JOURNAL OF BACTERIOLOGY, Sept. 1991, p. 5854–5860
0021-9193/91/185854-07$02.00/0

Vol. 173, No. 18

A Novel Locus Conferring Fluoroquinolone Resistance in *Staphylococcus aureus*

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Received 16 May 1991/Accepted 1 July 1991

Fluoroquinolones such as ciprofloxacin and ofloxacin are potent antimicrobial agents that antagonize the A subunit of DNA gyrase. We selected and mapped a novel fluoroquinolone resistance gene on the *Staphylococcus aureus* chromosome. Resistant mutants were selected with ciprofloxacin or ofloxacin and were uniformly localized to the A fragment of chromosomal DNA digested with *Sma*I and arrayed by pulsed-field gel electrophoresis. Several mutants (*cfxB*, *ofxC*) were genetically mapped between the *thr* and *trp* loci in the A fragment. A majority of A fragment fluoroquinolone resistance mutations were associated with reduced susceptibility to novobiocin, an antagonist of the B subunit of DNA gyrase. Two genes previously associated with fluoroquinolone resistance, the *gyrA* gene of DNA gyrase and the *norA* gene (associated with decreased drug accumulation), were localized to the G and D fragments, respectively. Thus, the fluoroquinolone resistance mutations in the A fragment are distinct from previously identified fluoroquinolone resistance mutations in *gyrA* and *norA*. Whether mutations in the A fragment alter a second topoisomerase or another gene controlling supercoiling or affect drug permeation is unknown.

Staphylococci have long been recognized as important agents of community- and hospital-acquired infections. When ciprofloxacin was released in the United States in 1987, it was hoped that it would be useful for the treatment of infections caused by multidrug-resistant strains of *Staphylococcus aureus*. The isolation of fluoroquinolone-resistant *S. aureus* (37), however, soon followed the introduction of these agents.

Little is known about resistance mechanisms in *S. aureus*, but the mechanisms of fluoroquinolone resistance in *Escherichia coli* have been well described. In *E. coli*, the enzyme DNA gyrase is the target of fluoroquinolone action (5, 9, 11, 34). DNA gyrase catalyzes the ATP-dependent supercoiling of DNA and the catenation and decatenation of DNA circles (4, 6). The enzyme is a tetramer of two A and two B subunits, encoded by the *gyrA* and *gyrB* genes, respectively. Mutations in either gene may confer quinolone resistance in *E. coli*. Distinct DNA gyrases have been isolated from the gram-positive species *Bacillus subtilis* (33) and *Micrococcus luteus* (18). DNA supercoiling activity has been identified in a cell lysate from one strain of *S. aureus* (35). A second mechanism of resistance to fluoroquinolones in *E. coli* is reduced drug accumulation associated with decreased expression of outer membrane porin protein OmpF (9, 10). *S. aureus* lacks an outer membrane; thus, drug resistance mechanisms must differ between these two species.

Two mechanisms of quinolone resistance have been proposed in *S. aureus*. In one study, a DNA fragment that confers quinolone resistance was cloned from *S. aureus* in *E. coli*. This fragment appears to encode a hydrophobic protein that may be membrane associated. Introduction of this locus (*norA*) on a plasmid into *E. coli* and *S. aureus* resulted in resistance and reduced accumulation of some quinolones (39). In a second study, point mutations in the *gyrA* gene of *S. aureus* were identified in quinolone-resistant members of susceptible and resistant pairs of clinical isolates (31).

No previous study of fluoroquinolone resistance in *S. aureus* has identified the chromosomal location of quinolone resistance loci in either clinical isolates or genetically defined laboratory strains. Instead, *S. aureus* genes have been inserted into *E. coli* or *S. aureus* on high-copy-number plasmids. *gyrA* mutations were found in clinically derived resistant isolates exposed to various concentrations of quinolones in vivo. The fluoroquinolone resistance in clinical isolates most likely results from multiple mutations in the *S. aureus* chromosome, leaving unclear the role of single copies of specific mutant chromosomal loci in fluoroquinolone resistance.

In this work, 34 single-step mutants were obtained by exposing a genetically defined laboratory strain to agar containing various concentrations of quinolone agents. Eleven of these mutants, judged to be independent by phenotype or selection in independent experiments, were then studied by physical mapping techniques. All 11 mapped to the A fragment of the *S. aureus* *Sma*I-digested chromosome. Three of these A-fragment fluoroquinolone-resistant (*flq*) mutations were mapped further by transformational analysis and found to be between the *thrB* locus and the *trp* operon. The fluoroquinolone resistance mutations described here are not in the structural genes for DNA gyrase (*gyr*) or the *norA* gene, which were localized to the G and D fragments, respectively, by DNA hybridization. Thus, our studies identified a novel *S. aureus* fluoroquinolone resistance gene (*flq*) located on the *Sma*I A chromosomal fragment.

MATERIALS AND METHODS

Bacterial strains and selection and scoring of mutants. The bacterial strains and plasmids used in this study are described in Table 1. Thirty-four mutants were obtained by plating *S. aureus* MT5 *nov* (novobiocin resistant) or ISP794 (novobiocin susceptible) (MIC = 0.25 μg ciprofloxacin or

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TABLE 1. Bacterial strains

Strain	Genotype	Origin or reference
S. aureus		
ISP86	8325[a] *uraA141 hisG15 nov-142 purA102 pig-131*	P. Pattee (14)
ISP225	Ps55	P. Pattee
ISP479	8325-4 [pI258 *blaI401 mer-14 repA36* Ω(Tn*551*)*1*] *pig-131*	P. Pattee (24)
ISP794	8325 *pig-131*	P. Pattee (32)
ISP1352	80CR3 *nov-142*(pTV1)	P. Pattee
ISP1541	8325 r1$^-$ m31$^+$ r2$^-$ m32$^+$ *pig-131* Ω1118(Tn*916*)	P. Pattee
ISP2133	8325 *pig-131 trp-489* Ω(Tn*917lac*)*2*	P. Pattee
ISP2134	8325 *pig-131 thrB494* Ω(Tn*917lac*)*1*	P. Pattee
MT5	8325 *nov-142 hisG15 pig-131*	This study; ISP86 DNA × ISP794
MT52222	8325 *nov-142 hisG15 pig-131 ofxC541*	This study; spontaneous Ofx^r isolate
MT5224c4	8325 *nov-142 hisG15 pig-131 cfxB542*	This study; spontaneous Cfx^r isolate
MT5224c9	8325 *nov-142 hisG15 pig-131 cfxB543*	This study; spontaneous Cfx^r isolate
MT52242	8325 *nov-142 hisG15 pig-131 ofx-544*	This study; spontaneous Ofx^r isolate
MT52184	8325 *nov-142 hisG15 pig-131 ofx-545*	This study; spontaneous Ofx^r isolate
MT522410	8325 *nov-142 hisG15 pig-131 ofx-546*	This study; spontaneous Ofx^r isolate
MT5224c3	8325 *nov-142 hisG15 pig-131 cfx-547*	This study; spontaneous Cfx^r isolate
MT111	8325 *pig-131 ofx-548*	This study; spontaneous Ofx^r isolate
MT201	8325 *pig-131 cfx-549*	This study; spontaneous Cfx^r isolate
MT211	8325 *pig-131 cfx-550*	This study; spontaneous Cfx^r isolate
MT221	8325 *pig-131 cfx-551*	This study; spontaneous Cfx^r isolate
MT5553	8325 *nov-142 hisG15 pig-131* Ω(Tn*551*)*1081 ofxC541*$^{+b}$	This study; ISP479 DNA × MT52222
MT5531	8325 *nov-142 hisG15 pig-131* Ω(Tn*551*)*1082 cfxB542*$^+$	This study; ISP479 DNA × MT5224c4
MT5452	8325 *nov-142 hisG15 pig-131* Ω(Tn*551*)*1083 cfxB543*$^+$	This study; ISP479 DNA × MT5224c9
MT5382	8325 *nov-142 hisG15 pig-131* Ω(Tn*551*)*1084 ofx-544*$^+$	This study; ISP479 DNA × MT52242
MT5310	8325 *nov-142 hisG15 pig-131* Ω(Tn*551*)*1085 ofx-545*$^+$	This study; ISP479 DNA × MT52184
MT5592	8325 *nov-142 hisG15 pig-131* Ω(Tn*551*)*1086 ofx-546*$^+$	This study; ISP479 DNA × MT522410
MT5416	8325 *nov-142 hisG15 pig-131* Ω(Tn*551*)*1087 cfx-547*$^+$	This study; ISP479 DNA × MT5224c3
MT1114	8325 *pig-131* Ω(Tn*551*)*1088 ofx-548*$^+$	This study; ISP479 DNA × MT111
MT2019	8325 *pig-131* Ω(Tn*551*)*1089 cfx-549*$^+$	This study; ISP479 DNA × MT201
MT2116	8325 *pig-131* Ω(Tn*551*)*1090 cfx-550*$^+$	This study; ISP479 DNA × MT211
MT2214	8325 *pig-131* Ω(Tn*551*)*1091 cfx-551*$^+$	This study; ISP479 DNA × MT221
MT5111	8325 *nov-142 hisG15 pig-131 cfxB543 trp-489* Ω(Tn*917lac*)*2*	This study; ISP2133 DNA × MT5224c9
MT4172	8325 *nov-142 hisG15 pig-131 ofxC541 thrB494* Ω(Tn*917lac*)*1*	This study; ISP2134 DNA × MT52222
MT292	8325 *nov-142 hisG15 pig-131 cfxB542 trp-489* Ω(Tn*917lac*)*2*	This study; ISP2133 DNA × MT5224c4
MT1222	8325 *pig-131* (Flq)	This study; spontaneous high-level fluoroquinolone resistance mutant
E. coli DH10B	F$^-$ *araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK mcrA* Δ(*mrr-hsdRMS-mcrBC*) *rpsL dor* φ80d*lacZ*M15 *endA1 nupG recA1*	Bethesda Research Laboratories

[a] *S. aureus* phage group III strain NCTC 8325 (25).
[b] + indicates that wild-type DNA has replaced the corresponding mutant allele after insertion of the transposon.

ofloxacin per ml for both strains) on brain heart infusion (Difco Laboratories) agar containing ciprofloxacin (two, four, or eight times the MIC) or ofloxacin (two, four, or eight times the MIC). Specific *flq* resistance alleles were, by convention, designated *cfx* if selected on ciprofloxacin and *ofx* if selected on ofloxacin. A highly fluoroquinolone-resistant strain, MT1222, was obtained by serial passage on brain heart infusion agar containing increasing concentrations of norfloxacin. Resistance to other agents was scored on brain heart infusion agar containing tetracycline (2 μg/ml), erythromycin (20 μg/ml), or novobiocin (10 μg/ml).

Media and reagents. Tryptone soya broth powder was purchased from Oxoid Limited (Hampshire, England). Complete defined synthetic agar was used to select and score nutritional markers (26).

Lysostaphin was purchased from ICN Biomedicals, Inc. (Costa Mesa, Calif.). Proteinase K, RNase, phenylmethylsulfonyl fluoride, and sterile normal rabbit sera were purchased from Sigma Chemical Co. Restriction endonucleases and *Hin*dIII-digested lambda DNA were purchased from Bethesda Research Laboratories.

Ciprofloxacin and ofloxacin were gifts from Miles Pharmaceuticals (West Haven, Conn.) and Ortho Pharmaceutical Corporation (Raritan, N.J.), respectively. Norfloxacin was from Merck Sharp & Dohme Research Laboratory. Sodium ampicillin, tetracycline, erythromycin, and carbenicillin were from Sigma Chemical Co.

Preparation of plasmid DNA. Small-scale plasmid DNA isolation from *S. aureus* was by the alkaline lysis method (29) except for the addition of lysostaphin (0.3 mg/ml) to allow lysis of the cells.

Transformations. High-molecular-weight transforming DNA was prepared by the method of Stahl and Pattee (32). Cells were made competent for transformation as described by Lindberg et al. (17), except that the original inoculum was grown in tryptone soya broth at 35°C overnight in a shaking water bath. Bacteriophage Φ55, maintained on strain ISP225, was used to render the *S. aureus* strains competent for transformation.

Preparation of transforming DNA containing random Tn*551* insertions. Strain ISP479 was used to prepare a pool of transforming DNA containing random Tn*551* insertions in the *S. aureus* chromosome by the method of Luchansky and Pattee (19). Eleven of the 34 original fluoroquinolone-resistant (Flq$^-$) mutants (alleles *ofx/cfx-541-551*) were transformed with this pooled transforming DNA selecting for

erythromycin resistance (Tn*551* insertion). The erythromycin-resistant transformants were then screened to identify transformants that were now fluoroquinolone susceptible (Flq^+) (Tn*551* insertion near the *flq* loci with recombination of flq^+ DNA). The strains containing Tn*551* linked to the *flq* loci were studied by pulsed-field gel electrophoresis after *Sma*I digestion as described below.

Pulsed-field gel electrophoresis (PFGE) of *Sma*I-digested *S. aureus* chromosomal DNA. The bacteria were grown in tryptone soya broth at 35°C to an optical density at 540 nm of 0.9. Cells were washed once with 10 ml of Tris-EDTA-NaCl buffer and resuspended in 20 ml of EC buffer (6 mM Tris, 1 M NaCl, 100 mM EDTA, 0.5% Brij, 0.2% Na deoxycholate, 0.5% Sarkosyl, pH 7.5). Two milliliters of cells was mixed with 2 ml of 2% low-temperature agarose and cast in a mold. The mold was chilled on ice, and the individual agarose inserts were ejected into EC buffer. Lysostaphin (0.1 mg/ml) and RNase (5 μg/ml) were added, and the inserts were incubated at 35°C for 24 h. The inserts were then treated with ES buffer (0.5 M EDTA, 1% Sarkosyl, pH 9) and proteinase K (20 mg/ml) at 50°C for 48 h followed in sequence by two incubations each (2 h, ambient temperature) with TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA) containing phenylmethylsulfonyl fluoride and with TE buffer alone. The inserts were then incubated with restriction enzyme *Sma*I in restriction enzyme buffer overnight at ambient temperature.

Inserts were placed in the wells of a 0.9% agarose gel. Electrophoresis was done with a CHEF-DR II system (Bio-Rad Laboratories) in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) at 4°C (22). The electrophoresis was at 180 V for 27 h, with a pulse time of 27 s.

Cloning of fluoroquinolone resistance locus. *S. aureus* MT1222 containing multiple mutations in the chromosome affecting fluoroquinolone resistance was used to prepare genomic DNA by the method of Matsuhashi et al. (20). Plasmid pUC19 DNA was prepared by CsCl-ethidium bromide density gradient centrifugation. Genomic and pUC19 DNAs were digested with the restriction enzyme *Hin*dIII and ligated with T4 DNA ligase. The ligated fragments were used to transform supercompetent DH10B cells (Bethesda Research Laboratories), selecting for fluoroquinolone and carbenicillin resistance (the latter encoded by the pUC19 vector).

Oligonucleotide synthesis. Synthesis was performed on a Pharmacia Gene Assembler Plus with beta-cyanoethyl chemistry (Polymer Core, Massachusetts General Hospital, Charlestown, Mass.).

DNA-DNA hybridizations. Southern transfers of restriction endonuclease-digested DNAs were done on GeneScreen Plus (NEN Research Products) (30). DNA probes were labeled with ^{32}P by the primer extension method (2, 3) (Amersham Corp.), and oligonucleotide probes were end labeled with ^{32}P by a 5′-end-labeling system (29) (NEN Research Products) according to the instructions of the manufacturer. Hybridizations were performed at 60°C in aqueous buffer.

RESULTS

Selection and characterization of fluoroquinolone resistance mutants. Single-step Flq^- mutants of *S. aureus* MT5 *nov* were obtained at frequencies of 5×10^{-9} for ciprofloxacin and at 3×10^{-7} to 4×10^{-12} for ofloxacin, with frequency decreasing with increasing concentration of drug. Similar mutation frequencies (6×10^{-6} to 7×10^{-9} for ciprofloxacin and 6×10^{-7} for ofloxacin) were found when fluoroquinolone resistance mutants of ISP794 (nov^+) were selected. Mutants selected with one fluoroquinolone were cross resistant to other fluoroquinolones. Susceptibilities to many other agents (β-lactams, nitrofurantoin, erythromycin, vancomycin, clindamycin, chloramphenicol, and tetracycline) were unchanged as determined by disk diffusion (1).

Mutants of MT5 had 2- to 8-fold-increased fluoroquinolone resistance, and 29 of 34 (85%) had 2- to 32-fold-decreased novobiocin resistance (Table 2). This phenotypic interaction between the *nov* and *flq* loci was also seen when the *nov* locus was wild type. In one of four *flq* mutants of ISP794 (nov^+) examined, there was a fourfold decrease in novobiocin MIC for the mutant compared with the parent strain ISP794. The phenotypic interaction between *flq* and *nov*/nov^+ was confirmed in experiments in which random Tn*551* insertions were introduced into *flq* strains by transformation. In all instances, when *flq* strains became fluoroquinolone susceptible (Flq^+) after transformation, the level of novobiocin resistance returned to that of the parent strain (Table 2). Eleven of these mutants, judged to be independent by phenotype or because of selection in separate experiments, were chosen for further study.

Mapping of resistance loci. Mapping of *flq* loci on the *S. aureus* chromosome occurred in two stages. First, these loci were physically localized to 1 of the 13 fragments of the *S. aureus* chromosome created by *Sma*I restriction digestion (Fig. 1). Second, finer genetic mapping was accomplished by transformational analysis.

Physical mapping. Selection of transposon (Tn) insertions linked to the genes of interest and physical mapping of the locations of the transposons on the chromosome have been used for gene mapping in *S. aureus* (19). High-molecular-weight DNA from ISP479, a strain containing transposon Tn*551* (conferring erythromycin resistance [Em^r]) randomly inserted into the chromosome, was transformed into 11 independent *flq* strains selecting for Em^r and screening for Flq^+ transformants. Cotransformation frequencies between the individual flq^+ loci and single linked independent Tn*551* insertions ranged from 5 to 90%, as determined by transformation of each Em^r Flq^+ transformant back into the original *flq* mutant.

The location of Tn*551* in these strains was then determined by *Sma*I digestion of chromosomal DNA, PFGE of DNA fragments (a representative PFGE gel stained with ethidium bromide is shown in Fig. 2, lanes 1 to 4), and Southern hybridization with a DNA probe containing Tn*917*. Plasmid pTV1 containing Tn*917* (40), which cross-hybridizes with Tn*551* (23), was isolated from *S. aureus* ISP1352. The purified *Hpa*I-*Eco*RI 5.5-kb Tn*917* restriction fragment was labeled with ^{32}P by the primer extension method and used as a probe for hybridization. A positive signal was demonstrated in the A fragment of chromosomal DNA from 11 of 11 independent single-step mutant strains (representative Southern blot shown in Fig. 2, lanes 5 to 7). MT5 *nov* and ISP794 (which served as negative controls) gave no signal, and the purified probe Tn*917* (the positive control) gave a strongly positive signal (data not shown). These findings served to identify the location of the *flq* loci in the A fragment, for the purposes of further genetic mapping.

Genetic mapping. The locations of 3 of the 11 independent *flq* loci physically mapped to the A fragment were then determined more precisely by transformations with selectable auxotrophic and transposon markers at known locations (Table 3). For example, strain MT5224c4 (*cfxB542*) was transformed with DNA from strain ISP2134 [8325 *thrB494* Ω(Tn*917lac*)*1*]. Five of 107 Em^r transformants (4.7%) were

TABLE 2. Relationship of fluoroquinolone and novobiocin resistance

Strain		MIC (μg/ml)	
Independent *flq* mutant	Derivative containing linked Tn*551* insertions	Cfx[a]	Nov[a]
Parent strain (MT5 [*nov*])		0.25	80
MT52222 (*nov ofxC541*)		2	80
	MT5553 [*nov* Ω(Tn*551*)*1081 ofxC541*$^{+b}$]	0.25	80
MT5224c4 (*nov cfxB542*)		2	80
	MT5531 [*nov* Ω(Tn*551*)*1082 cfxB542*$^{+}$]	0.25	80
MT5224c9 (*nov cfxB543*)		2	2.5
	MT5452 [*nov* Ω(Tn*551*)*1083 cfxB543*$^{+}$]	0.25	80
MT52242 (*nov ofx-544*)		1	20
	MT5382 [*nov* Ω(Tn*551*)*1084 ofx-544*$^{+}$]	0.25	80
MT52184 (*nov ofx-545*)		8	20
	MT5310 [*nov* Ω(Tn*551*)*1085 ofx-545*$^{+}$]	0.25	80
MT522410 (*nov ofx-546*)		1	2.5
	MT5592 [*nov* Ω(Tn*551*)*1086 ofx-546*$^{+}$]	0.25	80
MT5224c3 (*nov cfx-547*)		1	20
	MT5416 [*nov* Ω(Tn*551*)*1087 cfx-547*$^{+}$]	0.25	80
Parent strain (ISP794 [wild type])		0.25	0.16
MT111 (*ofx-548*)		2	0.16
	MT1114 [Ω(Tn*551*)*1088 ofx-548*$^{+}$]	0.25	0.16
MT201 (*cfx-549*)		1	0.04
	MT2019 [Ω(Tn*551*)*1089 cfx-549*$^{+}$]	0.25	0.16
MT211 (*cfx-550*)		2	0.16
	MT2116 [Ω(Tn*551*)*1090 cfx-550*$^{+}$]	0.25	0.16
MT221 (*cfx-551*)		8	0.16
	MT2214 [Ω(Tn*551*)*1091 cfx-551*$^{+}$]	1	0.16

[a] Cfx, ciprofloxacin; nov, novobiocin.
[b] + indicates that wild-type DNA has replaced the corresponding mutant allele after insertion of the transposon.

Cfx^{+} (ciprofloxacin susceptible), and 74.8% were Thr^{-}. Similar transformations with ISP2134 DNA and *ofxC541* and *cfxB543* mutants produced cotransformation frequencies with Em^{r} of 9.9 and 11.2%, respectively (Table 3). Additional transformations with DNA from ISP2133 [8325 *trp-489* Ω(Tn*917lac*)*2*] (representative cross in Table 3) produced cotransformation frequencies with Em^{r} and quinolone susceptibility of 29.1% for *cfxB542*, 20.6% for *cfxB543*, and 42.5% for *ofxC541*.

The gene order, *thrB cfxB/ofxC trp-489* (Fig. 3), was determined in two independent crosses (Table 4). In the first

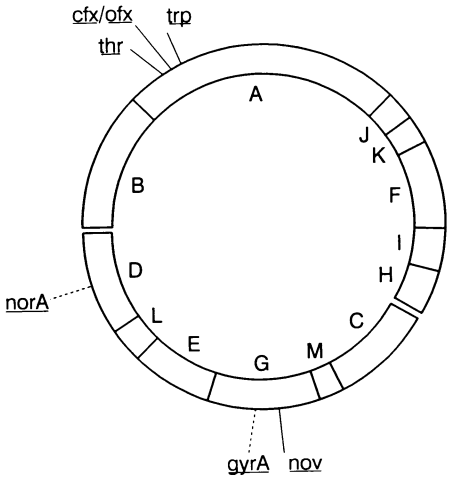

FIG. 1. Chromosome map of *S. aureus* modified from Pattee (25). The letters identify fragments obtained after *Sma*I digestion of the chromosome: A, 673 kb; B, 361 kb; C, 324 kb; D, 262 kb; E, 257 kb; F, 208 kb; G, 175 kb; H, 135 kb; I, 117 kb; J, 80 kb; K, 76 kb; L, 44 kb; M, 36 kb. The map locations of *thr*, *trp*, and *nov* are known positions. The location of *cfx/ofx* was established in this study. The locations of *norA* and *gyrA* were identified in this study by physical mapping to the identified fragment.

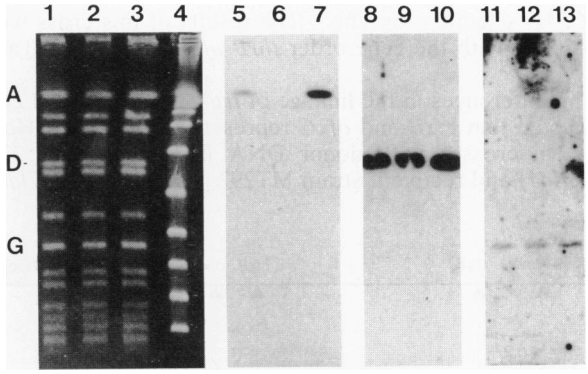

FIG. 2. PFGE in agarose gel and Southern hybridization analysis of quinolone resistance loci. PFGE gel after staining with ethidium bromide (lanes 1 to 4). Hybridization analysis of a PFGE gel using as probes ^{32}P-labeled Tn*917* (lanes 5 to 7), cloned 5.3-kb *Hin*dIII fragment containing *norA* (lanes 8 to 10), and *S. aureus gyrA* oligonucleotide (lanes 11 to 13). Strains: lane 1, MT5382 [*nov* Ω(Tn*551*)*1084 ofx-544*$^{+}$]; lane 2, MT5310 [*nov* Ω(Tn*551*)*1085 ofx-545*$^{+}$]; lane 3, MT5 (*nov*); lane 4, lambda ladder; lane 5, MT5592 [*nov* Ω(Tn*551*)*1086 ofx-546*$^{+}$]; lane 6, ISP794 (wild type); lane 7, MT5553 [*nov* Ω(Tn*551*)*1081 ofxC541*$^{+}$]; lane 8, MT5452 [*nov* Ω(Tn*551*)*1083 cfxB543*$^{+}$]; lane 9, MT2019 [Ω(Tn*551*)*1089 cfx-549*$^{+}$]; lane 10, MT5553 [*nov* Ω(Tn*551*)*1081 ofxC541*$^{+}$]; lane 11, MT2214 [Ω(Tn*551*)*1091 cfx-551*$^{+}$]; lane 12, MT5531 [*nov* Ω(Tn*551*)*1082 cfxB542*$^{+}$]; lane 13, MT1114 [Ω(Tn*551*)*1088 ofx-548*$^{+}$]. Letters at the left identify the *Sma*I fragments of the *S. aureus* chromosome.

TABLE 3. Linkage of *thrB* and *trp* to *flq* loci as determined by transformation[a]

Cross	Donor		Recipient		Transformant genotype class[b]			No. in class
	Strain	Genotype	Strain	Genotype	*thr*	*trp*	*cfx*	
1	ISP2134	*thrB494* Ω(Tn*917lac*)*1*	MT5224c4	*cfxB542*	−		− (r)	77
					+		− (r)	25
					−		+ (s)	3
					+		+ (s)	2
2	ISP2134	*thrB494* Ω(Tn*917lac*)*1*	MT5224c9	*cfxB543*	−		− (r)	67
					+		− (r)	28
					−		+ (s)	6
					+		+ (s)	6
3	ISP2134	*thrB494* Ω(Tn*917lac*)*1*	MT52222	*ofxC541*	−		− (r)	153
					+		− (r)	47
					−		+ (s)	12
					+		+ (s)	10
4	ISP2133	*trp-489* Ω(Tn*917lac*)*2*	MT5224c9	*cfxB543*		−	− (r)	197
						−	+ (s)	52
						+	− (r)	3
						+	+ (s)	0

[a] Selection was for erythromycin resistance.
[b] +, inheritance of the wild-type allele; −, inheritance of the mutant allele; r, resistant; s, susceptible.

cross, transforming DNA was prepared from strain MT5111 [*trp-489* Ω(Tn*917lac*)*2 cfxB543*] and was used to transform recipient ISP2134 [*thrB494* Ω(Tn*917lac*)*1*] with selection for Thr$^+$. *trp-489* and *cfxB543* were scored as unselected markers. The least frequent class had the *trp-489* phenotype of the donor and the sensitive Flq$^+$ allele of the recipient. Assuming that this class of recombinants resulted from four crossover events and that the more frequent genotypes resulted from only two crossover events, *cfxB543* must lie between *thrB* and *trp-489*. The cotransformation frequencies found for *thrB* with *cfxB543* (20%) and *thrB* with *trp-489* (5.6%) were also most consistent with the gene order *thrB cfxB trp-489*. The second three-factor transformational cross used DNA from strain ISP2133 [*trp-489* Ω(Tn*917lac*)*2*] to transform double mutant MT4172 [*thrB494* Ω(Tn*917lac*)*1 ofxC541*] selecting for Thr$^+$. The results of this cross were consistent with the gene order *thrB ofxC541 trp-489* (Table 4).

The differences in the linkage of *trp-489* to *ofxC* and *cfxB* suggested that *cfxB* and *ofxC* represent distinct loci. However, in crosses with donor DNA from strain MT52222 (*ofxC541*) and recipient strain MT292 [*trp-489* Ω(Tn*917lac*)*2 cfxB542*] with selection for Trp$^+$ and scoring for fluoroquinolone resistance, none of 295 transformants either was susceptible or had additive resistance. Thus, it is not yet possible to determine whether *cfxB* and *ofxC* represent distinct loci.

Determination that the *flq* mutations in the A fragment are not in *norA*, *gyrA*, or *gyrB*. (i) Localization of *norA* by PFGE. Whether *cfxB* and *ofxC* were mutations in *gyrA*, *gyrB*, or *norA* was evaluated as follows. *S. aureus* MT1222 constructed by serial passage of ISP794 on agar containing increasing concentrations of norfloxacin to contain multiple mutations affecting fluoroquinolone resistance was used to prepare genomic DNA. This strain had high-level resistance to fluoroquinolone agents, with MICs of 256 μg of norfloxacin per ml (512-fold increase), 64 μg of ciprofloxacin per ml (256-fold increase), and 8 μg of ofloxacin per ml (16-fold increase). Genomic and pUC19 DNA were digested with the restriction enzyme *Hin*dIII and ligated with T4 DNA ligase. The ligated fragments were used to transform supercompetent DH10B cells (Bethesda Research Laboratories), and a 5.3-kb fragment of *S. aureus* DNA was isolated by selection for fluoroquinolone and carbenicillin resistance (the latter encoded by the pUC19 vector). This cloned fragment had a

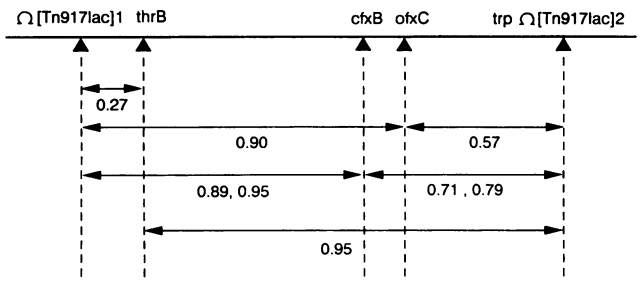

FIG. 3. Linkage relationship of fluoroquinolone resistance loci (*cfxB* and *ofxC*), *thrB*, and *trp*. Map distances are calculated from the data in Tables 3 and 4. Ω(Tn*917lac*)*1-thrB494* linkage data reflect cumulative data from crosses 1 to 3 in Table 3. *thrB494-trp-489* linkage data reflect cumulative data from crosses in Table 4. Map distances between two markers A and B are expressed as 1 minus the estimated cotransformation frequency (*C*), where *C* = frequency of cotransformation of two markers A and B (22).

TABLE 4. Linkage between *trp*, *thrB*, and *cfxB* or *ofxC*[a]

Cross	Donor		Recipient		Transformant genotype class[b]		No. in class
	Strain	Genotype	Strain	Genotype	*trp*	*cfxB/ofxC*	
1	MT5111	*trp cfxB*	ISP2134	*thrB*	+	+ (s)	167
					+	− (r)	36
					−	− (r)	7
					−	+ (s)	5
2	ISP2133	*trp*	MT4172	*ofxC thrB*	+	− (r)	203
					−	+ (s)	6
					+	+ (s)	5
					−	− (r)	3

[a] Selection was for Thr$^+$.
[b] +, inheritance of the wild-type allele; −, inheritance of the mutant allele; r, resistant; s, susceptible.

restriction enzyme map (with *Kpn*I, *Hae*III, *Hin*dIII, *Bam*HI, *Hin*cII, and *Eco*RI) highly similar to that of the *norA* genes cloned in several laboratories (21, 38, 39; data not shown). The *norA* DNA hybridized with a strong, specific signal to fragment D of the *Sma*I-digested *S. aureus* chromosome (Fig. 2, lanes 8 to 10). Thus, the *norA* locus was located on a different fragment of the *S. aureus* chromosome from the A fragment mutants.

(ii) Localization of *gyrA* by PFGE. A 39-bp synthetic oligonucleotide was prepared based on the sequence of a highly conserved region of the *S. aureus gyrA* gene, including the codon for the presumed active site (nucleotides 349 to 388) (12), labeled with ^{32}P by phosphorylation with bacteriophage T4 polynucleotide kinase (29), and used to probe the *Sma*I-digested *S. aureus* chromosome. A positive signal was obtained in the G fragment only (Fig. 2, lanes 11 to 13). This localizes the *gyrA* gene and the *gyrB* gene (which is contiguous to *gyrA* [12]) to the G fragment. Thus, the *cfxB* and *ofxC* mutations are in a gene(s) in the A fragment and are not in *norA*, *gyrA*, or *gyrB*, which are located in different fragments.

DISCUSSION

On the basis of fluoroquinolone resistance mechanisms in *E. coli*, other workers have cloned and partially sequenced the *gyr* genes of *S. aureus* (31) and identified point mutations associated with resistance. These studies were performed with *S. aureus* clinical isolates that may contain multiple mutations contributing to fluoroquinolone resistance. Thus, the relationship between resistance and the point mutations found is circumstantial and rests on the finding of inferred amino acid changes in the gyrase A protein from the post-treatment resistant isolates that are analogous to those found to cause resistance in *E. coli*.

Yoshida et al. (39) and Ohshita et al. (21) have identified a gene, *norA*, which confers fluoroquinolone resistance when it is present on a high-copy-number plasmid. Based on the DNA sequence, *norA* appears to encode a membrane protein. A chromosomal *norA* mutation has not been examined, leaving uncertain the effect of single copies of the gene on resistance. Although the loci previously described may contribute to fluoroquinolone resistance, this deduction has not been confirmed by genetic analysis of mutant loci on the *S. aureus* chromosome.

Using genetic analysis and physical mapping techniques, we identified a novel gene that confers fluoroquinolone resistance in *S. aureus*. The linkage of these loci to *thrB* and *trp* suggests that *ofxC* is a genetically distinct gene from *cfxB*, but this hypothesis could not be confirmed by a direct cross between *ofxC* and *cfxB*. The cloning and sequencing of these mutations will determine whether they represent a single or closely linked genes.

The product of the *ofxC* and *cfxB* gene(s) and its mechanism of action are not yet known. The phenotypic interaction of these and other A-fragment mutants with the *nov* locus suggests that the gene products interact. In *B. subtilis* and *E. coli*, *nov* (or *cou*) resistance loci are usually alleles of *gyrB*, and in *B. subtilis* (7, 15, 16) and *S. aureus* (12), the *gyrB* and *gyrA* genes are contiguous. Because the *nov* locus (25) in *S. aureus* is on the same *Sma*I-digested DNA fragment as the *gyrA* gene, the *nov* locus in *S. aureus* may be *gyrB*. The phenotypic interaction of *nov* and the A-fragment loci further suggest that the A fragment mutations are in the gene for topoisomerase I (*topA*) (36). DNA gyrase and bacterial topoisomerase I have antagonistic effects on the supercoiling of DNA, which may affect gene expression (28), and thus a mutation in one may be counterbalanced by a mutation in the other, as occurs in *E. coli* (27). Alternatively, the A-fragment mutations may alter the gene(s) of yet another topoisomerase (such as topoisomerase IV [13]) or a nontopoisomerase gene altering supercoiling (such as *osmZ* [8]) or may coordinately affect the permeation of both quinolones and novobiocin in opposite ways. Because mechanisms of quinolone resistance identified thus far are due either to alterations in DNA gyrase or alterations affecting permeation of quinolones but not novobiocin, the *flq* locus in the A fragment appears to involve a novel mechanism of quinolone resistance.

In this study, three loci associated with quinolone resistance were localized to distinct areas of the *S. aureus* chromosome. Our A-fragment mutations are genetically distinct from these previously described loci and thus are novel. Particularly striking is the finding that all 11 of the single-step mutants (selected with ciprofloxacin and ofloxacin) mapped by PFGE in the A fragment. Studies to clone, sequence, and identify the product of the fluoroquinolone resistance loci are ongoing.

ACKNOWLEDGMENTS

We especially thank Peter Pattee for his generous assistance in supplying bacterial strains, phage stocks, unpublished procedures, and critical reading of the manuscript. We thank Steve Beverley for his assistance with PFGE procedures and Morton Swartz for stimulating discussions.

This work was supported in part by grants from the United States Public Health Service and National Institutes of Health (RO1 AI23988), Lederle Laboratories (Pearl River, N.Y.), and The Robert Wood Johnson Pharmaceutical Research Institute (Raritan, N.J.). M.T. was supported by infectious diseases training grant AI07061 to Harvard Medical School from the National Institutes of Health.

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