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Genetics and Regulation of Outer Membrane Protein Expression by Quinolone Resistance Loci *nfxB*, *nfxC*, and *cfxB*

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Quinolone resistance mutations (*cfxB1*, *marA1*, and *soxQ1*) that reduce porin outer membrane protein OmpF map near 34 min on the *Escherichia coli* chromosome. Another such mutation, *nfxC1*, was found in strain KF131 (*nfxB*, 19 min). *nfxC1* and *cfxB1* mutants (selected with quinolones) differed slightly but reproducibly from *marA1* (selected with tetracycline) and *soxQ1* (selected with menadione) mutants in quinolone resistance and linkage to *zdd2208*::Tn*10kan* (33.7 min). For *nfxB nfxC1* and *cfxB1* mutants, as previously shown for *marA* mutants, resistance and reduced OmpF required the *micF* locus encoding an antisense RNA complementary to *ompF* mRNA and were associated with increased *micF* expression.

The development of bacterial resistance to fluoroquinolones has been increasingly recognized in clinical settings (18). Two mechanisms of resistance, alterations in the target enzyme DNA gyrase (8, 16, 21, 28–30) and decreased drug accumulation associated with changes in the bacterial outer membrane (2, 4, 5, 17, 20) have been characterized. In *Escherichia coli*, several resistant mutants with the latter mechanism (*nfxB* [21], *cfxB1* [19], *marA* [5, 10], and *norB* [17]) are pleiotropic (also having resistance to tetracycline, chloramphenicol, and some β-lactams) and involve interactions of several genetic loci (6, 20). *cfxB1* (selected with ciprofloxacin) and *marA* (selected with tetracycline) mutations are located around 34 min on the *E. coli* genetic map. Other quinolone resistance mutations in this region are now known and include *soxQ1* (selected with the naphthoquinone menadione) (12) and *nfxC1*, reported here in strain KF131 (*nfxB*), selected with norfloxacin.

Fluoroquinolone resistance appears to occur by reduction in drug accumulation that results from the interaction of fluoroquinolone efflux at the inner membrane and reduced OmpF porin channels in the outer membrane (4, 5, 20). The expression of *ompF*, which encodes OmpF protein, is regulated at the level of both transcription and translation (7, 25, 27). When overexpressed, the *micF* locus, which encodes an antisense RNA complementary to the 5′ end of the *ompF* message (1, 26), reduces *ompF* translation, likely because of its destabilization of *ompF* mRNA binding to the ribosome (1). *nfxB*, *cfxB1*, and *marA* mutants reduce *ompF* expression after transcription (6, 20), and we report here the involvement of *micF* in resistance and *ompF* expression in *nfxB nfxC1* and *cfxB1* mutants, as was reported in *marA* mutants (6).

Media included Mueller-Hinton and Luria-Bertani broth and agar (24) and, for experiments with *lacZ* fusion strains, A medium (24). The MIC was the lowest concentration of a doubling series at which no growth occurred on agar plates inoculated with a Steers device. The *E. coli* strains used, their relevant genotypes, and their sources are listed in Table 1.

Transformation was performed by the method of Lederberg and Cohen (22), and P1*vir* transduction was by the method of Miller (24). Selections for transposon insertions were done with 30 μg of tetracycline per ml (Tn*10*) or 20 μg of kanamycin per ml (Tn*10kan*). β-Galactosidase specific activity was measured by the method of Miller (24), and preparation of outer membrane proteins and urea-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were as previously described (21).

Prior experiments (20) in which resistance of KF131 was abolished by the presence of Tn*5*::*marA* (34 min) suggested a requirement for an intact *marA* gene. To determine if KF131 contained a mutation in the 34-min region, a P1 phage lysate grown on strain PLK1253 (*zde-234*::Tn*10*, 34.2 min) was used to transduce KF131 (*nfxB*) and EN226-8 (*cfxB1*), selecting for tetracycline resistance encoded by Tn*10*. Twenty-eight of 43 (65%) KF131 transductants and 39 of 47 (83%) EN226-8 transductants had wild-type susceptibility to norfloxacin, indicating that KF131, like EN226-8 (19), contained a mutation (termed *nfxC1*) near 34 min.

These findings were confirmed by transduction of *zdd2208*::Tn*10kan* (33.7 min) from strain JHC1075 into KF131. In 52 of 53 kanamycin-resistant transductants (98%), norfloxacin resistance returned to the wild-type level (0.08 μg/ml).

To compare *nfxC1*, *cfxB1*, *marA*, and *soxQ1*, we performed outcrosses of these loci by linkage to *zde-234*::Tn*10* and to *zdd2208*::Tn*10kan*. P1 lysates of MB320 (*nfxB nfxC1 zde-234*::Tn*10*), MB310 (*cfxB1 zde-234*::Tn*10*), and MB330 (*marA zde-234*::Tn*10*) (5) were used to transduce wild-type strain KL16, selecting for Tn*10*. Twenty-eight of 48 (58%) transductants from a lysate of MB320, 26 of 48 (54%) transductants from a lysate of MB310, and 0 of 32 transductants from a lysate of MB330 had complete norfloxacin resistance (MIC, 0.32 μg/ml). Eighteen of 32 (56%) transductants from the MB330 lysate, however, had an intermediate level of norfloxacin resistance (MIC, 0.16 μg/ml). Thus, the linkages of *nfxC1*, *cfxB1*, and *marA* to *zde-234*::Tn*10* were similar, but the *marA* allele of AG100-Tc2.5-1 conferred a lower level of norfloxacin resistance than *nfxC1* and *cfxB1*. Because outcross of the *nfxC1* mutation produced the same level of quinolone resistance as KF131 (*nfxB nfxC1*), the role of *nfxB* in resistance is uncertain.

To exclude possible linkage artifacts caused by tetracy-

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† Deceased 11 October 1991. This paper is dedicated to the memory of John S. Wolfson.

TABLE 1. *E. coli* strains

Strain	Genotype	Source or reference
KL16	Hfr *thi-1 relA spoT1* lambda$^-$	B. Bachman
KF131	KL16 *nfxB nfxC1*	This laboratory (21)
EN226-8	KL16 *cfxB1*	This laboratory (19)
AG100	*argE thi-1 rpsL xyl mtl* Δ(*gal-uvrB*) *supE44* lambda$^-$	S. B. Levy (10)
AG100-Tc2.5-1	AG100 *marA*	S. B. Levy (5)
MB101	KL16 Tn*5*::Δ*micF*	This study
MB102	KF131 Tn*5*::Δ*micF*	This study
MB103	EN226-8 Tn*5*:Δ*micF*	This study
MH621	MH20 Φ(*ompF-lacZ*) *16-21* (Hyb)	T. J. Silhavy (15)
MB201	MH621 Tn*5*::Δ*micF*	This study
MB202	MH621 *nfxB/nfxC1*	This study
MB203	MB202 Tn*5*::Δ*micF*	This study
MB204	MH621 *cfxB1*	This study
MB205	MB204 Tn*5*::Δ*micF*	This study
MB310	EN226-8 *zde-234*::Tn*10*	This study
MB320	KF131 *zde-234*::Tn*10*	This study
MB330	AG100-Tc2.5-1 *zde-234*::Tn*10*	This study
JHC1075	Δ*lac4169 zdd2208*::Tn*10kan*	B. Demple
JHC1072	Δ*lac4169 zdd2208*::Tn*10kan soxQ1*	B. Demple
JHC1069	Δ*lac4169 zdd2208*::Tn*10kan cfxB1*	B. Demple
JHC1113	Δ*lac4169 zdd2207*::Tn*10kan marA1*	B. Demple
DH115	KF131 *zdd2208*::Tn*10kan*	This study
MH20	F$^-$Δ(*lac*)*U169 rpsL relA thiA flbB*	T. J. Silhavy (15)
MH450	MH20 *ompF*::Tn*5* 1	T. J. Silhavy (15)
PLK1253	*trpR trpA9605 his-29 ilv pro arg thyA deoB* or *deoC tsx* Δ*rac zdd-230*::Tn*9 zde-234*::Tn*10*	L. McMurry (3)
SM3001	F$^-$ Δ*lacU169 araD rpsL relA thi flbB* Δ*micF1*	S. Mizushima (23)

cline selection of Tn*10*, the tetracycline resistance of which overlaps that of *nfxC1*, *cfxB1*, and *marA*, we compared linkages to *zdd2208*::Tn*10kan* (or *zdd2207*::Tn*10kan* for *marA*) located at 33.7 min. P1 lysates prepared from strains JHC1069 (*cfxB1 zdd2208*::Tn*10kan*), JHC1072 (*soxQ1 zdd2208*::Tn*10kan*), JHC1113 (*marA1 zdd2207*::Tn*10kan*), and DH115 (*nfxC1 zdd2208*::Tn*10kan*) were used to transduce KL16, selecting for kanamycin resistance. The linkages of *cfxB1* (85 of 104 [81.7%]) and *nfxC1* (124 of 153 [81.0%]) were similar but differed somewhat from those of *soxQ1* (98 of 104 [90.7%]) and *marA* (74 of 100 [74%]), suggesting possible differences in the location of these mutations. The level of resistance conferred also differed, with MICs of norfloxacin for *cfxB1* and *nfxC1* transductants of 0.32 μg/ml and for *marA* and *soxQ1* transductants of 0.16 μg/ml. Clear distinction of whether these mutations are alleles of the same or highly linked genes will likely require cloning and DNA sequencing.

To determine the role of *micF* in the resistance and reduction of OmpF caused by *nfxB/nfxC1* and *cfxB1*, we transduced Tn*5*::Δ*micF* from strain SM3001 into KL16; KF131; EN226-8; MH621, which contains an *ompF-lacZ* protein fusion; and previously constructed derivatives of MH621 containing *nfxB/nfxC1* (MB202) and *cfxB1* (MB201) (20). Transductants of KF131 and EN226-8 had return of norfloxacin MICs to the level for KL16 but above that for KL16Δ*micF* (Table 2). Introduction of Tn*5*::Δ*micF* also resulted in increased amounts of OmpF in the outer membrane in KF131 and EN226-8 (Fig. 1) and return of the β-galactosidase levels from the *ompF-lacZ* fusions of MB202 and MB201 to the level of MH621 (Table 3). These findings indicate a requirement for *micF* for both resistance and reduced *ompF* expression caused by *nfxB/nfxC1* and *cfxB1* but suggest that a small remaining effect (twofold) on norfloxacin resistance may be independent of *micF*. Similarly, in the *nfxB/nfxC1* but not the *cfxB1* mutant, resistances to tetracycline and chloramphenicol were abolished by Δ*micF* (Table 2), suggesting that *cfxB1* may also regulate these resistances independently of *micF*.

To determine the effects of *nfxB nfxC1* and *cfxB1* on *micF* expression, plasmid pMicB21, which contains a *micF-lacZ* operon fusion, was transformed into MB101, MB102, and MB103 (genotypes in Table 2). β-Galactosidase activity was undetectable in MB101 lacking pMicB21. In the presence of pMicB21, β-galactosidase activity was readily detected and was increased 8- to 12-fold in the presence of *nfxB/nfxC1* and *cfxB1*. These differences did not appear to be due to the copy number of pMicB21, because agarose gel electrophoresis of limiting dilutions of minipreps of plasmid DNA from these strains revealed minimal to no differences in the amounts of plasmid DNA (data not shown). Thus, these mutations apparently increase transcription of *micF* RNA.

There are many similarities in the phenotypes of these mutations located around 33.8 min. *marA* (5, 6, 9) and *soxQ1*

TABLE 2. Effects of a *micF* deletion on antimicrobial agent resistance of *nfxB* and *cfxB* mutants

Strain	Genotype	MIC (μg/ml)		
		Norfloxacin	Tetracycline	Chloramphenicol
KL16	Wild type	0.08	4.0	8.0
MB101	KL16 Δ*micF*	0.04	4.0	8.0
KF131	KL16 *nfxB nfxC1*	0.32	16.0	32.0
MB102	KF131 Δ*micF*	0.08	4.0	8.0
EN226-8	KL16 *cfxB1*	0.32	8.0	32.0
MB103	EN226-8 Δ*micF*	0.08	8.0	16.0

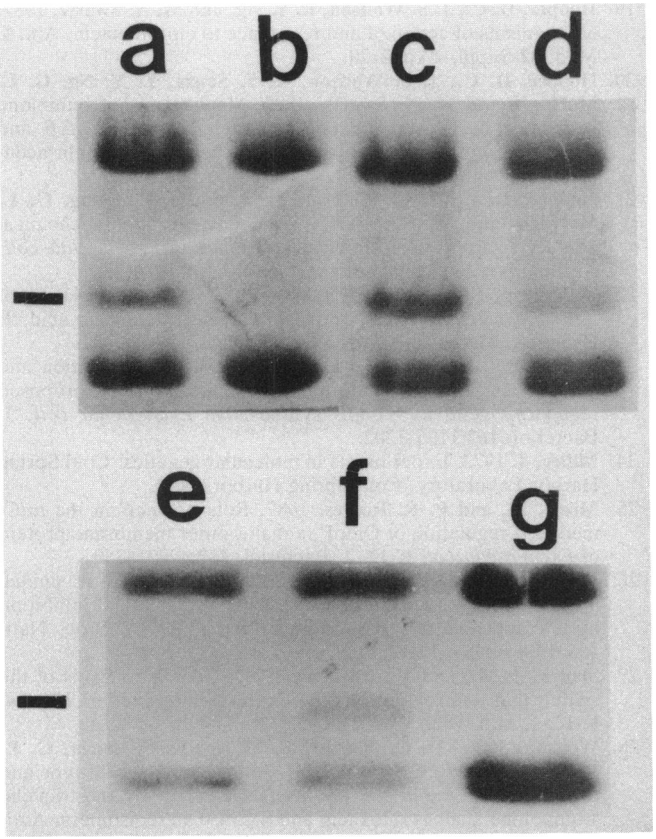

FIG. 1. Urea-SDS-polyacrylamide gels of outer membrane proteins of strains MB102 (KF131 Δ*micF*) (a), KF131 (*nfxB nfxC1*) (b), MB101 (KL16 Δ*micF*) (c), KL16 (wild type) (d), EN226-8 (*cfxB1*) (e), MB103 (EN226-8 Δ*micF*) (f), and MH450 (*ompF*::Tn*5*) (g). The region of OmpF, OmpC, and OmpA is shown, and OmpF is indicated by the marker on the left.

(11), like *cfxB1*, also mediate quinolone resistance in a manner dependent on the *micF* locus. Both *soxQ1* and *cfxB1* mutations result in increased expression of a similar array of proteins distinguished by two-dimensional polyacrylamide gel electrophoresis (11).

Despite substantial overlap in phenotypes, however, there were differences among the *soxQ1*, *cfxB1*, *nfxC1*, and *marA* mutations. The level of norfloxacin resistance conferred by *cfxB1* and *nfxC1* was twofold higher than that of *marA* and *soxQ1*, which is consistent with the earlier findings for nalidixic acid resistance in *cfxB1*, *marA*, and *soxQ1* mutants (11). *soxQ1* and *cfxB1* also differ in their effects on the cellular levels of endonuclease IV and glucose-6-phosphate dehydrogenase, and the *marA1* mutation has the properties of a weak allele of *soxQ1* (11). In addition, *cfxB1* is dominant to $cfxB^+$ in merodiploids (20), whereas *marA* exhibits only partial dominance to $marA^+$ (10).

The recent cloning and sequencing of the *marA* region has also revealed a potentially complex operon of at least three genes (13, 14). Further DNA sequence analysis will be required for dissection of these loci and further elucidation of their regulatory functions. The pleiotropic effects of these mutations and their selection with distinct compounds suggest that they are part of overlapping networks of genes that allow the cell to respond to a range of environmental insults, including a synthetic class of compounds, such as the quinolones.

Specific subcomponents of such networks may have distinct final effectors, and those that affect quinolone resistance have in common reduced OmpF, which is thought to decrease the rate of diffusion of quinolones across the outer membrane (4, 20). Reduced fluoroquinolone accumulation in these mutants is reversed by energy inhibitors and, thus, appears to result from the interactions of diminished OmpF with an energy-dependent mechanism, which appears to involve a saturable quinolone efflux system shown in everted inner membrane vesicles of wild-type and mutant bacteria (4, 5, 20). Because quinolone efflux was unchanged in a *marA* mutant, proof of its role in resistance awaits identification of other mutants with altered efflux. Because the *cfxB1* and *nfxC1* mutants have greater resistance to norfloxacin than the *marA1* mutant and have residual resistance in the absence of *micF*, investigations to determine if the *cfxB1* and *nfxC1* mutations also augment quinolone efflux are under way.

TABLE 3. Interactions of *micF* with *nfxB* and *cfxB* on expression of an *ompF-lacZ* protein fusion

Strain	Genotype	β-Galactosidase units (Mean ± SD)	% Relative to wild type
MH621	Wild type	1,015 ± 166	100
MB201	MH621Δ*micF*	732 ± 56	72
MB202	MH621 (*nfxB*)[a] *nfxC1*	98 ± 5.9	10
MB203	MB202 Δ*micF*	1,088 ± 30	107
MB204	MH621 *cfxB1*	56 ± 2.6	6
MB205	MB204 Δ*micF*	1,015 ± 232	100

[a] MB202 and MB204 were constructed by P1 transduction to MH621 from KF131 (*nfxB nfxC1*) and EN226-8 (*cfxB1*), selecting for norfloxacin resistance. It is uncertain whether MB202 contains *nfxC1* alone or in combination with *nfxB*.

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