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Endogenous Active Efflux of Norfloxacin in Susceptible *Escherichia coli*

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***Escherichia coli* was shown to have an energy-dependent reduced uptake of the fluoroquinolone antimicrobial agent norfloxacin. Studies of everted inner membrane vesicles suggested that this reduced accumulation involved a carrier-mediated norfloxacin active efflux generated by proton motive force with an apparent K_m of 0.2 mM and a V_{max} of 3 nmol min^{-1} mg of $protein^{-1}$. Other hydrophilic, but not hydrophobic, quinolones competed with norfloxacin for transport. Porin (OmpF)-deficient *E. coli* cells were twofold less susceptible to norfloxacin and showed twice as much energy-dependent reduction in drug uptake. However, active efflux assayed in everted vesicles from the OmpF strain was unchanged compared with that in the parental strain. These findings suggest that in the OmpF mutant decreased outer membrane permeability, combined with active efflux across the inner membrane, in some manner results in decreased steady-state uptake of norfloxacin and lowered drug susceptibility.**

The fluoroquinolones are newly introduced broad-spectrum synthetic antimicrobial agents. They have greater activity against gram-positive and gram-negative bacteria than do the older quinolone analogs nalidixic acid and oxolinic acid (11, 28). Resistance to fluoroquinolones has been infrequent and limited to chromosomal mutations primarily affecting DNA gyrase and outer membrane permeability (28), with no reports of plasmid-mediated or transferable resistance. In *Escherichia coli*, the fluoroquinolones are thought to cross the outer membrane primarily through the OmpF porin (9), and indeed, fluoroquinolone-resistant mutants with a reduction or loss of OmpF have been isolated (1, 10, 12).

In studying fluoroquinolone transport, we unexpectedly observed an endogenous active efflux for norfloxacin in plasmid-free susceptible *E. coli*. This is the second endogenous efflux for an antimicrobial agent seen in susceptible bacterial cells, the first being one described for the tetracyclines (18). In this study, we characterized norfloxacin efflux in wild-type strains and in an OmpF-deficient mutant. Our results suggest that the active efflux acts in concert with decreases in outer membrane permeability and could become a factor allowing for the emergence of strains resistant to the fluoroquinolones.

MATERIALS AND METHODS

Bacterial strains. Plasmid-free *E. coli* K-12 strain AG100 (7); its *ompF* derivative LM218, constructed by P1 transduction (20) of *ompF*::Tn*5* from strain MH450 (8); and *E. coli* ML strain ML308-225 (27) were used in this study.

Antimicrobial susceptibility. MICs of norfloxacin (Merck & Co., Inc., Rahway, N.J.) were determined by agar dilution (26) on Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) at 30°C with inocula of 2×10^5 to 2×10^6 CFU per spot. Inhibition of growth was assessed after 48 h of incubation.

Uptake of [^{3}H]norfloxacin by whole cells and spheroplasts. Cells were grown at 30°C in M9 medium (20) supplemented with 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.2% glucose, and 0.001% B_1 (required by AG100) to an A_{530} of 0.75, washed in growth medium, and resuspended in medium to an A_{530} of 60. Spheroplasts were prepared from strain ML308-225 by using the lysozyme-EDTA method of Kaback (13). Norfloxacin uptake was assayed at 30°C upon addition at time zero of [^{3}H]norfloxacin (74 mCi/mg; uniformly labeled in the piperazine ring; generously provided by Merck & Co.) to a final concentration of 0.125 μM, adjusted to a specific activity of 3.6 mCi/mg by addition of unlabeled norfloxacin (stock solution; 1.0 mg/ml in 0.02 N NaOH). When used, 2,4-dinitrophenol (DNP) was added to a final concentration of 2 mM. Samples of 0.04 ml each were vacuum filtered through glass fiber GF/C filters (Whatman Inc., Clifton, N.J.) and washed three times with 2-ml volumes of assay medium. Filters were air dried, and the radioactivity was assayed by liquid scintillation counting in Betafluor (National Diagnostics, Somerville, N.J.) at an efficiency of approximately 20%. Nonspecific binding of [^{3}H]norfloxacin to the filters was subtracted. Protein concentration was estimated by the method of Lowry et al. (15) by using bovine serum albumin as a standard. For norfloxacin, 1 μM = 0.319 μg/ml.

Preparation of everted membrane vesicles. Cells were grown in 750 ml of medium A (4) containing 0.4% Casamino Acids, 0.2% glucose, and 0.001% B_1 with shaking at 30°C and harvested at an A_{530} of 0.9. Cells were washed once with 100 mM potassium phosphate–10 mM sodium EDTA, pH 6.6, at 4°C and resuspended in 20 ml of the same buffer. Everted membrane vesicles were prepared with a French pressure cell at 5,000 lb/in^2 as previously described (19). Final vesicle pellets were gently suspended in 0.5 ml of 50 mM potassium phosphate, pH 6.6, and stored at −70°C.

Uptake of [^{3}H]norfloxacin by everted membrane vesicles. Accumulation of [^{3}H]norfloxacin in everted membrane vesicles was assayed at 30°C in vesicle assay buffer (50 mM potassium phosphate, pH 7.5) with a vesicle protein concentration of 0.5 mg/ml. Unless otherwise noted, norfloxacin was at 2 μM, 2.3 mCi/mg. Lithium lactate was added at 20

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mM. Samples of 0.05 ml each were diluted into 10 ml of 0.1 M lithium chloride–0.1 M potassium phosphate wash buffer, pH 7.5, vacuum filtered through a GN-6 Metricel (mixed esters of cellulose) 0.45-μm-pore-size membrane (Gelman Sciences, Inc., Ann Arbor, Mich.), washed with 4 ml of the same buffer, and dried. Radioactivity was measured as described for whole cells.

For kinetic studies, vesicles were energized with lactate for 5 min before [^{3}H]norfloxacin addition. [^{3}H]norfloxacin was premixed with appropriate amounts of unlabeled norfloxacin and added to a final concentration of 1.54 μCi/ml at time zero. Samples (0.05 ml each) were taken at 1 min after addition of norfloxacin. Uptake was linear during this 1 min (data not shown). The amount of norfloxacin retained after subsequent deenergization of vesicles with 0.1 mM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was subtracted from total accumulation to give the energy-dependent component.

For competition studies, unlabeled norfloxacin, ciprofloxacin (Miles Pharmaceuticals, West Haven, Conn.), ofloxacin (Ortho Pharmaceuticals, Raritan, N.J.), and enoxacin (Warner-Lambert, Ann Arbor, Mich.) were prepared at concentrations of 1 mg/ml in 0.02 N NaOH. Nalidixic acid and oxolinic acid were dissolved at 10 mg/ml in 0.1 N NaOH. Different amounts of unlabeled drugs were diluted in water and combined with the [^{3}H]norfloxacin before addition to vesicle assay buffer-containing vesicles. Lactate was used in the assay, which was as described in the preceding paragraph. To determine whether these compounds interfered with the ability of the vesicles to generate a proton gradient, we examined the energy-dependent quenching of acridine orange (22). Vesicles (60 μg of protein per ml) were energized with 20 mM lithium lactate or 5 mM NADH in 50 mM potassium phosphate buffer, pH 7.5, containing 2 μM acridine orange. The highest drug concentration used in the competition assays was tested for its effect on fluorescence quenching by using an LS-5 fluorescence spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) with an excitation and emission pair of 490 and 530 nm.

RESULTS

Effect of energy inhibition on uptake of norfloxacin in *E. coli* cells and spheroplasts. [^{3}H]norfloxacin accumulation was assayed in whole-cell preparations of plasmid-free *E. coli* K-12 strain AG100 and its *ompF* derivative LM218. The insertion mutation indeed caused loss of outer membrane porin OmpF as judged by sodium dodecyl sulfate-polyacrylamide-urea gel electrophoresis (16) (data not shown); such a loss has been associated with reduced susceptibility to norfloxacin and reduced uptake (9, 10, 12). The MIC of norfloxacin for strain AG100 was 0.25 μM, whereas that for LM218 was 0.50 μM. In both strains, norfloxacin accumulated rapidly and reached equilibrium within 5 min (Fig. 1). Strain LM218, the OmpF-deficient mutant, accumulated about 50% less norfloxacin than did its OmpF$^+$ parent. When DNP, a protonophore that destroys proton motive force (23), was then added to the labeled cultures, norfloxacin accumulation increased rapidly in both strains to reach higher plateau levels of almost equal amounts (Fig. 1). This increase was more dramatic for strain LM218, since its initial accumulation of norfloxacin was much lower than that in the isogenic strain AG100. CCCP had an effect similar to that of DNP (data not shown). AG100 and LM218 incubated with DNP before norfloxacin addition gave similar high levels of accumulation (data not shown). These findings together

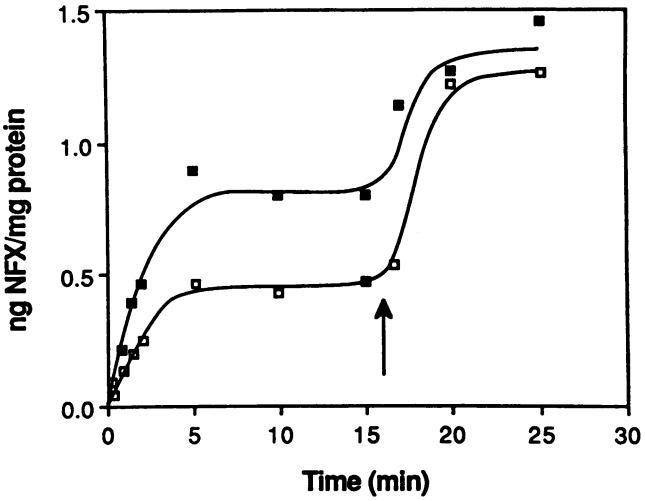

FIG. 1. Accumulation of norfloxacin (NFX) by whole cells. [^{3}H]norfloxacin uptake by strains AG100 (■) and LM218 (□) was assayed at 30°C after addition of [^{3}H]norfloxacin at time zero. DNP was added to a final concentration of 2 mM at the time indicated by the arrow.

indicated that *E. coli* cells used energy to reduce norfloxacin accumulation and that the reduction was enhanced by an *ompF* mutation.

To identify the membrane location of the energy-dependent reduction in norfloxacin accumulation, we used *E. coli* ML308-225 (27), in which the outer membrane is easily removed to form spheroplasts (13). Whole cells of strain ML308-225 showed norfloxacin accumulation similar to that of strain AG100 (data not shown). In the energized state, lysozyme-EDTA-derived spheroplasts (13), like cells, accumulated approximately half as much norfloxacin as they did when deenergized with the protonophore CCCP (0.6 versus 1.0 ng/mg of protein). Therefore, the outer membrane was probably not important for the energy-dependent reduction in accumulation of norfloxacin.

Energy-dependent uptake of norfloxacin in everted membrane vesicles. To determine whether an active efflux system existed in the inner membrane and to determine whether such an efflux was augmented in the *ompF* strain, we prepared everted membrane vesicles from cultures of strains AG100, LM218, and ML308-225. In such vesicles, cellular active efflux energized by proton motive force is measured as active uptake. In the absence of an exogenous energy source, norfloxacin accumulated in the vesicles to approximately 3 ng/mg of vesicle protein (Fig. 2). This value corresponded to an equilibration with the external norfloxacin concentration, assuming an internal vesicle volume of 8.4 μg/mg of vesicle protein (19). Upon addition of lactate to generate proton motive force (23), the vesicles rapidly took up three times more norfloxacin from the medium than they had in the preenergized state. Approximately the same amount of norfloxacin accumulated in vesicles from all three strains. Upon addition of CCCP, the internal norfloxacin concentration of the vesicles returned to the preenergized level, indicating that the drug accumulated with energy was free and not irreversibly bound in the vesicles (Fig. 2). NADH (5 mM) served equally well as an energy source, and DNP (2 mM) was an energy inhibitor (data not shown). These results with everted vesicles suggested the presence of an energy-dependent efflux system for norfloxacin in the inner membrane of whole cells. Both K-12 (AG100) and

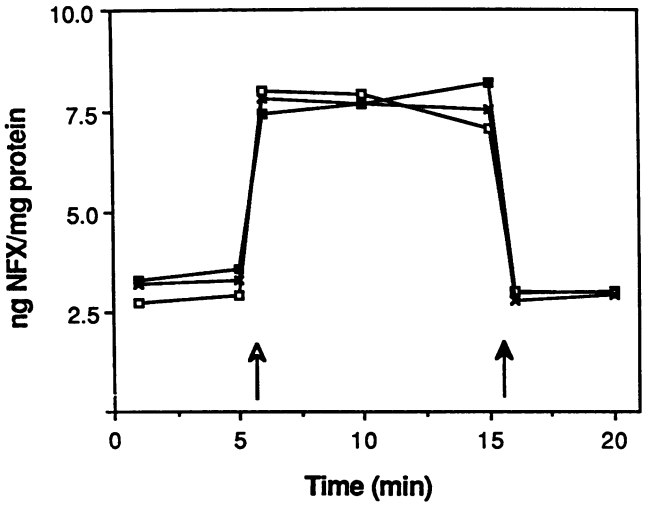

FIG. 2. Accumulation of norfloxacin (NFX) by everted membrane vesicles from *E. coli*. Uptake of [^{3}H]norfloxacin (added at 2 μM [2.3 mCi/mg]) was assayed in everted membrane vesicles from strains AG100 (□), LM218 (×), and ML308-225 (■). Lactate (20 mM) and CCCP (0.1 mM) were added at the times indicated by the open and closed arrows, respectively.

non-K-12 (ML308-225) strains showed the efflux. There was no difference in the kinetics or magnitude of this efflux between everted membrane vesicles of strains LM218 and AG100. Therefore, the lower accumulation of norfloxacin by energized *ompF* cells compared with energized wild-type cells could not be attributed to a larger active efflux in *ompF* cells at the inner membrane.

Saturation kinetics of norfloxacin efflux. To determine whether the energy-dependent uptake of norfloxacin by everted membrane vesicles was carrier mediated, we examined the effect of external norfloxacin concentration upon the rate of uptake in vesicles derived from strain AG100. With increasing concentrations of norfloxacin, saturation of uptake was seen (Fig. 3A). These data, analyzed in the Lineweaver-Burke form with an assumption of Michaelis-Menton kinetics (24), revealed that the vesicle uptake system had a K_m of approximately 0.2 mM and a V_{max} of approximately 3 nmol min^{-1} mg of protein^{-1} (Fig. 3B). The range of K_ms from three different experiments was 0.17 to 0.26 mM, and the V_{max} ranged from 2.7 to 3.4 nmol min^{-1} mg of protein^{-1}. The saturability of the system suggested that efflux of norfloxacin was carrier mediated. The highest concentration of norfloxacin tested had no effect on the proton gradient in energized vesicles, as judged by the acridine orange fluorescence quenching assay (data not shown).

Three other hydrophilic fluoroquinolones, ciprofloxacin, enoxacin, and ofloxacin, were tested at three concentrations (5, 50, and 200 μM) for the capacity to compete with [^{3}H]norfloxacin at 2 μM for transport into everted vesicles. All three drugs showed an inhibition of accumulation of [^{3}H]norfloxacin similar to that of unlabeled norfloxacin (Fig. 4A). If this inhibition was competitive, the affinity of these analogs for the system would be similar to that for norfloxacin. These analogs had no effect on membrane energization as assayed by fluorescence quenching of acridine orange (data not shown). The more hydrophobic quinolone analogs nalidixic acid and oxolinic acid had no effect on norfloxacin accumulation, even at a concentration of 0.5 mM (Fig. 4B). The structurally unrelated drugs tetracycline (for which an

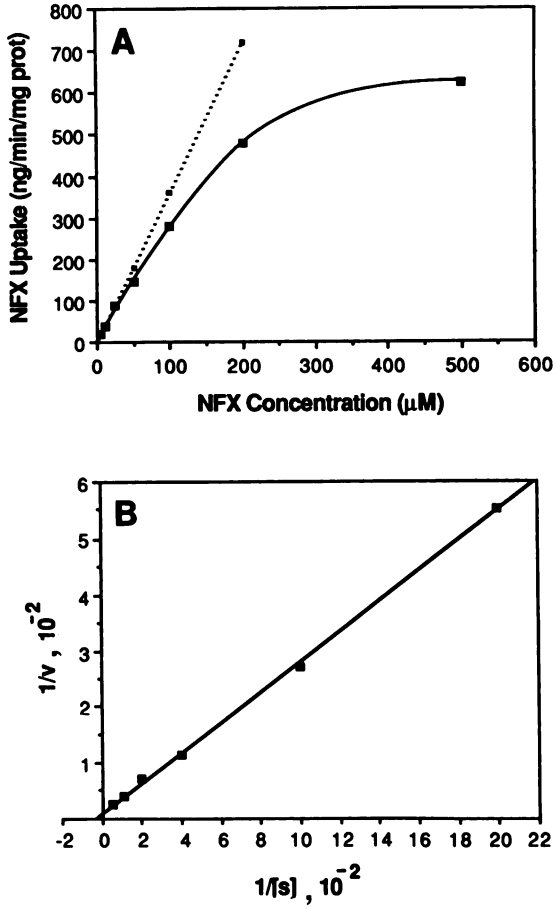

FIG. 3. (A) Rate of uptake of norfloxacin (NFX) as a function of external norfloxacin concentration. The dotted line shows the expected uptake for a nonsaturable process, based on values obtained with low concentrations of norfloxacin (2, 5, and 10 μM). (B) Lineweaver-Burke plot (24) of the kinetic data.

endogenous efflux system has been described [18]) and chloramphenicol also failed to interfere with norfloxacin accumulation in everted vesicles at concentrations up to 0.5 mM (data not shown).

Energy requirements for norfloxacin uptake in everted membrane vesicles. Since norfloxacin uptake in everted vesicles was supported by lactate and NADH and since DNP and CCCP inhibited this uptake, the energy was presumably derived from proton motive force. We examined norfloxacin uptake into everted vesicles in the presence of nigericin, a potassium/proton antiporter which dissipates the proton gradient across the membrane in the presence of potassium (23), and valinomycin, a potassium ionophore which destroys the electrical potential across the membrane (23). At pH 7.5, nigericin completely inhibited uptake; valinomycin decreased accumulation 30 to 40% (Fig. 5). These findings were consistent with the dependence of drug efflux on proton motive force and suggested that both proton gradient and electrical potential components were involved; however, measurements of these components would be required to verify this hypothesis.

DISCUSSION

Our results suggest the presence in susceptible *E. coli* of a carrier-mediated active efflux for norfloxacin with apparent

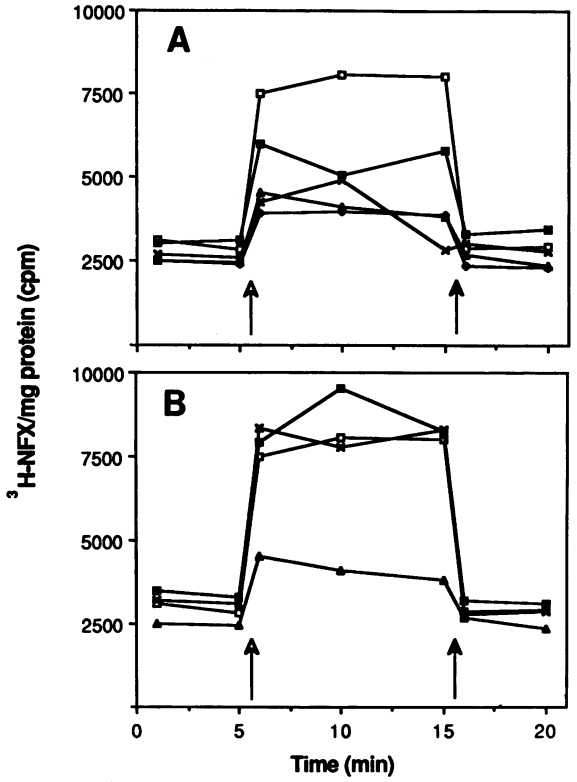

FIG. 4. Effects of other quinolones on norfloxacin (NFX) accumulation by everted membrane vesicles of strain AG100. [^{3}H]norfloxacin, premixed with appropriate amounts of unlabeled quinolones, was added at time zero to 1.54 μCi/ml of (2 μM) norfloxacin. Lactate (20 mM) and CCCP (0.1 mM) were added at the times indicated by the open and closed arrows, respectively. (A) Symbols: □, 2 μM norfloxacin; ▲, 200 μM norfloxacin; ◆, 200 μM ciprofloxacin; ×, 200 μM ofloxacin; ■, 200 μM enoxacin. (B) Symbols: □, 2 μM norfloxacin; ▲, 200 μM norfloxacin; ×, 500 μM nalidixic acid; ■, 500 μM oxolinic acid.

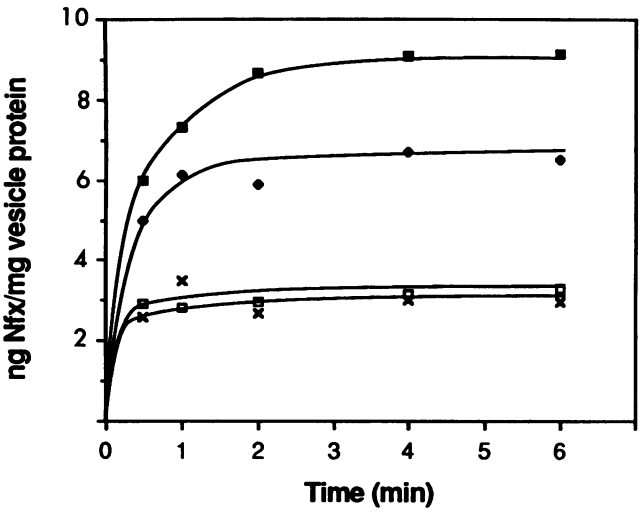

FIG. 5. Effects of nigericin and valinomycin on norfloxacin (Nfx) accumulation in everted membrane vesicles. Everted vesicles derived from strain AG100 were energized with lactate and pretreated for 5 min at 30°C with nigericin or valinomycin at 2 μg/ml before addition of [^{3}H]norfloxacin at 10 μM. Symbols: □, no lactate; ■, lactate; ×, lactate plus nigericin; ◆, lactate plus valinomycin.

specificity for several related fluoroquinolones. The efflux was seen both in a plasmid-free K-12 strain (AG100) and in a non-K-12 strain (ML308-225) of independent origin. Other investigators have shown that the fluoroquinolone enoxacin entered *E. coli* cells by diffusion as opposed to active uptake (2). We found also that norfloxacin rapidly diffused into energy-depleted cells but that this accumulation was reduced in energized cells. In the enoxacin report (2) one can note a small increase in enoxacin accumulation in deenergized cells compared with energized cells. This would be consistent with efflux of enoxacin and with our observation of competition by enoxacin for norfloxacin efflux. The presence of active norfloxacin efflux already in place in bacterial cells suggests potential ways by which these cells could become resistant to these new fluoroquinolone antibacterial agents—by increasing the gene dosage or expression of the carrier or by increasing the affinity of the efflux system for the fluoroquinolones.

Without noting energy dependence, other reports have also indicated less accumulation of norfloxacin in OmpF-deficient mutants (1, 9, 10). Increased resistance to norfloxacin in OmpF-deficient mutants has been noted in the present study and in previous work (9, 10, 12). We saw that whole cells lacking OmpF had a greater energy-dependent decrease in norfloxacin accumulation than did the parental strain (Fig. 1). A simple decrease in outer membrane permeability to norfloxacin due to OmpF loss does not appear to explain these data; OmpF loss should cause both entry and exit rates of norfloxacin across the outer membrane to decline, and the net effect on steady-state accumulation would be zero. Apparently, a simple model in which norfloxacin is actively effluxed across the inner membrane into the periplasm while crossing the outer membrane in both directions through OmpF may be inadequate. Just how a decrease in OmpF results in lower net steady-state norfloxacin accumulation is not evident. One possibility is that the efflux in all strains somehow directs norfloxacin to exit the outer membrane at sites other than OmpF, so that entry rate across the outer membrane is lowered in *ompF* cells while the exit rate is unaffected.

Efflux as a basis for resistance to chemotherapeutic agents is being increasingly recognized. Tetracycline-specific efflux carrier proteins are responsible for resistance specified by a number of different, exogenously acquired tetracycline resistance determinants (19). Extrachromosomal genes are responsible for efflux of arsenate compounds in resistant bacterial cells (21, 25). Among drug-resistant mammalian tumor cells (3, 14), the malaria parasite *Plasmodium falciparum* (17), and the fungus *Aspergillus nidulans* (5), energy-dependent drug efflux has been associated with at least some of the resistances.

That such efflux systems exist even in cells regarded as susceptible has also been reported (5, 6, 18). Wild-type strains of *A. nidulans* possess an efflux system for the sterol synthesis inhibitor fenarimol (5). One efflux carrier protein for anthracyclines in resistant mammalian tumor cells is also expressed in lower levels in normal tissues (6). An endogenous efflux of tetracyclines in susceptible *E. coli* is evident in whole cells only when they are grown under certain culture conditions (18). Our present description of efflux of norfloxacin in susceptible *E. coli* is also the first report of an endogenous efflux system specific for a fully synthetic antimicrobial agent. This suggests that the norfloxacin efflux system normally has a different transport function.

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