Mechanisms of Quinolone Resistance in *Escherichia coli*: Characterization of *nfxB* and *cfxB*, Two Mutant Resistance Loci Decreasing Norfloxacin Accumulation

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Two genetic loci selected for norfloxacin (*nfxB*) and ciprofloxacin (*cfxB*) resistance were characterized. Both mutations have previously been shown to confer pleiotropic resistance to quinolones, chloramphenicol, and tetracycline and to decrease expression of porin outer-membrane protein OmpF. *nfxB* was shown to map at about 19 min and thus to be genetically distinct from *ompF* (21 min), and *cfxB* was shown to be very closely linked to *marA* (34 min). *cfxB* was dominant over *cfxB* in merodiploids, in contrast to other quinolone resistance mutations. The two loci appear to interact functionally, because *nfxB* was not expressed in the presence of *marA*:Tn5. Both *nfxB* and *cfxB* decreased the expression of *ompF* up to 50-fold at the posttranscriptional level in strains containing *ompF-lacZ* operon and protein fusions. Both mutations also decreased norfloxacin accumulation in intact cells. This decrease in accumulation was abolished by energy inhibitors and by removal of the outer membrane. These findings, in conjunction with those of Cohen et al. (S. P. Cohen, D. C. Hooper, J. S. Wolfson, K. S. Souza, L. M. McMurry, and S. B. Levy, Antimicrob. Agents Chemother. 32:1187–1191, 1988), suggest a model for quinolone resistance by decreased permeation in which decreased diffusion through porin channels in the outer membrane interacts with a saturable drug efflux system at the inner membrane.

Two mechanisms of resistance to quinolone antibacterial agents have been recently characterized: alteration of the target enzyme DNA gyrase and decreased drug accumulation. Drug resistance mutations in the *gyrA* and *gyrB* genes of DNA gyrase are specific to quinolones and have been identified in *Escherichia coli* (3, 12, 19, 21–23, 37, 39, 43, 47, 48), *Pseudomonas aeruginosa* (20, 24, 34, 35), and *Citrobacter freundii* (2) strains exposed to nalidixic acid, pipemidic acid, norfloxacin, ciprofloxacin, and ofloxacin.

Quinolone resistance caused by decreased drug accumulation is conferred by mutations in genes other than *gyrA* or *gyrB*. Several such genes have been characterized in *E. coli* (5, 9, 14, 19, 21, 22) and *P aeruginosa* (20, 24, 35). In *E. coli*, mutations in *nfxB* (22), *D. C. Hooper, J. S. Wolfson, K. S. Souza, and M. N. Swartz, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 944, 1986), *norB* (19), *norC* (19) selected for norfloxacin resistance, and *cfxB* (21) selected for ciprofloxacin resistance have been associated with pleiotropic resistance to nonquinolone antimicrobial agents, decreased porin outer-membrane protein OmpF, and decreased drug binding. An *E. coli* strain selected for resistance by serial passage on increasing concentrations of enoxacin also had decreased amounts of OmpF and decreased enoxacin accumulation (5). Mutations in the structural gene for OmpF result in modest (twofold or less) decreases in norfloxacin susceptibility and accumulation (18). These findings and those characterizing norfloxacin (18) or ciprofloxacin (21) uptake in *Salmonella* or *E. coli* strains have suggested that one route of hydrophilic quinolone permeation into these bacteria is via porin outer-membrane proteins, in particular OmpF.

Similarly, in *P. aeruginosa* strains selected for norfloxacin resistance (20) and ciprofloxacin resistance (35), pleiotropic resistance has been associated with alterations in outer-membrane proteins (20, 35) and drug uptake (20). In this organism, however, in some resistant isolates a new outer-membrane protein was detected (20), and outer-membrane protein changes have been seen with selective (nonpleiotropic) quinolone resistance (11).

Recently an energy-dependent system located at the bacterial inner membrane has been found to diminish norfloxacin accumulation in wild-type *E. coli* (9). This system was saturable with excess norfloxacin in everted membrane vesicles, and in intact cells with an *ompF* mutation it decreased net norfloxacin accumulation.

The purpose of our studies was to evaluate in more depth the genetics and mechanism of resistance of *nfxB* and *cfxB*. *nfxB* was shown to be genetically distinct from the structural gene *ompF*, and *cfxB* (21) was shown to be very closely linked to *marA* (13, 14), a previously described mutant locus selected with tetracycline or chloramphenicol and causing pleiotropic resistance, including resistance to nalidixic acid. *nfxB* and *cfxB* were also shown to decrease expression of OmpF at the posttranscriptional level and to diminish norfloxacin accumulation by an energy-dependent process that requires an intact outer membrane.

**MATERIALS AND METHODS**

**Chemicals.** Norfloxacin was obtained from Merck & Co., Inc., Rahway, N.J., and ciprofloxacin was obtained from Miles Inc., Pharmaceutical Division, West Haven, Conn. Both were dissolved (1 mg/ml) in 0.02 N NaOH. [3H]Norfloxacin (74 mCi/mg) uniformly labeled in the pipera-azine ring was generously provided by Merck & Co. Rifampin was purchased from Calbiochem-Behring, La Jolla, Calif. Chloramphenicol, tetracycline hydrochloride, 2,4-dinitrophenol, carbonyl cyanide m-chlorophenylhydrazine

* Corresponding author.
TABLE 1.  E. coli strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KL16</td>
<td>Hfr thi-1 relA spoT1 &amp;</td>
<td>B. Bachmann</td>
</tr>
<tr>
<td>KF130</td>
<td>KL16 gyrA</td>
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<td>KF131</td>
<td>KL16 ncsB</td>
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<td>EN226-8</td>
<td>KL16 cfsB</td>
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<td>RW1229</td>
<td>F- pro aroA galK</td>
<td>R. Weisberg</td>
</tr>
<tr>
<td>JC10240</td>
<td>thr-300 recA srl-300::Tn10</td>
<td>B. Bachmann</td>
</tr>
<tr>
<td></td>
<td>relA1 rpsE3200 (Spc')</td>
<td></td>
</tr>
<tr>
<td>GMS407</td>
<td>spoT1 lyv-318 thi-1</td>
<td>S. B. Levy (14)</td>
</tr>
<tr>
<td></td>
<td>acl-lacY1 galK2 manA4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mtl-1 tss-29 supE44 widd</td>
<td></td>
</tr>
<tr>
<td>KF700</td>
<td>GMS407 Rif' cfxB</td>
<td>This study</td>
</tr>
<tr>
<td>KF701</td>
<td>KF700 recA srl-300::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>KF702</td>
<td>GMS407 Rif' recA srl-300: :Tn10</td>
<td>This study</td>
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<td>J5550</td>
<td>lacY1 galK2 manA4 aroD</td>
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<tr>
<td></td>
<td>gyrA12 recA1 rpsL100</td>
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<td>mtl-1 argE3 supE44 F'506</td>
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<td></td>
<td>fbbB</td>
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<td></td>
<td>(HYB.)</td>
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<td>T. Silhavy (15)</td>
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<td>AG100</td>
<td>argE thi-1 rpsL xyl ml galK</td>
<td>S. B. Levy (14)</td>
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<tr>
<td></td>
<td>(gal-uvrB) supE44 &amp;</td>
<td></td>
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<tr>
<td>AG1065</td>
<td>AG100 marA::Tn3</td>
<td>S. B. Levy (14)</td>
</tr>
<tr>
<td>PLK1253</td>
<td>trpR trpA9605 his-29 lyv pro</td>
<td>L. McMurry (6)</td>
</tr>
<tr>
<td></td>
<td>arg thyA deoB or deoC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tss rac zdd-230::Tn9 zde-234::Tn10</td>
<td></td>
</tr>
<tr>
<td>ML308-225</td>
<td>lacI lacY lacZ</td>
<td>L. McMurry (46)</td>
</tr>
</tbody>
</table>

(CCCP), and lysozyme were obtained from Sigma Chemical Co., St. Louis, Mo.

Bacteriologic media. Rich media included Mueller-Hinton broth and agar and L broth and agar (27). In experiments using ompF-lacZ fusion strains, cells were grown in A medium (27) and in experiments measuring norfloxacin accumulation, cells were grown in M9 broth supplemented with Casamino Acids (0.5%), glucose (0.2%), and thiamine (5 μg/ml) (9).

Bacterial strains and bacteriophages. The E. coli strains used, their relevant genotypes, and their sources are listed in Table 1. Bacteriophages P1 vir and P1 Tn9 clrl00 were gifts from G. Jacoby and L. J. Rosner, respectively.

Conjugation and bacteriophage P1 transduction. Conjugation and P1 vir transduction were done by the protocol of Miller (27), except that direct selection for resistance to norfloxacin or ciprofloxacin in transductions required a 2- to 3-h delay before addition of a second drug-containing top agar (16, 22). Lysozymization, induction, and transduction with P1 Tn9 clrl00 were done by the procedures of Rosner (36) and Silhavy et al. (38).

Dominance studies. To test the dominance of cfxB in relation to cfxB' , GMS407 Rif' was selected by plating GMS407 on agar containing 100 μg of rifampin per ml. cfxB was then transferred to GMS407 Rif' by P1 transduction with selection for ciprofloxacin resistance. The presence of cfxB in this strain (KF700) was confirmed by scoring for resistance to tetracycline and chloramphenicol. Strain JC10240 recA srl-300::Tn10 was then lysogenized with P1 Tn9 clrl00 with selection for chloramphenicol resistance at 30°C, and a P1 lysate was induced by incubation at 43°C. This P1 lysate was used to transduce KF700 and GMS407 Rif' with selection for tetracycline (30 μg/ml) resistance. Transductants were scored for susceptibility to UV irradiation as a marker for transfer of the recA mutation, which is linked to Tn10. One recA transductant of KF700 (cfxB) was designated KF701, and one such transductant of GMS407 Rif' (cfxB' ) was designated KF702.

Assessment of OmpF expression with ompF-lacZ fusion strains. The nfsB and cfxB mutations were introduced by P1 vir transduction with direct norfloxacin or ciprofloxacin selection into strains MH1531 and MH621, which contain chromosomal ompF-lacZ operon and protein fusions, respectively. Assays of β-galactosidase in these and the parent strains were performed by the method of Miller (27).

Preparation of spheroplasts. Spheroplasts of strains KL16 and KF131 were prepared by suspension of logarithmic-phase cells in a buffer of 50 mM Tris (pH 8)–20% sucrose, followed by treatment with EDTA (10 mM) and freshly prepared lysozyme (0.3 mg/ml) for 30 min at ambient temperature in a manner similar to that previously described (26). The spheroplasts were then suspended in spheroplast assay buffer (see below). Clumping of spheroplasts of these strains necessitated gentle dispersion with a Dounce homogenizer. Spheroplast formation was confirmed by the presence of osmotic fragility (>90% decrease in optical density upon dilution in water) and by rounding of cells examined by light microscopy.

Measurement of norfloxacin accumulation. (i) Intact cells. Bacteria were grown at 37°C to an optical density at 530 nm of 0.75, washed in medium, and suspended in fresh medium to an optical density at 530 nm of 60. [3H]norfloxacin (0.04 μg/ml, specific activity adjusted to 3.6 mCi/mg with unlabelled norfloxacin) was added, and samples (40 μl) were taken at specified times and collected on GF/C filters (Whatman, Inc., Clifton, N.J.) presoaked with medium. Filters were washed immediately with three 2-ml portions of medium, dried, and counted by liquid scintillation (Betalfluor; National Diagnostics, Somerville, N.J.). Binding of the label to filters in the absence of cells was subtracted from binding in the presence of cells. Cell protein was determined by the method of Lowry et al. (25), with bovine serum albumin as the standard. In comparisons of different bacterial strains, the results were expressed in nanograms of norfloxacin bound per milligram of cell protein. In later experiments comparing intact wild-type and mutant bacteria and using spheroplasts, bacteria were grown and incubated in the presence of norfloxacin at 30°C. At this lower temperature, differences in uptake between wild-type and mutant bacteria were more readily detected.

(ii) Spheroplasts. After the spheroplast formation procedure, cells were washed and suspended at an optical density at 530 nm of 20 to 40 in spheroplast assay buffer containing 50 mM potassium phosphate (pH 6.0), 20% sucrose, 10 mM magnesium sulfate, and 20 mM lithium lactate (26). The procedure was similar to that for intact cells, except that [3H]norfloxacin of higher specific activity (25 mCi/mg) was used at the same total drug concentration (0.04 μg/ml) and the spheroplasts were collected on membrane filters (Millipore HA; 0.45-μm pore size; Millipore Corp., New Bedford, Mass.) and washed with three 2-ml portions of spheroplast assay buffer in which LiCl (0.1 M) replaced lithium lactate.

RESULTS

Further mapping of nfxB. Previous mapping of the nfxB locus by gradient of transfer and cotransduction with aroA (20 min) had located nfxB at about 20 min on the E. coli
genetic map (22). ompF (21 min), the structural gene for porin OmpF, cotransduced 50% with pyrD (21 min), and nfxB did not cotransduce with pyrD (22). It remained possible, however, that nfxB was an allele of ompF and that a genetic rearrangement had occurred in the 19- to 21-min region of the chromosome of strain RW1229 (nfxB araA), which was used in the mapping experiments. To exclude this possibility, we used a P1 vir lysate grown on strain MH450 (ompF::Tn5) to transduce strains RW1229 (nfxB araA) and RW1229 (araA), selecting for kanamycin resistance. Cotransduction of araA with Tn5 was 32% (10 of 31) in recipient RW1229 (nfxB araA) and 33% (10 of 30) in recipient RW1229 (araA). Thus, introduction of nfxB into RW1229 did not result in a major genetic rearrangement in the 20- to 21-min region, and nfxB is likely genetically distinct from ompF.

Possible interactions of nfxB and cfxB. nfxB and cfxB have virtually identical phenotypes but are genetically distinct. cfxB has been previously mapped by interrupted mating to the region of 32 min on the E. coli chromosome (21). Locus marA (34 min) confers resistance to nalidixic acid, tetracycline, chloramphenicol (10, 13), and new quinolone agents (S. P. Cohen, L. M. McMurry, D. C. Hooper, J. S. Wolfson, and S. B. Levy, manuscript in preparation), as do cfxB and nfxB (21, 22). Disruption of marA by insertion of Tn5 is known to result in loss of drug resistance (14).

Transduction of marA::Tn5 into EN2226-8 (cfxB) also resulted in loss of the CfxB phenotype (21), suggesting that cfxB is an allele of marA. To assess the potential interaction of locus nfxB with cfxB or marA, strain KF131 (nfxB) was transduced with a lysate of P1 vir grown on AG1065 (marA::Tn5), with selection for kanamycin resistance. Of 94 transductants, 93 had wild-type resistances to norfloxacin, tetracycline, and chloramphenicol, suggesting that a functional marA locus is necessary for expression of nfxB. To confirm that Tn5 remained in the marA region after transduction, P1 vir was grown on a KF131 (nfxB marA::Tn5) transductant. This lysate was used to transduce strain PLK1253 (zdd-230::Tn9 zde-234::Tn10), with selection for Tn5 and scoring for Tn9 (33.3 min) and Tn10 (34.2 min). Tn5 was linked 44% (12 of 27) to Tn10 and 22% (6 of 27) to Tn9, indicating that Tn5 was located in the marA region in the KF131 transductant. This same lysate was then used to outcross the nfxB locus into strain KL16, with selection for norfloxacin resistance. Despite multiple attempts, no norfloxacin-resistant transductants of KL16 could be obtained. This finding suggested that nfxB had been altered in the KF131 (nfxB marA::Tn5) transductant, possibly by reversion to nfxB+, and that nfxB and marA::Tn5 might each be unstable in the presence of the other.

Loss of the cfxB phenotype in 96 of 97 clones of EN2226-8 (cfxB) transduced with marA::Tn5 after selection for kanamycin resistance had been considered evidence that cfxB and marA were allelic or very closely linked. The foregoing experiments called this interpretation into question, because introduction of marA::Tn5 may in some cases reverse the phenotype of genetically distant mutant loci. We therefore transduced EN2226-8 (cfxB) with a P1 vir lysate grown on PLK1253, selecting for zdd-230::Tn9 (33.3 min) or zde-234::Tn10 (34.2 min) and scoring for cfxB+. cfxB was 65% linked to Tn10 (32 of 49 Tn10 transductants were cfxB+) and 8% linked to Tn9 (4 of 49 Tn9 transductants were cfxB+), linkages similar to those reported for marA with each of these transposon insertions (14). Thus, cfxB is closely linked to marA.

Studies of dominance of cfxB. Dominance studies were performed by using strains KF700 (recA+ cfxB) and KF701 (recA cfxB). KF701 was fourfold more resistant to ciprofloxacin than KF702 (recA cfxB+) but was also more susceptible to ciprofloxacin than KF700 because of the presence of the recA mutation (Table 2). Thus, the cfxB mutation still conferred relative quinolone resistance in a recA genetic background.

The dominance of cfxB in relation to cfxB+ was determined by conjugation of F+506 (14; mat+) strains 31 to 37 min of the E. coli chromosome and thus includes the cfxB gene) from JE5550 into KF700 and into KF701 with rifampin (25 µg/ml) counterselection and selection for mannose as a sole carbon source. With the recA+ recipient, 7 of 16 transconjugants had a CfxB+ phenotype, confirming that F+506 contained the cfxB+ gene (Table 2), which had recombined with the cfxB mutant allele on the chromosome. With the recA recipient, five of five transconjugants which were presumed to be merodiploids carrying the cfxB+ allele on the F+ plasmid and the cfxB allele on the chromosome had the CfxB (drug resistance) phenotype of KF701 (Table 2). These findings indicated that cfxB is dominant over cfxB+, in contrast to previously reported quinolone resistance mutations in gyrA (16).

Effects of nfxB and cfxB on ompF expression. Both nfxB and cfxB result in decreased amounts of OmpF on polycrylamide gels of purified outer membranes (21, 22). To investigate further the effects of these mutations on ompF expression, we transduced each locus into strains MH513 and MH621, which contain chromosomal ompF-lacZ operon and protein fusions, respectively. Both nfxB and cfxB resulted in reproducible decreases (30 to 45%) in β-galactosidase activity in the operon fusion strain, but much larger decreases (20-fold) in β-galactosidase activity occurred in the protein fusion strain (Table 3). These findings, therefore, indicate that the major downregulation of ompF expression by these mutations was at the posttranscriptional level.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>MIC (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>GMS407a</td>
<td>Parent</td>
<td>0.01</td>
</tr>
<tr>
<td>KF700</td>
<td>recA+ cfxB</td>
<td>0.08</td>
</tr>
<tr>
<td>KF701</td>
<td>recA cfxB</td>
<td>0.01–0.02</td>
</tr>
<tr>
<td>KF702</td>
<td>recA cfxB+</td>
<td>0.0025</td>
</tr>
<tr>
<td>KF700-1⁴</td>
<td>F506</td>
<td>0.01</td>
</tr>
<tr>
<td>KF700-2⁵</td>
<td>F506</td>
<td>0.04–0.08</td>
</tr>
<tr>
<td>KF701-1⁶</td>
<td>F506</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* Rifampin-resistant derivative.
⁴ Transconjugants of JE5550 and KF700; KF700-1 is representative of one class (7 of 16 transconjugants) and KF700-2 is representative of a second class (9 of 16 transconjugants).
⁵ Transconjugants of JE5550 and KF701 (five of five).

[¹H]norfloxacin uptake by intact cells and spheroplasts. (i) Studies with wild-type cells. As previously reported for other E. coli strains (9), intact cells of wild-type strain KL16 in the presence of drug concentrations around the MIC rapidly accumulated norfloxacin, reaching a plateau within 5 min after addition of the drug. This plateau level of the cell-associated drug was proportional to the amount of the labeled drug added and to the concentration of cells used in the assay (data not shown). Uptake was not saturable with a 500-fold excess of unlabeled norfloxacin (Fig. 1). This finding could not be attributed to the binding of a radiolabeled species other than norfloxacin, because at least 10% of the

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total label added was bound to cells and the \(^3\text{H}\) norfloxacin preparation used was shown to be 99% radiochemically pure by thin-layer and high-performance liquid chromatography (H. H. Gadebusch, Merck Institute for Therapeutic Research, personal communication).

When more of the drug was added after attainment of the plateau, proportional additional binding was observed (Fig. 2), indicating that the plateau of drug binding was a steady state and did not result from an irreversible reaction of the cells to the presence of the drug. Norfloxacin was also rapidly eluted from the cells after 100-fold dilution of the drug (Fig. 3), indicating that binding was rapidly reversible.

Decreases in pH and increases in magnesium concentrations are known to increase the MICs of norfloxacin and other quinolones (4, 7, 40). These conditions resulted in a fourfold increase in the MIC and also produced 2- to 2.5-fold decreases in the plateau level of norfloxacin accumulation (Table 4), suggesting that the effects of these conditions on the MIC result from diminished drug accumulation.

(ii) Studies with \(nfxB\) and \(cfxB\) mutants. Norfloxacin uptake by KF131 \((nfxB)\) and EN226-8 \((cfxB)\) differed greatly from that of their parent strain, KL16. The rate of initial uptake was sixfold lower, and the plateau level of accumulation was four- to fivefold lower for both mutants than for the parent strain (Fig. 4). Uptake of norfloxacin in KF130 (22), which contains a \(gyrA\) mutation selected for norfloxacin resistance, and in a \(cfxB\) mutant, which was transduced with \(marA::\text{Tn5}\) (thereby resulting in loss of resistances conferred by \(cfxB\)), was similar to that of KL16 (data not shown).

The role of energy in the diminished drug accumulation seen in the \(nfxB\) and \(cfxB\) mutants was studied. Addition of CCCP (50 \(\mu\)M) resulted in a rapid increase in drug accumulation in the mutant strains, such that drug binding in mutant and wild-type bacteria was equivalent after CCCP treatment (Fig. 4). This finding indicated that the diminished steady-state level of norfloxacin binding in these mutants was dependent on an energy-requiring process.

The greater accumulation of norfloxacin in the wild type than in the \(nfxB\) mutant was also shown to be dependent on

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Operon fusion (MH513)</th>
<th>Protein fusion (MH621)</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SEM U of β-galactosidase activity(^a)</td>
<td>Mutant/ wild-type ratio</td>
</tr>
<tr>
<td>Wild type</td>
<td>314 ± 9.8</td>
<td>0.69</td>
</tr>
<tr>
<td>(nfxB)</td>
<td>217 ± 11</td>
<td>1,374 ± 32</td>
</tr>
<tr>
<td>Wild type</td>
<td>358 ± 39</td>
<td>0.55</td>
</tr>
<tr>
<td>(cfxB)</td>
<td>198 ± 21</td>
<td>0.60</td>
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</table>

\(^a\) The values are means of three separate experiments.

FIG. 1. Binding of \(^3\text{H}\) norfloxacin to intact cells of \(E.\ coli\) KL16 and its lack of saturability. The standard conditions were as described in Materials and Methods. The same amount of \(^3\text{H}\) norfloxacin was present in both binding assays, but the total concentrations of norfloxacin were adjusted with the unlabeled drug to 0.04 \(\mu\)g/ml (○) (standard conditions) and 200 \(\mu\)g/ml (●).

FIG. 2. Binding of additional \(^3\text{H}\) norfloxacin to intact cells after attainment of an initial steady-state level of binding. Standard conditions, as described in Materials and Methods, were used for both assays (○ and ●). Additional norfloxacin equal to that added at time zero was added at the time indicated by the arrow to one (●) of a pair of identical cultures.

FIG. 3. Reversibility of \(^3\text{H}\) norfloxacin binding to intact cells of \(E.\ coli\) KL16. Standard conditions, as described in Materials and Methods, were used. Identical binding assays (○ and ●) were sampled (40 \(\mu\)l) at the times indicated. In one of the assays (○), the entire assay mixture was diluted 100-fold into fresh medium (arrow) and additional samples (4 ml) were taken.
TABLE 4. Effects of changes in pH and magnesium concentration on the binding and antimicrobial activity of norfloxacin in E. coli KL16

<table>
<thead>
<tr>
<th>pH</th>
<th>Mg²⁺ concn (mM)</th>
<th>Mean ± SEM norfloxacin binding (% of control)</th>
<th>MIC (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>6.0</td>
<td>1.0</td>
<td>48 ± 3</td>
<td>0.32</td>
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<td>7.0</td>
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<td>100</td>
<td>0.08</td>
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<tr>
<td>7.7</td>
<td>1.0</td>
<td>98 ± 2</td>
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<td>116 ± 6</td>
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<td>7.0</td>
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<td>7.0</td>
<td>14.0</td>
<td>39 ± 11</td>
<td>0.32</td>
</tr>
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</table>

* Binding was determined at the plateau level at time = 5 min. Control, M9 broth (pH 7.0) containing 1 mM Mg²⁺.

DISCUSSION

Quinolone resistance loci nfxB and cfxB have been shown to be regulatory loci which, when mutated, cause a major decrease in the expression of ompF at a posttranscriptional level. nfxB was selected with norfloxacin (22) and maps at 19 min on the E. coli chromosome and thus is distinct from the ompF gene at 21 min. cfxB was selected with ciprofloxacin (21) and is closely linked to marA (34 min), a mutation initially selected for resistance to tetracycline or chloramphenicol (14). marA mutations selected in this manner also confer resistance to nalidixic acid (10, 13), norfloxacin, ciprofloxacin, and ofloxacin (Cohen et al., in preparation) and have decreased OmpF (10).

A functional relationship appears to exist between the nfxB and marA loci, because inactivation of marA by transposon insertion resulted in loss of the ability to recover the nfxB mutation. The details of these interactions are unknown. Transposon insertion inactivates the drug resistance of the marA mutant allele, suggesting that marA+ encodes a factor(s) which negatively regulates expression of ompF; drug resistance mutations in this locus might thus result in an increase in the expression of this factor. In support of this hypothesis, we have shown that cfxB is dominant over cfxB+. In other studies (14), however, a marA mutant selected for tetracycline resistance was only partially dominant over marA+. Whether mutation in nfxB results in an increase in a negative regulatory factor or loss of a positive factor required for expression of ompF is unknown, but a functional marA gene appears to be required for cell viability when nfxB is mutant. Quinolone resistance determined by genetically dominant mutations is potentially transferable on plasmids, but plasmid-mediated quinolone resistance of this type has not been identified.

Earlier studies (14) have also identified additional loci that interact with marA, including a gene(s) in the 16- to 31-min region of the chromosome. Whether this gene(s) is nfxB is unknown.

The major decrease in expression of ompF with ompF-lacZ fusion strains took place at the posttranscriptional level. This down regulation might occur during translation, during transport through the cell inner membrane en route to the outer membrane, or during both (41). The micF locus, linked to and expressed in parallel with the ompC gene, encodes an antisense RNA which hybridizes with portions of the ompF transcript (29). When present in multiple copies, micF decreases expression of ompF, but the role of single chromosomal copies of micF in regulating ompF expression remains uncertain (1). marA appears to diminish OmpF by increasing expression of a single chromosomal copy of micF (10). In addition, lysozymes of phage PA-2, which encodes a new outer-membrane protein (33), and strains with tolC (66 min) mutations (30) have amounts of OmpF reduced by posttranscriptional mechanisms (28, 33). nfxB and cfxB represent loci that should be added to those known to regulate ompF expression.

No changes are detected in the amounts of OmpA, OmpC, and LamB in electrophoresis of outer-membrane proteins isolated from the nfxB mutant (22), raising the possibility that the nfxB mutation limits the increase in ompC expression that occurs when ompF expression is diminished under other circumstances, such as changes in osmolality (15, 45) and pH (17).

Studies of the uptake of norfloxacin and other quinolones (5, 19) have been hampered by the necessity to use extremely high drug concentrations, which might generate artifactual effects. Conditions allowing evaluation of norfloxacin at concentrations equal to or below the MIC were used for the first time for wild-type (9) and mutant quinolone-resistant cells in the studies presented here. Under these conditions, norfloxacin rapidly bound to and eluted from

FIG. 4. Binding of [³H]norfloxacin to intact cells of E. coli KL16 (wild type) (△), KF131 (nfxB) (●), and EN226-8 (cfxB) (○) and the effect of CCCP. Standard conditions, as described in Materials and Methods, were used. CCCP (50 µM) was added at the times indicated by the arrows, and additional samples were taken.

TABLE 5. Differences in norfloxacin uptake between E. coli KL16 and KF131 (nfxB) eliminated by spheroplast production

<table>
<thead>
<tr>
<th>Condition</th>
<th>Strain</th>
<th>Mean ± SEM norfloxacin bound (ng/mg of cell protein) at plateau</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cells</td>
<td>KL16 (wild type)</td>
<td>0.77 ± 0.024</td>
</tr>
<tr>
<td></td>
<td>KF131 (nfxB)</td>
<td>0.34 ± 0.033</td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>KL16 (wild type)</td>
<td>0.38 ± 0.039</td>
</tr>
<tr>
<td></td>
<td>KF131 (nfxB)</td>
<td>0.41 ± 0.041</td>
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</table>
intact cells. The lack of saturation of uptake in intact cells was in contrast to the saturability of norfloxacin uptake by everted inner-membrane vesicles (9) and suggested that in intact cells the drug rapidly equilibrated with a cellular compartment, possibly the periplasm or outer membrane, because in the wild-type strain spheroplasts bound about half as much of the drug as intact cells did (Table 5).

If it is assumed that all of the drug bound is intracellular, then norfloxacin appears to be concentrated within intact bacterial cells. If cellular protein is about 0.55 of cellular dry weight (31) and cellular volume is 2.5 \( \mu \)g/ml of dry weight (42), the amount of cell-bound norfloxacin (0.8 ng/mg of cell protein; Table 5) would result in a cellular concentration of 0.18 \( \mu \)g/ml ((0.8 ng/mg of protein) \times 0.55 mg of protein per mg of dry weight)/(2.5 \( \mu \)g/mg of dry weight)), a level 4.5-fold greater than that present in the extracellular fluid (0.04 \( \mu \)g/ml). A similar value of cell-associated norfloxacin was obtained by using another method of cell collection, centrifugation through silicone oil (Cohen et al., in preparation).

For enoxacin binding to \textit{E. coli} (5), similar calculations are also consistent with the concentration of enoxacin within cells. The meaning of this possible concentration of quinolones within cells is uncertain, but these findings suggest that certain cellular compartments or components are capable of trapping quinolones.

Increasing concentrations of magnesium are known to cause a decrease in the antibacterial potency of quinolones (40), and it has been suggested that these agents are chelators of this and possibly other divalent cations (8, 44). Increasing concentrations of magnesium were found also to diminish substantially the steady-state level of drug binding (Table 4; 8). Whether magnesium binds to norfloxacin to generate a complex which permeates the cell less well through porin outer-membrane proteins or stabilizes the outer membrane in the presence of quinolone (8) is unknown.

Decreasing pH has effects on norfloxacin activity (40, 50) and uptake similar to those of increased magnesium concentrations. The pK values for the carboxyl group and piperazine 4'-nitrogen of norfloxacin are 6.2 to 6.4 and 8.7 to 8.9, respectively (Gadebusch, personal communication). A change in the pH of the medium from 7.0 to 6.0 thus would result in a higher fraction of the compound having a single positive charge rather than a dipolar ionic configuration. Dipolar ionic compounds are known to penetrate rapidly through porin channels, but cationic species do as well (32, 49). The decreased potency of norfloxacin may be related to this difference in charge, but the exact mechanism is unknown.

In strains KF131 (nfxB) and EN226-8 (cfxB), the rates of norfloxacin uptake were sixfold slower than that in their parent strain, KL16, a finding compatible with the decrease in the OmpF diffusion channel seen in these mutant strains. In addition, however, the steady-state levels of the drug in the mutants was fivefold lower than that in the parent strain, a decrease of a magnitude possibly sufficient to account for the four- to eightfold difference in drug resistance seen in these mutants. Decreased rates of passive drug diffusion through the outer membrane alone, however, cannot account for decreased cellular drug concentrations at steady state unless the half-time of diffusion exceeds the doubling time of the organism, a condition not seen in our experiments. The additional factor, however, may be the saturable energy-dependent drug efflux system recently discovered at the inner membrane of wild-type cells (9) or a smaller periplasmic space.

Differences in uptake between nfxB and wild-type cells were not seen in spheroplasts, indicating that an intact outer membrane is also necessary for the difference in drug binding seen in the mutant. These findings, taken together with those of Cohen et al. (9), suggest a model in which norfloxacin resistance results from an altered permeation in which the cell-associated drug is determined at least in part by the combination of diminished influx by diffusion through porin pathways in the outer membrane and a saturable drug efflux system at the inner membrane (9). Because nfxB and cfxB are regulatory mutations and produce both greater resistance and a greater reduction in drug accumulation than ompF mutant cells (9), these resistance loci may also regulate another factor in addition to ompF expression that is important for quinolone permeation. The nature of this other factor(s) is uncertain.

The presence of this efflux system predicts the possibility of another class of drug resistance mutations, such as that found with the tetracycline efflux systems, in which the drug efflux system has enhanced efficiency (13). We did not identify such a class of mutants for the quinolones. The nfxB mutant (Table 5) appears not to have alterations in its efflux system, because drug uptake by spheroplasts of KL16 versus those of KF131 (nfxB) did not differ.

Our studies demonstrate the dependence of diminished uptake in nfxB and cfxB mutant strains on proton motive force in a fashion similar to that of the quinolone efflux system most clearly demonstrated in membrane vesicles (9). Other workers (5, 8) have found little effect of energy inhibitors on the uptake of enoxacin or floxacin in intact wild-type bacteria. As shown here (Fig. 4) and by Cohen et al. (9), increases in norfloxacin uptake seen in wild-type bacteria were small but reproducible (9) and might easily be missed. In nfxB and cfxB mutants, however, the effects of energy inhibitors were clearly evident. Differences in uptake between mutant and wild-type bacteria, as well as the effects of energy inhibitors, were more readily demonstrated at 30°C than at 37°C (9; S. P. Cohen, D. C. Hooper, J. S. Wolfson, L. M. McMurry, and S. B. Levy, unpublished data).

Studies of drug uptake may be difficult to perform. Use of drug concentrations at or below the MIC for the test organism is likely to be particularly important to avoid possible artifacts with use of highly bactericidal agents such as the quinolones. The findings that two pleiotropic mutants conferring quinolone resistance and two conditions that diminish the activity of norfloxacin (low pH and increased magnesium) diminish drug uptake suggest that measurements of cell uptake as performed in this study have physiologic relevance.

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