit gene. Amino acid substitutions in GyrB correlating with fluoroquinolone resistance include Asp437→Asn and Arg458→Glu (8). The remaining mechanism of resistance involves overexpression of *norA*, the gene encoding the NorA protein. NorA is a membrane-based multidrug efflux protein capable of transporting fluoroquinolones as well as several other structurally unrelated compounds from the cell (11, 12, 25). Topoisomerase- and NorA-mediated resistance mechanisms can occur alone or in combination, but *grlA* mutations always have been found to precede those in *gyrA*, suggesting that topoisomerase IV is the primary target of fluoroquinolones in *S. aureus* (5, 6, 16, 24).

The accumulation of resistance-conferring mutations in a single strain can lead to very high MICs of some fluoroquinolones. Topoisomerase mutations result in cross-resistance to all members of the class, but newer compounds such as clinafloxacin or trovafloxacin, which have higher intrinsic activity against *S. aureus* than older compounds such as ciprofloxacin or norfloxacin, may still have clinically relevant activity against strains expressing this type of resistance (4, 10). Also, the NorA protein appears to have a predilection for hydrophilic fluoroquinolones (i.e., ciprofloxacin or norfloxacin), and thus its activity affects the MICs of such compounds to a greater degree than those of hydrophobic drugs such as sparfloxacin (25).

We have previously reported on our work with *S. aureus* strains derived from the same parent strain (SA-1199) that express NorA-type resistance in either a constitutive or an inducible manner (SA-1199B and SA-1199-3, respectively [9, 11, 12]). Several additional fluoroquinolone-resistant mutants of SA-1199 were produced, and all of these strains were analyzed for their mechanisms of fluoroquinolone resistance. We found that most of the known resistance mechanisms can be produced in derivatives of a single parent strain and that they can be present individually or in combination. We also found that mutations resulting in topoisomerase- and NorA-mediated fluoroquinolone resistance can arise, alone or in combination, in a single step.

MATERIALS AND METHODS

Bacterial strains. SA-1199 is a methicillin- and fluoroquinolone-susceptible clinical isolate. SA-1199A and SA-1199B are fluoroquinolone-resistant mutants of this strain that were recovered from the blood and cardiac vegetations of rabbits that had experimental endocarditis with SA-1199 and had failed ciprofloxacin therapy given for treatment of this infection. SA-1199R and SA-1199O are fluoroquinolone-resistant mutants of SA-1199 selected on gradient plates containing ciprofloxacin or ofloxacin, respectively. SA-1199C and SA-1199-3 are single-step fluoroquinolone-resistant mutants of SA-1199 recovered on solid media containing clinafloxacin at twofold or ciprofloxacin at fivefold the MIC for the parent strain, respectively. Details on the production of these mutants are given elsewhere; the frequencies at which they were recovered were 4.6×10^{-8} for clinafloxacin and 1×10^{-9} for ciprofloxacin (10, 12). The mutants were maintained on drug-free Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.), and in all cases fluoroquinolone resistance was stable.

Determination of antimicrobial susceptibilities. Unless otherwise noted, all reagents were the highest grade available and were obtained from Sigma Chemical Co., St. Louis, Mo. Norfloxacin was obtained from Merck, Rahway, N.J. MICs were determined on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) according to the guidelines of the National Committee for Clinical Laboratory Standards (13). The effect of reserpine (final concentration, 20 μg/ml) on selected MICs also was determined.

Uptake of [^{14}C]enoxacin. Uptake studies were performed using whole cells as described previously (12). [^{14}C]enoxacin (specific activity, 15.9 μCi/mg) was provided by Parke-Davis Pharmaceutical Research, Ann Arbor, Mich. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (final concentration, 100 μM) was used to dissipate the proton motive force across the cytoplasmic membrane.

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PCR procedures. Codons 7 to 146 of *gyrA* and 2 to 257 of *grlA*, encompassing the QRDR of each gene, were amplified from genomic DNA by using primers and PCR parameters as described by Sreedharan et al. and Ferrero et al., respectively (5, 21). Codons 378 to 496 of *gyrB*, a region that includes the codons for which mutations correlating with fluoroquinolone resistance have been described previously (8), and the homologous region of *grlB* (codons 382 to 509) also were amplified from genomic DNA. The primers used for *gyrB* were 5′-TCGCACGTACAGTGGTTG-3′ (forward) and 5′-CGCTAGATCAAAGTCGCC-3′ (reverse), and those used for *grlB* were 5′-GCACAACAAGCAAGG-3′ (forward) and 5′-CGCACCATCAGTATCAGC-3′ (reverse). PCR parameters for *grlB* and *gyrB* amplification were 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 0.5 min.

The *gyrA* and *grlA* PCR products were purified from agarose gels by using a QIAquick gel extraction kit according to the protocol of the manufacturer (Qiagen Inc., Chatsworth, Calif.) and then cloned into pUC19 for sequence determination. The *gyrB* and *grlB* fragments were purified by ammonium acetate precipitation and then subjected to restriction fragment length polymorphism (RFLP) analysis (2) as described below.

The *norA* genes of SA-1199C and SA-1199O were amplified from genomic DNA and then purified and cloned into pUC19 for sequence determination (11).

Northern blotting. PCR was employed to produce a 790-bp internal fragment of *norA* for use as a probe as described previously (12). Protoplasts of SA-1199, SA-1199B, SA-1199C, and SA-1199O were prepared by exposing organisms to lysostaphin (30 μg/ml) in SMM buffer (0.5 M sucrose; pH 6.8) for 45 min on ice (17). Total cellular RNA was isolated by the method of Chomczynski (3). Equivalent amounts of RNA (30 μg) from each strain were applied to and separated in a formaldehyde-containing agarose gel. The RNA was transferred to a nylon membrane, and hybridization with the *norA* probe was carried out under high-stringency conditions (42°C, 50% formamide) (20).

DNA sequence determination. Nucleotide sequences were determined by the dideoxy chain-termination method using ^{35}S-dATP (1,000 Ci/mmol; New England Nuclear, Boston, Mass.) (19). At least three independently generated PCR products were sequenced to control for the possibility of polymerase-induced errors.

RFLP analysis. RFLP analysis of *gyrB* and *grlB* PCR products was carried out by digesting them with *Hin*fI (recognition site, GANTC), *Mnl*I (recognition site, NNNNNNGAGG), or *Bsa*AI (recognition site, pyrimidine-ACGT-purine) according to the guidelines of the manufacturer (New England Biolabs, Inc., Beverly, Mass.). Undigested (control) and digested fragments were separated in agarose gels and visualized following staining with ethidium bromide.

Digestion of the *gyrB* PCR product (357 bp) with *Hin*fI produces fragments of 178, 121, 33, and 25 bp. Loss of the recognition site that includes codons 437 and 438 ([GAC-TC]T) results in fragments of 211, 121, and 25 bp. Any mutation at position 1 or 2 of either codon results in an amino acid substitution and will be detected; these positions include the codon 437 mutation that correlates with fluoroquinolone resistance (GAC→AAC, resulting in an Asp→Asn change) (8). *Mnl*I normally digests the PCR product twice, generating fragments of 181, 122, and 54 bp. Loss of the site that includes codons 456 to 459 (C[CA-TTA-CGA-GG]T) results in fragments of 235 and 122 bp. Changes at positions 2 and 3 of codon 458 and positions 1 and 2 of codon 459 will be detected; alterations at all of these positions except position 3 of codon 458 result in either amino acid substitutions or the introduction of a stop codon. The previously described change correlating with fluoroquinolone resistance lies in codon 458 (CGA→CAA, resulting in an Arg→Gln change) (8).

As with *gyrA* and *grlA*, there is significant similarity between *gyrB* and *grlB*. To date, no *grlB* mutations resulting in fluoroquinolone resistance have been identified in *S. aureus*. However, a *parE* mutation causing fluoroquinolone resistance in *Streptococcus pneumoniae* has recently been described (18). The resulting ParE amino acid substitution (Asp→Asn at position 435) is homologous to the Asp→Asn substitution at position 437 in the *S. aureus* GyrB protein. Accordingly, it is logical to predict that in *S. aureus*, mutations at sites homologous to those in *gyrB* that have been associated with fluoroquinolone resistance also may occur in *grlB*. At the amino acid level, the *S. aureus* GyrB and GrlB proteins are 52% identical, but between GyrB residues 437 to 458 and the homologous region of GrlB (residues 432 to 453) there is 77% identity. GrlB amino acids 432 and 453, which are homologous to residues 437 and 458 of GyrB (the positions at which mutations correlating with fluoroquinolone resistance are found), are identical to the GyrB residues.

*Hin*fI digests the wild-type *grlB* PCR product (384 bp) once, generating fragments of 233 and 151 bp. The fragment remains uncut if the *Hin*fI site that includes codons 432 and 433 ([GAT-TC]T) is lost. Changes at the first two positions of either codon will be detected, and all lead to amino acid substitutions. *Bsa*AI has two sites in the wild-type PCR product, generating fragments of 189, 170, and 25 bp. Loss of the site spanning codons 452 to 454 (T[TA-CGT-G]GT) results in fragments of 359 and 25 bp. Changes to any base at position 3 and to any base except cytosine at position 2 of codon 452 will be detected. All but one of these possible changes in codon 452 (TTA→TTG, both of which code for Leu) will result in an amino acid substitution or the introduction of a stop codon. Any change in codon 453 also will be detected. All substitutions at positions 1 and 2 result in an amino acid substitution; any change in position 3 results in loss of the recognition site but not an amino acid substitution. A change to anything other than adenine at position 1 of codon 454 will be detected and results in an amino acid substitution.

TABLE 1. MICs for study strains

Strain	MIC (μg/ml)			
	NOR[a]	NOR + R[b]	EtBr[c]	EtBr + R
SA-1199	1	0.5	8	2
SA-1199A	32	8	8	2
SA-1199B	64	8	64	8
SA-1199C	256	32	64	16
SA-1199O	32	8	4	2
SA-1199R	32	8	8	2
SA-1199-3	8	1	64	4

[a] NOR, norfloxacin.
[b] R, reserpine (20 μg/ml).
[c] EtBr, ethidium bromide.

RESULTS AND DISCUSSION

MICs of norfloxacin and ethidium bromide, and the effect of reserpine on those MICs, are shown in Table 1. A fourfold difference was observed in the norfloxacin MICs for SA-1199B and SA-1199C despite the fact that the two strains were found to possess the identical fluoroquinolone resistance mechanisms (see below). The simplest explanation for this apparent discrepancy is that SA-1199C likely possesses an additional resistance mechanism(s) not identified in our study. Possibilities include mutations at sites in *grlA* and/or *gyrA* not included in our sequencing, *grlB* or *gyrB* mutations undetected by RFLP analysis, or as-yet-undescribed mechanisms of fluoroquinolone resistance.

High-level resistance to ethidium bromide was seen only in strains possessing NorA-mediated efflux (SA-1199B, SA-1199C, and SA-1199-3; see below). This is not unexpected, as ethidium bromide is known to be a good substrate of NorA (12, 14). The MICs of ethidium bromide for each of the strains expressing NorA-mediated resistance were the same, and this MIC was reduced by reserpine to within 1 dilution of that for SA-1199 in the absence of reserpine. However, the reduction by reserpine was not complete, as the ethidium bromide MICs in the presence of reserpine remained two- to eightfold above that for SA-1199 under the same testing conditions. One possible explanation for this observation is that reserpine may not interfere completely with the function of NorA. On the basis of the structural and functional homology between NorA and the *Bacillus subtilis* multidrug transporter Bmr, it is possible that the mechanism by which reserpine impedes NorA function is through competitive inhibition with substrate (1). Perhaps substrates such as ethidium bromide and norfloxacin have a greater affinity for the substrate recognition site(s) and are capable of competing effectively with reserpine for that site(s). Further work characterizing the NorA substrate recognition site(s) and the interaction of that site(s) with both substrates and inhibitors is required to understand the above findings more completely.

Enoxacin uptake profiles for all strains except SA-1199-3 are shown in Fig. 1. The profile for SA-1199R was identical to that for SA-1199. SA-1199B had reduced uptake reversed completely by the addition of CCCP, consistent with its known efflux-related resistance mediated by NorA (9). SA-1199-3 previously unexposed to a NorA substrate has been shown to have a curve similar to that of SA-1199 and when preexposed to a NorA substrate during growth (induced) has a curve similar to that of SA-1199B and is responsive in the same way to CCCP

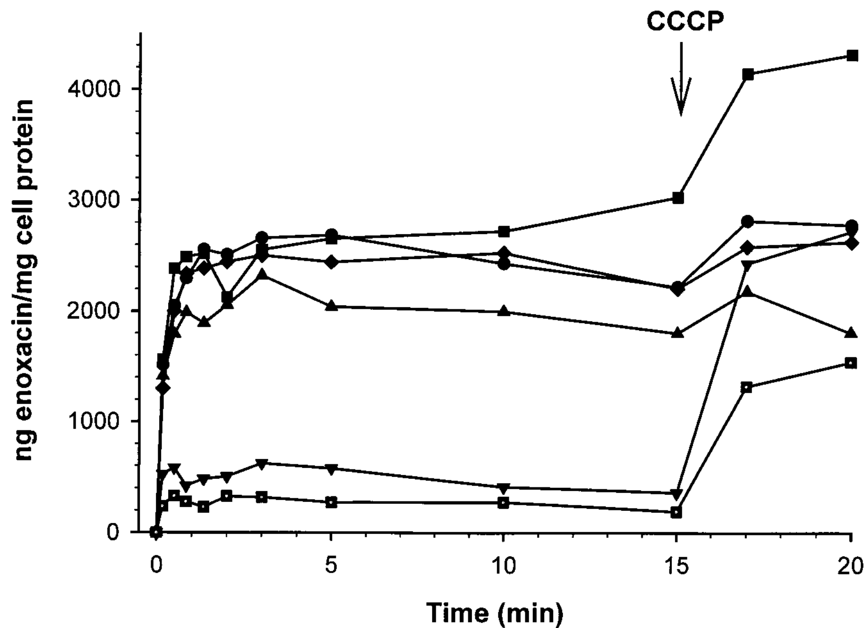

FIG. 1. [^{14}C]enoxacin uptake profiles. CCCP (final concentration, 100 μM) was added at the indicated time (arrow). ●, SA-1199; ■, SA-1199A; ▼, SA-1199B; ◘, SA-1199C; ▲, SA-1199O; ◆, SA-1199R.

(12). SA-1199C demonstrated reduced uptake reversed by CCCP similar to that observed for SA-1199B, consistent with an efflux-mediated process. The uptake profiles for SA-1199A and SA-1199O were qualitatively similar to that for SA-1199 and were considered wild type in nature.

We have shown previously that the quantity of *norA* mRNA is increased in SA-1199B compared with that in SA-1199 (11). We found that the quantity of *norA* mRNA in SA-1199C was at least as large as that seen in SA-1199B; strains SA-1199 and SA-1199O possessed much smaller, but equivalent, amounts of *norA* mRNA (data not shown). These findings are consistent with the conclusion that *norA* expression is increased in SA-1199B and SA-1199C.

No nucleotide changes resulting in amino acid substitutions were found within the coding region of NorA in strains SA-1199O and SA-1199C. We have reported the same to be true for SA-1199-3 (12). However, a thymine-to-adenine transversion was found in the *norA* promoter region of SA-1199C. We have reported an identical mutation in SA-1199B previously, and another group has described a mutation at this location in a different *S. aureus* strain expressing NorA-mediated fluoroquinolone resistance (11, 15). The site of this mutation is 89 bp upstream of the *norA* initiation codon and 11 bp downstream of the −10 promoter motif (Fig. 2). It has been proposed that a mutation at this position may be responsible for increased *norA* transcription (15). The facts that both SA-1199B and SA-1199C possess a mutation at this position and demonstrate increased expression of *norA* are consistent with this hypothesis. However, as SA-1199-3 does not have this mutation but does have increased *norA* expression (albeit inducible), there likely is an additional factor(s) involved in *norA* regulation (12). There is a perfect 8-bp inverted repeat encompassing the −10 motif of the *norA* promoter (Fig. 2); this region may function as a binding site for a regulatory protein. It is intriguing to hypothesize that a mutation in a *norA*-regulatory protein plus the above-described promoter region mutation, which may alter the binding of a regulatory protein to its recognition site, is required for the constitutive up-regulation of *norA* (as observed for SA-1199B and SA-1199C), whereas the inducible nature of SA-1199-3 may be due to a mutation(s) in the gene for a regulatory protein only. This hypothesis is plausible especially for SA-1199B, which was selected in vivo. Clearly, further work with these strains to better define *norA* regulation is in order.

Table 2 shows the amino acid changes resulting from mutations in the QRDRs of *gyrA* and *grlA*. Changes at each of the previously described GrlA positions correlating with fluoroquinolone resistance were found in our series. The only GyrA alteration found was the rather uncommon Glu88→Gly mutation (23). In no case was a mutation in GyrA found in the absence of a GrlA mutation. This observation has also been described by others and is evidence supporting the conclusion that GrlA is the primary target of fluoroquinolones in *S. aureus* (5, 6, 16, 24).

No *grlB* alterations were detected by RFLP. A mutation(s) in codon 458 and/or 459 of *gyrB* was identified by the loss of an

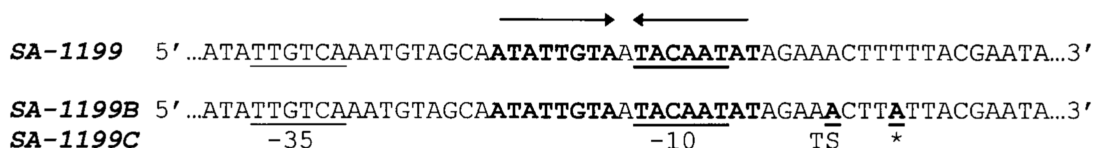

FIG. 2. *norA* promoter region. The −35 and −10 motifs, an 8-bp perfect inverted repeat encompassing the −10 motif, the transcriptional start site (TS), and the T→A transversion found in SA-1199B and SA-1199C (*) are indicated.

TABLE 2. Sequencing results for QRDRs

Strain	NOR MIC (μg/ml)[a]	Sequence change[b]	
		GrlA	GyrA
SA-1199	1	None (wt)	None (wt)
SA-1199A	32	Glu84→Lys	None (wt)
SA-1199B	64	Ala116→Glu	None (wt)
SA-1199C	256	Ala116→Glu	None (wt)
SA-1199O	32	Ala116→Glu	None (wt)
SA-1199R	32	Ser80→Tyr	Glu88→Gly
SA-1199-3	8	None (wt)	None (wt)

[a] NOR, norfloxacin.
[b] All strains have Met instead of Ile at position 45 (see reference 5). wt, wild type.

*Mnl*I site in all strains; the fact that this mutation(s) was present in SA-1199 indicates that it is inconsequential with respect to fluoroquinolone resistance. Barring the presence of undetected resistance mechanisms, the substitutions Glu84→Lys (SA-1199A) and Ala116→Glu (SA-1199O) in GrlA confer equivalent levels of fluoroquinolone resistance. The double-mutant strain SA-1199R, which possesses a Ser80→Tyr mutation in GrlA and a Glu88→Gly mutation in GyrA, has no greater resistance to norfloxacin than the single mutants described above. Others have shown that the Ser80→Tyr mutation results in a level of resistance equivalent to that seen in each of our single GrlA mutants (6). It has been shown that the more common Glu88→Lys GyrA mutation, when added to the Ser80→Tyr GrlA mutation, results in a step-up in norfloxacin resistance compared to that of a strain having only the GrlA mutation (6). It seems plausible to hypothesize that the effect of the Glu88→Gly change, which results in the substitution of an uncharged residue for a negatively charged one, on fluoroquinolone-GyrA interaction is minimal, whereas the effect of the Glu88→Lys change, which substitutes a positively charged residue for a negatively charged one, is significant. Earlier work done to assess the effect of the Glu88→Gly mutation in GyrA on fluoroquinolone susceptibility did not address possible concomitant GrlA mutations (23). Additional strains with combined Ser80→Tyr GrlA and Glu88→Gly GyrA mutations would have to be analyzed to establish the contribution of this mutation to fluoroquinolone resistance.

The Ala116→Glu mutation in GrlA is intriguing. Ng et al. previously have reported a mutation at this position correlating with fluoroquinolone resistance (16). Ala116 lies in a highly conserved region; the AAMRYTE motif is completely conserved in the GyrA proteins of *Escherichia coli*, *S. aureus*, and *B. subtilis* and the ParC and GrlA proteins of *E. coli* and *S. aureus*, respectively. The tyrosine residue of this motif is known to lie in the active site of GyrA, at one point forming a covalent bond with DNA (22). We agree with Ng et al. that the homologous tyrosine at position 119 of GrlA is quite likely to lie in the active site of that enzyme. It is possible that the substitution of a charged residue (Glu) for an uncharged one (Ala) so near the active site of GyrA may alter the fluoroquinolone-gyrase interaction by affecting hydrogen bonding between the drug and protein that may be required for an inhibitory action to be observed.

Using a laboratory strain of *S. aureus* exposed to fourfold the MIC of ciprofloxacin for that strain, Ferrero et al. found that single *grlA* mutations resulting in fluoroquinolone resistance could be selected at a frequency of approximately 10^{-8} (6). NorA-type and *gyrA* mutants appeared only when these first-step mutants were exposed to fourfold the ciprofloxacin MICs for those strains, again at a frequency of approximately 10^{-8}. We found that single and combined mechanisms of fluoroquinolone resistance can develop in a single step, as illustrated by SA-1199-3 and SA-1199C, respectively. It is possible that there is strain specificity with respect to the propensity for the emergence of fluoroquinolone resistance by any of the mechanisms discussed here, with SA-1199 having an increased likelihood of acquiring the mutation(s) necessary for NorA-type resistance compared to that of other strains. Additional strains would need to be studied to address this issue.

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