ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1986, p. 639–644
0066-4804/86/040639-06$02.00/0
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Vol. 29, No. 4

Genetic and Biochemical Characterization of Norfloxacin Resistance in *Escherichia coli*

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Received 2 October 1985/Accepted 10 January 1986

In *Escherichia coli* the frequency of spontaneous single-step mutation to high levels of resistance to the newer 4-quinolone agent norfloxacin was confirmed to be over 300-fold lower than that to the older agent nalidixic acid. Serial passage on incremental concentrations of drug was necessary to produce mutants highly resistant to norfloxacin. Genetic analysis of one such highly resistant strain identified two mutations conferring drug resistance. One mutation, *nfxA*, mapped around 48 min on the *E. coli* genetic map and was shown to be an allele of *gyrA* by studies demonstrating an increased drug resistance of DNA gyrase reconstituted with the gyrase A subunit isolated from the mutant strain. These findings also identified the DNA gyrase A subunit as a target of norfloxacin. The second mutation, *nfxB*, mapped between 20 and 22 min and was associated with additional resistances to tetracycline, chloramphenicol, and cefoxitin and with decreases in outer membrane porin protein OmpF. The *nfxA* and *nfxB* mutations together accounted for most, but not all, of the norfloxacin resistance phenotype of this strain.

The newer 4-quinolone antimicrobial agents such as norfloxacin (NFX) are structurally related to nalidixic acid (NAL) but have substantially increased potency and are broader in spectrum. The newer agents, therefore, show promise for use in treatment of a variety of bacterial infections. A potentially serious limitation to this usefulness, however, might be the development of bacterial resistance, a problem that occurred with NAL in certain settings (28).

Resistance of bacteria to NAL and the 4-quinolones results from chromosomal mutations, but has not been found to be carried on plasmids or transposons (1). A target of NAL action is the essential bacterial enzyme DNA gyrase (9). The structure and functions of DNA gyrase have been the subject of several reviews (3, 9, 35). Mutations in the gene [*gyrA* (*nalA*)] encoding the A subunit of the enzyme DNA gyrase confer on the bacterium and the enzyme high-level resistance to NAL. In addition to two A subunits, this enzyme contains two B subunits, the products of the *gyrB* (*cou*) gene. Purified DNA gyrase has a variety of activities in vitro that are inhibited by NAL, including the introduction of negative superhelical twists into duplex circular DNA and the reversible interlocking of DNA circles like links in a chain. In the growing bacterium DNA gyrase is required for DNA replication, transcription of certain operons, DNA repair, and other processes, all of which are antagonized by NAL. The level of superhelical twisting of intracellular DNA is controlled at least in part by the activities of DNA gyrase and topoisomerase I, the product of the *topA* gene. Other genes in which mutations may cause altered levels of resistance to NAL include *nalB* (12), *nalC* (*gyrB*) (37), *nalD* (31, 37), *icd* (18), *cya* (18), and *crp* (17).

Less is known about the mechanisms of resistance of bacteria to NFX and other newer 4-quinolones. The frequency with which bacteria spontaneously become resistant to high concentrations of NFX is substantially lower than the frequency of spontaneous resistance to NAL with selection in vitro for single-step mutations (36). Serial passage of bacteria on incremental concentrations of NFX does, however, result in highly drug-resistant strains (33) that, by the nature of selection, likely contain multiple mutations. Although *gyrA* mutations in *Escherichia coli* selected for resistance to NAL also confer resistance to NFX (2), there has been no genetic characterization of mutants selected directly for resistance to NFX. We present here data on NFX resistance selected by passage on increasing NFX concentrations and a genetic and biochemical characterization of a highly drug-resistant strain. Mutations in the *gyrA* gene and a gene affecting OmpF outer membrane porin protein were identified and together account for most, but not all, of the drug resistance phenotype.

MATERIALS AND METHODS

Chemicals. NAL was obtained from Calbiochem-Behring, La Jolla, Calif. NFX and cefoxitin (CFX) were obtained from Merck & Co., Inc., Rahway, N.J. Tetracycline hydrochloride (TC) was obtained from Lederle Laboratories, Pearl River, N.Y., as a powder containing 2.5 g of ascorbic acid per g of TC. Chloramphenicol (CM), phosphonomycin, α-glycerol phosphate, dithiothreitol, tRNA, bovine serum albumin, spermidine hydrochloride, ATP, and agarose type II were purchased from Sigma Chemical Co., St. Louis, Mo. NAL (10 mg/ml) was dissolved in 0.1 N NaOH, and NFX (1 mg/ml) was dissolved in 0.02 N NaOH.

Bacterial strains, bacteriophages, and colicins. The bacterial strains and bacteriophages used and their sources are given in Table 1. Colicin A (6) was a gift from J. Foulds.

Selection of NFX-resistant mutants. *E. coli* KL16 was grown at 37°C on a series of Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) plates containing twofold increments in NFX concentration from 0.016 to 16 μg/ml as described previously (33). In sequence, growth from each plate was spread on a plate containing the next higher drug concentration, using a sterile cotton-tipped applicator. Two clones growing at each drug concentration were purified on a plate containing the same drug concentration and were later assessed for susceptibility to NFX, TC, CM, and CFX.

For determination of the frequency of resistance to NFX

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TABLE 1. Bacterial strains and bacteriophages

Strain or phage	Genotype or characteristic	Source (reference)
E. coli K-12 strain		
KL16	Hfr *thi-1 relA1 spoT1* λ⁻	B. Bachmann
KF111	KL16 NFX^r (*nfxA nfxB ?nfxC*)	By serial selection
KF131	KL16 *nfxB*	By transduction from KF111
KF130	KL16 *nfxA*	By transduction from KF111
C600	F⁻ *thi-1 thr-1 leuB6 lacY1 tonA21 supE44*	B. Bachmann
KF611	C600 *glpT13*	By transduction from ECL6, phosphonomycin selection
χ697	F⁻ λ⁻ *leuB6 purE42 trpE38 his-208 argG77 ilvA681 met-160 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 azi-6 rpsL104 tonA23 tsx-67 supE44*	B. Bachmann
JC10241	Hfr λ⁻ *thr-300 relA1 ilv-318 spoT1 thi-1 rpsE2300 srl-300*:Tn*10*	B. Bachmann
JF568	*aroA357 ilv-277 metB65 his-53 purE41 cyc-1 xyl-14 lac629 rpsL97 tsx63 proC24*	J. Foulds (7)
JF703	JF568 *ompF254 aroA*⁺	J. Foulds (7)
JF733	JF568 *ompA252*	J. Foulds
CS109	W1485F⁻	C. Schnaitman (30)
CS1253	CS109 Δ*ompC178 zei-298*::Tn*10*	C. Schnaitman (30)
KL164	KL16 *thyA24 deoB13 nalB14*	B. Bachmann
RW1229	F⁻ *pro his aroA galK*	R. Weisberg
KL185	F⁻ *pyrD34 trp-45 his-68 thi-1 galK35 malA1* (λ^r) *xyl-7 mtl-2 rpsL118*	B. Bachmann
N99	*galK2*	M. Gellert
MK47	*recA* Δ(*gal att* λ *bio*) (pMK47)	M. Gellert (23)
ECL6	*glpT13 phoA8 relA1 tonA22* T2^r *pit-10 spoT1 fadL701*	E. C. C. Lin (13)
Bacteriophage		
P1*vir*	For transduction	E. Brinkman
K20	Specific for OmpF	C. Schnaitman (30)
TuIb	Specific for OmpC	J. Foulds (7)
SS-4	Specific for OmpC	C. Schnaitman (30)
K-3	Specific for OmpA	J. Foulds

or NAL, strain KL16 was grown overnight in Mueller-Hinton broth at 37°C. The overnight culture was then plated directly on Mueller-Hinton agar containing NAL (20, 40, and 100 μg/ml) or was concentrated 25-fold by centrifugation and resuspension in saline and then plated on Mueller-Hinton agar containing NFX (0.4, 1.0, 4.0, and 10.0 μg/ml). Dilutions of this inoculum were plated on drug-free Mueller-Hinton agar to determine the inoculum size. The frequency of mutation to resistance was determined as the number of colonies appearing on the drug-containing plates divided by the inoculum applied to those plates.

Drug, colicin, and bacteriophage susceptibility tests. Susceptibilities to NFX, NAL, CM, TC, and CFX were determined by agar dilution on either Mueller-Hinton or Luria agar (22) plates at 37°C, using a Steers replicator to transfer cells from wells containing single colonies suspended in 0.4 ml of saline. These values were reproducible within one dilution and gave values similar to those when defined inocula of 2×10^5 to 2×10^6 CFU per spot were used. For bacteriophage and colicin susceptibility tests, bacteria were grown overnight in Mueller-Hinton broth, diluted in broth, spread on Mueller-Hinton agar, and allowed to grow for 1 h at 37°C. Dilutions of bacteriophages (1:10 in Luria broth), undiluted colicin A, or broth alone were then spotted (5 μl) on the plates, and inhibition of growth was assessed after incubation for 18 to 24 h at 37°C.

Genetic mapping. Hfr interrupted matings were performed as previously described (12), using streptomycin counterselection and selection for loss of histidine auxotrophy or for resistance to NAL or NFX. NAL and NFX resistance selections were performed by addition of a drug-containing second top agar (5 ml) after 2 h of incubation at 37°C, a method similar to that previously reported for selection of *gyrA* recombinants with NAL (12). Transduction using P1 *vir* was as described before (5) except that direct selection for resistance to NFX also required a 2-h delay before the addition of the drug-containing top agar. Gradient-of-transfer experiments followed the procedure of Foulds and Barrett (6).

Enzyme purification and assay. The DNA gyrase B subunit was purified by DEAE-Sepharose and hydroxylapatite chromatography from *E. coli* MK47 (courtesy of M. Gellert) containing a cloned *gyrB* gene (23). Gyrase A subunits from strains KL16 and KF130 were purified by polymin P (BDH Chemicals Ltd., Poole, England) and ammonium sulfate precipitations and by DEAE-Sepharose chromatography as described previously (14). For these experiments 1 U of DNA gyrase A subunit activity was defined as the smallest amount of protein which in the presence of excess gyrase B protein fully supercoiled 0.3 μg of relaxed pBR322 DNA in 60 min at 25°C. The concentration of NFX which when added to assays containing 1 U of enzyme reduced the intensity of the most fully supercoiled band by one-half was estimated as the 50% inhibitory concentration from the visual inspection of photographs of UV-transilluminated agarose gels stained with ethidium bromide (14).

Polyacrylamide gel electrophoresis of outer membrane proteins. Outer membrane protein fractions were extracted by the method of Matsuyama et al. (21). Discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed by the method of Lugtenberg et al. (19) and the method of Lundrigan and Earhart (20). The gels were fixed, stained, and destained by the method of Fairbanks et al. (4).

RESULTS

Selection of NFX resistance in vitro. Plating of *E. coli* KL16 on agar containing 0.4, 1, 4, or 10 μg of NFX per ml resulted

in detectable resistant clones only at 0.4 μg/ml (fivefold above the MIC) (6×10^{-11} per CFU plated). At the higher drug concentrations resistance frequencies were $<3 \times 10^{-11}$ per CFU plated. Similar findings were obtained when strain N99 was used. In contrast, KL16 clones resistant to 40 and 100 μg of NAL per ml (10- and 40-fold above the MIC) were obtained at frequencies of 1.1×10^{-8} to 2.0×10^{-8} per CFU plated. Serial passage of KL16 on agar containing increasing concentrations of NFX resulted in clones with progressive increases in drug resistance, reaching NFX MICs of ≥20 μg/ml. In addition, for some clones increases in resistance to TC and CM were seen. Some of the strains with the highest MICs grew poorly. We chose for further characterization one strain (KF111) purified from cells growing at 8 μg of NFX per ml (passage 9) which had multiple resistances and which grew well (Table 2). Resistance to NFX in KF111 was stably maintained during growth in drug-free broth for 90 generations.

Mapping of NFX resistance by interrupted mating. When KF111 (Hfr) was mated with χ697 (F^-) (MICs of 0.04 μg of NFX, 2 to 4 μg of TC, and 4 μg of CM per ml), NFX-resistant (selected with top agar containing 6.25 μg of NFX) and NAL-resistant (selected with top agar containing 500 μg of NAL) recombinants appeared concurrently and just prior to the appearance of His^+ recombinants (Fig. 1). Selection with a higher concentration of NFX (top agar containing 50 μg of NFX) resulted in the appearance of a lesser number of late NFX-resistant recombinants. Each class of NFX-resistant recombinants was further studied for drug susceptibilities. The MIC of NFX of the purified early recombinants (n = 13) selected with NFX was increased 32-fold (to 1.28 μg/ml) over that of χ697 without a change in MIC of TC or CM. In contrast, the purified late recombinants (n = 23) had two- to fourfold additional increases in the MIC of NFX (2.56 to 5.12 μg/ml) and, like the donor strain KF111, had increases in the MICs of TC (8 μg/ml) and CM (8 to 16 μg/ml). A point of gene entry just prior to *his* (about 44 min) suggested that the early recombinants resulted from transfer of a single gene, here designated *nfxA*, which was likely an allele of *gyrA* (48 min) conferring NFX and NAL resistance. The late recombinants were thought to result from transfer of *nfxA* and an additional gene(s), designated *nfxB*, responsible for an additional increment in NFX resistance and for resistances to TC and CM.

Mapping of NFX resistance by transduction. NFX resistance was transduced from KF111 into KL16 by using direct selection for drug resistance. Two classes of transductants were identified. One class (n = 9) had MICs of 0.64 to 1.28 μg of NFX and >128 μg of NAL per ml and no increase in resistance to TC or CM (strain KF130 is a member of this class); the second class (n = 3) had MICs of 0.32 μg of NFX and 16 μg of NAL per ml and increased resistances to TC, CM, and CFX (strain KF131 is a member of this class) (Table 2). From KF130 *nfxA* it was possible to cotransduce NFX resistance with *glpT* (49 min). Two of four $glpT^+$ transductants of KF611 (*glpT*) had increases in NFX MIC from 0.04 to 0.64 μg/ml, indicating that *nfxA* was closely linked to *glpT*, which is closely linked to *gyrA* (14). NFX resistance from KF111 was also cotransduced with *glpT*. Attempts to cotransduce NFX resistance from KF111 with genes closely linked to *gyrB* (selecting for $dnaA^+$), *nalB* (selecting for srl^+), and *topA* (selecting for $trpE^+$) were unsuccessful. In addition, we were unable to detect increments in resistance to NFX when P1 *vir* grown on KF111 was used to transduce KF130 *nfxA gyrB*(Cou^r) *dnaA*(Ts) selecting for temperature resistance. Temperature-resistant clones which had lost coumermycin resistance, indicating transduction of *gyrB* and *dnaA* genes from KF111, had no detectable change in the MIC of NFX relative to that of KF130 *nfxA*.

NFX resistance of DNA gyrase A subunit of KF130 (*nfxA*). In KF130 *nfxA* is closely linked to *glpT* and therefore also closely linked to *gyrA*. To prove that the *gyrA* gene product of KF130 had altered susceptibility to NFX, we partially purified the DNA gyrase A subunits from KF130 and its wild-type parent KL16. In the presence of an excess of wild-type DNA gyrase B subunit, the concentration of NFX

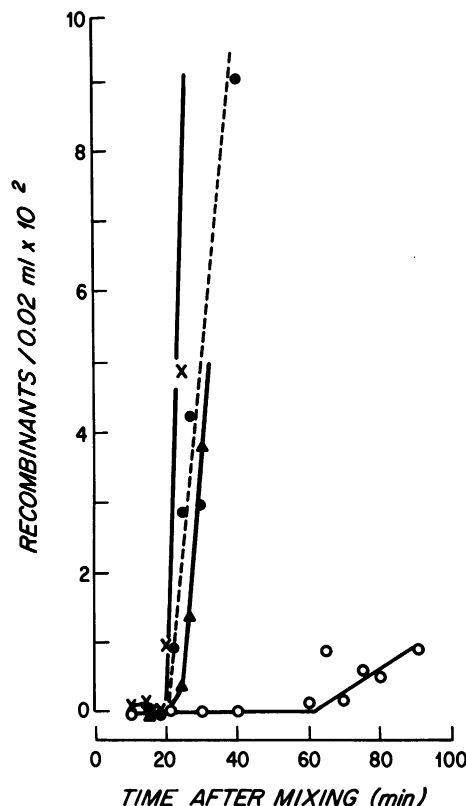

FIG. 1. Interrupted mating between KF111 (Hfr) and χ697 (F^-). Streptomycin (100 μg/ml) was used for counterselection. Selection included top agar overlays (5 ml) containing 100 μg of NAL per ml (×), 1.25 μg of NFX per ml (●), or 10 μg of NFX per ml (○) or omission of histidine (▲) from supplemented minimal medium.

TABLE 2. Antimicrobial agent susceptibilities of KL16, KF111, KF130, KF131, and selected other strains

Strain	Relevant genotype	MIC (μg/ml)				
		NFX	NAL	TC	CM	CFX
KL16	Wild type	0.08	4	4	8	4
KF111	*nfxA nfxB ?nfxC*	10–20	>128	16	>32	>32
KF130	*nfxA*	0.64	>128	4	4	8
KF131	*nxfB*	0.32	16	16	32	>32
JF568	Wild type	0.08	8–16	≤2	4	4
JF703	*ompF252*	0.16	8	4	8	16
JF733	*ompA*	≤0.04	4	≤2	≤2	≤2
CS109	Wild type	0.08	4	<2	4–8	4
CS1253	Δ*ompC*::Tn*10*	0.08	2–4	>16	4	≤2

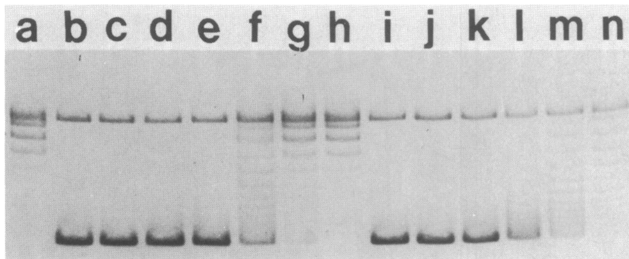

FIG. 2. Inhibition of DNA gyrase supercoiling activity by NFX. Reaction mixtures all contained 0.3 μg of pBR322 DNA. The mixture in lane a contained no enzyme. Those mixtures containing DNA gyrase (lanes b to n) had 2 to 4 U of the gyrase B subunit and 1 U of gyrase A subunit partially purified from NT525 *gyrA*$^+$ (lane b), KL16 (lanes c to h), or KF130 (lanes i to n). NFX concentrations present in the reaction mixtures were 0 (lanes b, c, i), 0.05 (lane d), 0.15 (lane e), 0.5 (lane f), 1.5 (lanes g, j), 5.0 (lanes h, k), 15 (lane 1), 25 (lane m), or 50 (lane n) μg/ml.

producing half inhibition of 1 U of DNA gyrase supercoiling activity was 30-fold higher with the gyrase A subunit from KF130 than with the gyrase A subunit from KL16 (Fig. 2). Similarly, the 50% inhibitory concentration for NAL, using enzyme reconstituted with the A subunit from KF130 (500 to 1,000 μg/ml), was substantially higher than that reconstituted with the A subunit from KL16 (25 μg/ml). These findings proved that *nfxA* is an allele of *gyrA* which confers resistance to NFX and NAL.

Mapping of *nfxB*. Genetic mapping of *nfxB* was more difficult than genetic mapping of *nfxA*. Because of the multiple drug resistances of *nfxB* strains, *nfxB* was thought likely to be a mutation affecting drug permeation. Attempts, however, to cotransduce NFX resistance from KF131 (*nfxB*) or KF111 (*nfxA nfxB*) directly to strains containing genes closely linked to *ompF* (selecting for *aroA*$^+$ or *pyrD*$^+$), *ompR* (selecting for *aroB*$^+$), or *nalB* (selecting for *srl*$^+$) were unsuccessful. It was, however, possible to cotransduce *nfxB*$^+$ and *aroA*$^+$. *nfxB* was transduced from KF131 into KL185 *pyrD* and RW1229 *aroA* by NFX selection. Transductants also exhibited increments in resistance to TC and CM concurrently. These strains (KL185 *nfxB pyrD* and RW1229 *nfxB aroA*) were used as recipients for transduction with Pl *vir* grown on wild-type KL16. Four of 20 *aroA*$^+$ transductants of RW1229 *nfxB* had decreases in resistances to NFX, TC, and CM to the level of RW1229. In contrast, none of 30 *pyrD*$^+$ transductants of KL185 *nfxB* had detectable changes in drug resistances. These findings locate *nfxB* to the region around *aroA* at 20 to 21 min on the *E. coli* genetic map. Gradient-of-transfer experiments (Fig. 3) also located *nfxB* in the region of 20 min. These findings placed *nfxB* in the region near *ompF* (21 min).

***nfxB* alteration of outer membrane porin OmpF expression.** *nfxB* conferred additional resistances to TC, CM, and CFX and mapped near *ompF*, which encodes an outer membrane porin protein, mutations in which confer similar antimicrobial resistances (10). We therefore assessed the expression of OmpF protein by specific bacteriophage and colicin susceptibility and by electrophoresis of outer membrane proteins. The parent strain KL16 was susceptible to OmpF-, OmpA-, and OmpC-specific bacteriophages and to colicin A. KF111 and KF131 (*nfxB*) were, however, resistant to bacteriophage K20 and to colicin A (both OmpF specific), indicating a specific decrease in functional OmpF protein (Table 3). With two separate buffer systems, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins from KL16, KF111, KF130, and KF131 also demonstrated a specific decrease in OmpF in KF111 and KF131. Representative results in one such buffer system (20) are shown in Fig. 4. Of additional interest is the finding that the decrease of OmpF appeared greater in KF111 than in KF131, suggesting that other mutations in KF111 may interact with the *nfxB* locus in altering *ompF* expression. That alterations of OmpF or its expression can affect susceptibility to NFX is further supported by the findings that the independently selected mutation *ompF254* in strain JF703 conferred a twofold increment in resistance to NFX over its parent strain JF568 (Table 2).

Inability of *nfxA* and *nfxB* to account for all of the resistance to NFX in KF111. To determine whether *nfxA* and *nfxB* mutations were additive in conferring phenotypic resistance to NFX, a *glpT* derivative of KF131 was constructed by transduction from ECL6 (*glpT13*) selecting for resistance to phosphonomycin. That the transductants were *glpT* was confirmed by the loss of the ability to grow on α-glycerol phosphate as a sole carbon source. *nfxA* was transduced from KF130 or KF111 into KF131 (*nfxB glpT*) by selection for *glpT*$^+$ (growth on α-glycerol phosphate). Of 32 *glpT*$^+$ transductants, 21 had an increase in NFX MIC to 2.56 μg/ml, a concentration four- to eightfold below the MIC for KF111. This finding indicated that *nfxA* and *nfxB* together account for most, but not all, of the resistance to NFX present in KF111.

DISCUSSION

Mutations in *gyrA* selected for resistance to NAL have been shown to confer resistance to NFX (2). Our studies

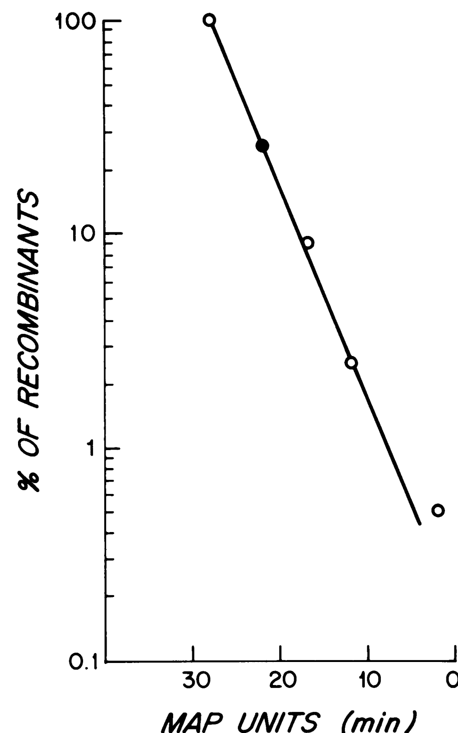

FIG. 3. Gradient of transfer mapping of *nfxB*. KF131 was mated with χ697. Trp$^+$ Str^r recombinants (n = 198; *trpE* = 28 min) were selected and scored for Gal$^+$ (*galK* = 17 min), Pur$^+$ (*purE* = 12 min), Leu$^+$ (*leuB* = 2 min) (○), and resistances to NFX, TC, and CM (●). The drug resistances did not segregate.

TABLE 3. Alterations of *nfxB*-containing and other strains in susceptibility to OmpF-specific bacteriophage and colicin

Strain	Relevant genotype	Bacteriophage[a]			Colicin A (OmpF specific)[a]
		K20 (OmpF specific)	SS-4/Tulb (OmpC specific)	K3 (OmpA specific)	
KL16	Wild type	S	ND/S	S	S
KF130	*nfxA*	S	ND/S	S	ND
KF131	*nfxB*	R	ND/S	S	R
KF111	*nfxA nfxB*	R	S/ND	S	R
JF568	Wild type	S	S/ND	ND	S
JF703	*ompF*	R	S/ND	ND	R
CS109	Wild type	S	S/ND	ND	S
CS1253	Δ*ompC*	S	R/ND	ND	S

[a] Spot tests on Mueller-Hinton agar. S, Susceptible; R, resistant; ND, not done.

represent the first proof that strains directly selected for resistance to NFX are *gyrA* mutants that produce a DNA gyrase A subunit with increased resistance to NFX, indicating that the *nfxA* mutation of KF111 resides within the *gyrA* structural gene, rather than in a nearby genetic sequence controlling its expression. These studies also prove that the A subunit of DNA gyrase is a target of NFX in *E. coli*.

Mutations in several additional genetic loci known to confer low levels of resistance to NAL in *E. coli* (12, 18, 37) do not include loci affecting the porin outer membrane proteins. Recently, in other bacterial species resistance to 4-quinolone agents such as NFX has been associated with increased resistance to nonquinolone antimicrobial agents (27, 29, 34) and changes in outer membrane protein profiles (11, 29, 34). Our experiments demonstrate for the first time that changes in the expression of a known outer membrane porin protein, OmpF, are associated with an increase in resistance to NFX, TC, CM, and CFX. *ompF* mutations are known to confer resistances to TC and CM (reviewed in reference 10). Interestingly, our *nfxB* mutation also confers low levels of resistance to NAL. These findings suggest that one route of NFX access to its target DNA gyrase is via the OmpF porin channel. The small molecular size of NFX and its existence as a zwitterion at neutral pH are compatible with this possibility (24).

Our findings do not exclude the possibility that the change in OmpF expression is secondary to changes in other outer membrane components (e.g., lipopolysaccharides) which are themselves responsible for altered drug permeation. The striking selectivity of the effect of *nfxB* on OmpF relative to OmpC or OmpA, however, makes this possibility seem less likely.

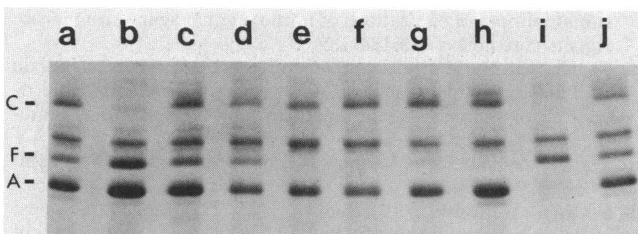

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins of KL16, KF111, KF130, KF131, and control strains by the method of Lundrigan and Earhart (20). The bacteria were grown at 37°C in Mueller-Hinton broth (lanes a to d, f to j) or Luria broth (lane e). The outer membrane proteins were isolated as described in Materials and Methods. Lanes contained 5 to 10 μg of protein for KL16, KF111, KF130, and KF131 and 5 to 20 μg of protein for the control strains. Strains included CS109 (lane a), CS1253 Δ*ompC* (lane b), KF130 *nfxA* (lane c), KL16 (lane d), KF111 *nfxA nfxB* (lanes e, f), KF131 *nfxB* (lane g), JF703 *ompF* (lane h), JF733 *ompA* (lane i), and JF568 (lane j). Band designations are as follows: C = OmpC; F = OmpF; A = OmpA.

The exact nature of our *nfxB* mutation(s) is uncertain. *nfxB* confers alterations in expression of functional OmpF at the cell surface, but we have been unable to contransduce *nfxB* with *pyrD*, a locus which cotransduces 50% with *ompF* (5; K. S. Souza and D. C. Hooper unpublished observations). *nfxB*, like *ompF*, is, however, cotransducible with *aroA* and also maps around 20 to 22 min by gradient-of-transfer experiments. Other known loci in this region which might affect NFX, TC, CM, and CFX resistances include *himD* (*hip*), a locus affecting integration of bacteriophage (15) and interacting with certain *gyrB* alleles (8); *cmlA*, a locus conferring resistance to CM (26); and *livR*, a locus affecting transport of amino acids (25) and possibly drugs. If *nfxB* is an allele of one of these genes, then it must also affect OmpF expression; alternatively, KF131 must contain an additional mutation affecting OmpF. It is also possible that *nfxB* is a previously unidentified locus affecting *ompF* expression. Further genetic studies are in progress.

Single-step spontaneous mutations to drug resistance were found to occur at substantially reduced frequencies (over 300-fold) with NFX selection relative to NAL selection at comparable levels above the MIC. The reason that single-step mutations to resistance to NFX occur less frequently than mutations to resistance to NAL when selected at comparable levels above the MIC is unknown. Perhaps the number of nonlethal *gyrA* mutations effectively decreasing drug binding to its target are many fewer with NFX than with NAL. Further studies are warranted.

Because there remained an additional four- to eightfold increment in NFX resistance in KF111 in comparison to KL16 genetically reconstructed to contain both *nfxA* and *nfxB*, an additional mutation(s) affecting susceptibility to NFX likely exists. Certain NAL resistance mutations mapping in *gyrB* have been shown to produce increased susceptibility to 4-quinolones, such as NFX, which contain 7-piperazine substituents (31, 37). In KF111 we were unable to detect an additional mutation in *gyrB* altering NFX susceptibility, despite transfer of *gyrB* and *nfxA* from KF111 into the same bacterial strain to allow a possible allele-specific interaction of the two gene products. We have, however, isolated single-step mutants of KF130 (*nfxA*) conferring high-level resistance to NFX, but which lack additional resistances to TC or CM. Analysis of these other mutations will perhaps provide additional insight into the mechanisms of NFX resistance and action.

ACKNOWLEDGMENTS

We thank M. Gellert, J. Foulds, C. Schnaitman, B. Bachmann, E. C. C. Lin, and R. Weisberg for providing bacterial strains, J. Foulds and C. Schnaitman for providing bacteriophages, and M. Gellert and J. Foulds for critical reading of the manuscript. Emma Teneriello provided excellent technical assistance, and Laura Surman helped in preparation of the manuscript.

This work was supported in part by a grant from the Merck Institute for Therapeutic Research.

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