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Active Efflux of Fluoroquinolones in *Mycobacterium smegmatis* Mediated by LfrA, a Multidrug Efflux Pump

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The *lfrA* gene cloned from chromosomal DNA of quinolone-resistant *Mycobacterium smegmatis* mc^2-552 conferred low-level resistance to fluoroquinolones when present on multicopy plasmids. Sequence analysis suggested that *lfrA* encodes a membrane efflux pump of the major facilitator family (H. E. Takiff, M. Cimino, M. C. Musso, T. Weisbrod, R. Martinez, M. B. Delgado, L. Salazar, B. R. Bloom, and W. R. Jacbos, Jr., Proc. Natl. Acad. Sci. USA 93:362–366, 1996). In this work, we studied the role of LfrA in the accumulation of fluoroquinolones by *M. smegmatis*. The steady-state accumulation level of a hydrophilic quinolone, norfloxacin, by *M. smegmatis* harboring a plasmid carrying the *lfrA* gene was about 50% of that by the parent strain but was increased to the same level as that of the parent strain by addition of a proton conductor, carbonyl cyanide *m*-chorophenylhydrazone. Norfloxacin efflux mediated by LfrA was competed for strongly by ciprofloxacin but not by nalidixic acid. Furthermore, we showed that portions of norfloxacin accumulated by starved cells were pumped out upon reenergization of the cells, and the rates of this efflux showed evidence of saturation at higher intracellular concentrations of the drug. These results suggest that the LfrA polypeptide catalyzes the active efflux of several quinolones.

The reemergence of tuberculosis worldwide has once again produced a major public health problem. Tuberculosis and other mycobacterial infections are often difficult to treat because mycobacteria are intrinsically resistant to most common antibiotics, apparently because of their extremely low cell wall fluidity and permeability (1, 13, 14, 18, 35). The situation is made worse by the dramatic increase in multidrug-resistant strains (6, 7). Fluoroquinolones inhibit bacterial DNA gyrase and are active against mycobacteria in vitro; thus, they seem to be promising for development as antituberculous agents. Unfortunately, fluoroquinolone-resistant clinical isolates of *Mycobacterium tuberculosis* have already appeared (3, 31, 33). Mutations conferring resistance to quinolones have been reported for several bacterial species (2), including the gram-negative bacteria *Escherichia coli* (5, 10, 22) and *Pseudomonas aeruginosa* (4, 16, 28) and the gram-positive bacterium *Staphylococcus aureus* (11, 23, 24, 36). Two principal mechanisms have been described: (i) alteration of DNA gyrase and (ii) decreased drug accumulation in the cell as a result of either decreased influx or increased efflux. Alteration of DNA gyrase is usually the result of a mutation in the *gyrA* or, less frequently, the *gyrB* gene. The genes that encode the DNA gyrase of *M. tuberculosis* H37Rv have been cloned and sequenced, and mutations associated with quinolone resistance have been found in the *gyrA* gene from clinical isolates (3, 33).

In gram-positive bacteria, it has been shown that active efflux pumps play an important role in the decreased accumulation of quinolones. An example is the *norA* gene product of *S. aureus*, which confers resistance to quinolones and appears to be a membrane-associated, energy-dependent efflux pump (23, 24, 36). In gram-negative bacteria, decreased drug accumulation has more often been ascribed to the loss of pore-forming outer membrane proteins (for a review, see reference 25). However, especially in quinolone-resistant strains of *P. aeruginosa*, unexpected increases in outer membrane proteins in the 50 to 54-kDa range have frequently been observed (summarized in references 9 and 19) and it is difficult to explain why the production of additional proteins could lower outer membrane permeability. Now that it is known that active multidrug efflux systems of many gram-negative bacteria contain outer membrane channel proteins of this size (16, 19, 27), it appears likely that the quinolone resistance of these strains was caused instead by the active efflux process.

To our knowledge, active efflux of drugs has not been demonstrated in mycobacteria. Recently, one of us cloned a gene, *lfrA*, from a ciprofloxacin-resistant strain, *Mycobacterium smegmatis* mc^2-552. When introduced into a susceptible strain, mc^2-155, on multicopy plasmids, this gene conferred low-level resistance to hydrophilic fluoroquinolones such as ciprofloxacin and ofloxacin but not resistance to hydrophobic quinolones such as sparfloxacin (32). The predicted protein sequence indicated that LfrA is homologous to QacA from *S. aureus*, TcmA from *Streptomyces glaucescens*, and ActII and Mmr from *Streptomyces coelicolor*; thus, it corresponds to a putative membrane efflux pump of the major facilitator family (32). In this work, we extended this study by measuring the accumulation of norfloxacin in strain mc^2-155 with or without the presence of plasmids carrying the *lfrA* gene. Furthermore, the norfloxacin that accumulated in starved cells was shown to be pumped out upon reenergization of cells, following Michaelis-Menten-type kinetics. Our results provide strong evidence that LfrA is indeed an active efflux transporter, with apparent specificity for several related fluoroquinolones.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *M. smegmatis* high-frequency transformation strain mc^2-155 (12), which is susceptible to fluoroquinolones, was used as the host strain into which plasmids carrying the *lfrA* gene were introduced. Strain mc^2-552 is a ciprofloxacin-resistant mutant strain derived from mc^2-155 (32). The net influx of quinolones appeared to be lower in mc^2-552, from which the *lfrA* fragment in pMN1 was isolated. The strain also contains a *gyrA* point mutation. pMN1 is composed of the shuttle vector pYUB53 containing a

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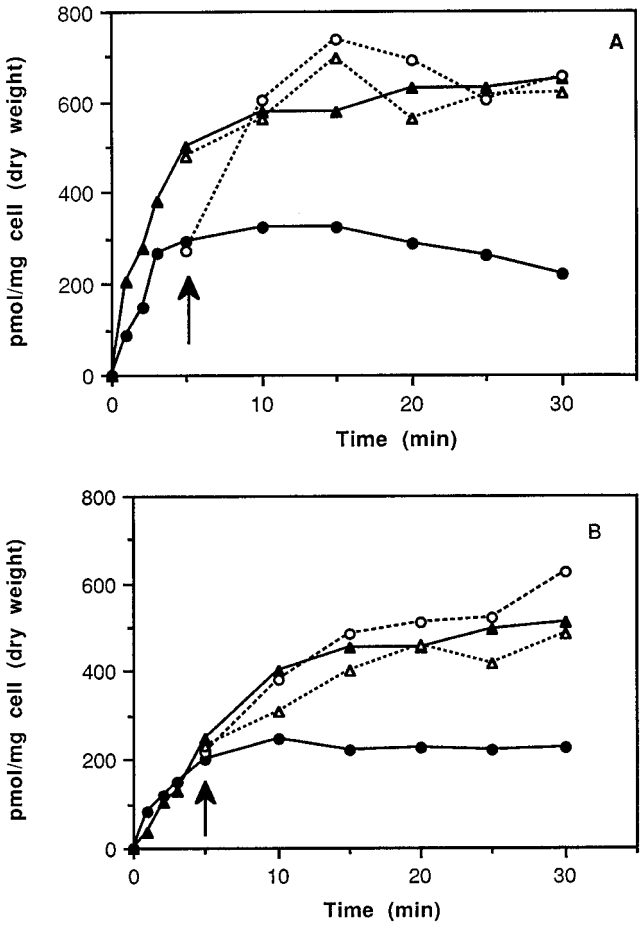

FIG. 1. Accumulation of norfloxacin by whole cells of *M. smegmatis*. To 1.5-ml cell suspensions, 25 μM [^{14}C]norfloxacin was added at time zero. At the time indicated by the arrow (5 min), 0.6 ml of the cell suspension was transferred into a new vessel and 0.1 mM CCCP was added. (A) Conditions: mc^2-155/pMN1 (●), mc^2-155/pMN1 plus 0.1 mM CCCP (○); mc^2-155/pYUB53 (▲), and mc^2-155/pYUB53 plus 0.1 mM CCCP (△). (B) Conditions: mc^2-552 with (○) or without (●) CCCP and mc^2-155 with (△) or without (▲) CCCP.

strong Kan^r promoter and a 3.0-kb *Msp*I fragment of genomic DNA containing the *lfrA* gene cloned into the *Cla*I site just downstream of the Kan^r promoter. pSB51 was constructed by subcloning of pMN1 fragments into vector pMV203 and contained the 2.3-kb full-length *lfrA* gene. A detailed description of these plasmids and strains has previously been given (32).

Bacteria were grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with 10% Middlebrook OADC enrichment (Difco) and 0.2% glycerol. Cells were harvested in the midexponential phase of growth.

Chemicals. [^{14}C]norfloxacin (specific activity, 14.8 mCi/mmol) was a gift from Merck & Co. (Rahway, N.J.). Norfloxacin, ciprofloxacin, enoxacin, nalidixic acid, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were from Sigma Chemical Co.

Assay of norfloxacin accumulation. Uptake experiments were performed essentially as described previously (20). All such experiments were repeated at least twice to make certain that the results were reproducible. Cells were harvested by centrifugation at room temperature, washed, and resuspended in 0.1 M K-phosphate (pH 7.0) to a density of 5.0 mg (dry weight)/ml. Addition of the carbon source, 0.2% glycerol, to the assay buffer did not change the results, presumably because of the presence of intracellular energy reserve compounds; thus, glycerol was omitted unless indicated otherwise. Portions of 1 or 1.5 ml were preincubated for 5 to 10 min at 23°C in an Erlenmeyer flask with vigorous aeration by shaking, and the assay was started by addition of [^{14}C]norfloxacin to a final concentration of 25 μM. At various time points thereafter, 50 μl of the suspension was removed and diluted into 1 ml of 0.1 M K-phosphate (pH 7.0) buffer containing 0.1 M LiCl and the mixture was immediately filtered through a cellulose acetate membrane filter (0.22-μm pore size; Millipore). The filter was washed with 5 ml of the same buffer. The radioactivity retained on the filter was then determined in a Beckman LS7000 liquid scintillation counter by using Ecolume (ICN Biomedicals) scintillation cocktail. In all cases, counts from label binding to filters alone were subtracted from the measured values.

For competition experiments, unlabeled norfloxacin, ciprofloxacin, enoxacin, and nalidixic acid were prepared at a concentration of 1 mg/ml in 0.02 N NaOH. Unlabeled and labeled drugs were combined at a molar ratio of 4:1 and then added to the cell suspensions to start the uptake assay.

For kinetic studies of efflux, cell suspensions were preincubated with 0.1 mM CCCP in the phosphate buffer with gentle shaking at 37°C for 1 h so that the endogenous energy reserve of the cells was exhausted. These starved cells were then washed three or more times with the phosphate buffer at room temperature to remove CCCP. The uptake assay was started by addition of [^{14}C]norfloxacin to 25 μM as described above. After 20 min of incubation to allow norfloxacin accumulation to reach a high steady-state level, cells were reenergized by addition of an energy source, 0.2% glycerol. The decrease in the intracellular level of norfloxacin was monitored by filtering portions of the suspension at short time intervals. The rate of efflux was calculated from the datum points in the first 3 min, during which the curves were approximately linear.

RESULTS

Accumulation of norfloxacin in intact cells of *M. smegmatis*. The time course of accumulation of [^{14}C]norfloxacin is shown in Fig. 1. For all of the strains studied, norfloxacin accumulation reached steady-state levels after approximately 10 min. Although the curves are rather in similar shape, the steady-state accumulation level in mc^2-155 containing the *lfrA* gene on pMN1 was only one-half of that of its parent strain, mc^2-155, containing only the vector pYUB53 (Fig. 1A). Similarly, mc^2-155 with another *lfrA*-containing plasmid, pSB51, accumulated about 50% less than did the parent strain, mc^2-155, containing the vector alone (data not shown). The norfloxacin accumulation level in mc^2-552, from which the *lfrA* gene was cloned, was also lower than that in the parent strain, mc^2-155 (Fig. 1B).

To study the effect of membrane energization on the uptake of norfloxacin, CCCP, a protonophore that destroys proton motive force, was also added to the labeled cultures. Upon addition of CCCP, norfloxacin accumulation increased rapidly in strains mc^2-155/pMN1, mc^2-155/pSB51, and mc^2-552 and reached higher plateau levels that were almost equal to the levels observed in the parent strain, mc^2-155, or in mc^2-155 containing the vectors alone. Because CCCP has been predicted to produce a slight increase in norfloxacin accumulation in an acidic medium, even in the absence of active efflux (26), we also added the same concentration of CCCP to the three

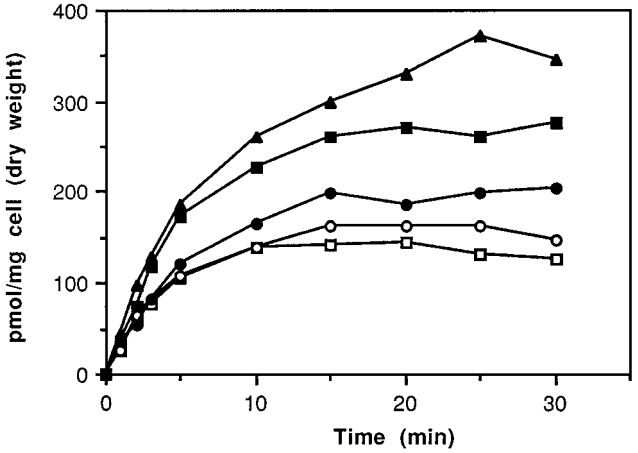

FIG. 2. Accumulation of norfloxacin by whole cells of *M. smegmatis* mc^2-155/pMN1 in the presence of unlabeled quinolones. Unlabeled quinolones at 50 μM were premixed with 12.5 μM [^{14}C]norfloxacin and added to the cultures at time zero. Symbols: □, 12.5 μM [^{14}C]norfloxacin; ■, 12.5 μM [^{14}C]norfloxacin plus 50 μM norfloxacin; ▲, 12.5 μM [^{14}C]norfloxacin plus 50 μM ciprofloxacin; ●, 12.5 μM [^{14}C]norfloxacin plus 50 μM enoxacin; ○, 12.5 μM [^{14}C]norfloxacin plus 50 μM nalidixic acid.

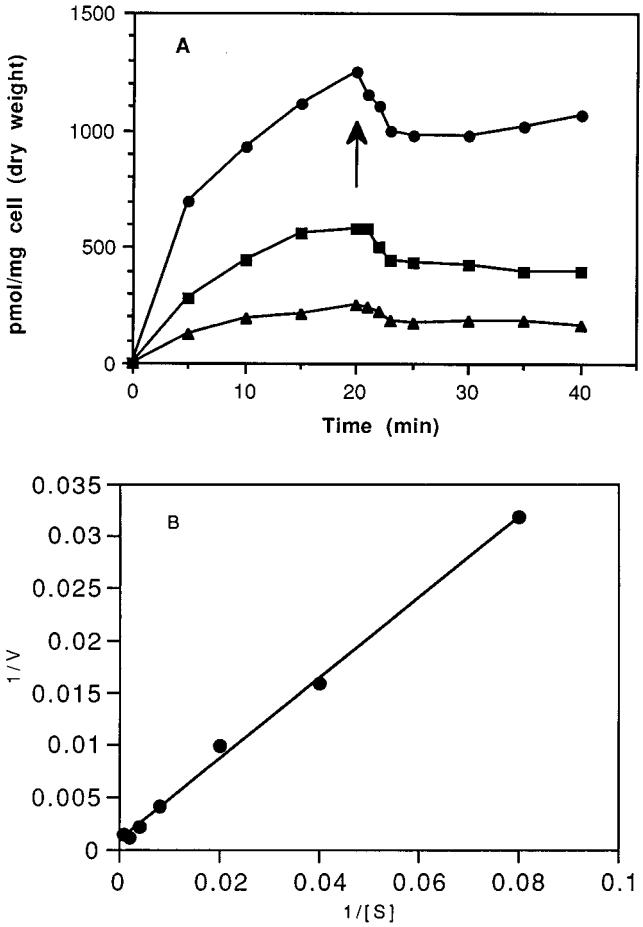

FIG. 3. (A) Kinetic experiments. Cells of strain mc^2-155/pMN1 were pretreated with 0.1 mM CCCP and washed. Glycerol was added to a final concentration of 0.2% at the time indicated by the arrow. Norfloxacin at various concentrations was added to aliquots of the cell suspension. The uptake curves of three concentrations of norfloxacin are shown here as examples. [^{14}C]norfloxacin concentrations: 12.5 μM (▲), 25 μM (■), and 50 μM (●). (B) Rate of efflux as a function of the external norfloxacin concentration plotted in the Lineweaver-Burke form. The external norfloxacin concentrations tested were 5, 10, 20, 50, 100, 200, and 500 μM. Velocity (V) is expressed as picomoles per minute per milligram of dry weight, and the substrate concentration [S] is expressed in micromolar units.

parent strains. Under our conditions, which used phosphate buffer at pH 7.0, CCCP (0.1 mM) had no significant effect on the norfloxacin accumulation in strains carrying vectors without *lfrA* inserts, e.g., mc^2-155/pYUB53, and wild-type parent strain mc^2-155 (Fig. 1). These data indicated that LfrA pumped out norfloxacin in an energy-dependent process, presumably by utilizing proton motive force.

Substrate specificity of LfrA. To determine the substrate specificity of LfrA, three other fluoroquinolones were tested for the ability to compete with [^{14}C]norfloxacin for transport by LfrA in mc^2-155/pMN1. We measured the transport of these quinolones indirectly because only norfloxacin was available to us in radiolabeled form. The result is shown in Fig. 2. The accumulation of 12.5 μM [^{14}C]norfloxacin alone and that of the sample diluted with a fourfold excess (50 μM) of unlabeled norfloxacin were determined as controls. The accumulation level of [^{14}C]norfloxacin was significantly higher in the presence of unlabeled norfloxacin, presumably because unlabeled norfloxacin competed with labeled norfloxacin for excretion by LfrA, a catalyzed process, whereas there was no competition for influx, a nonspecific, passive diffusion process. If other fluoroquinolones competed with norfloxacin for LfrA, then the accumulation level of [^{14}C]norfloxacin would also be increased. One of the fluoroquinolones, ciprofloxacin, showed such competition; in fact, its affinity for LfrA appeared to be slightly higher than that of norfloxacin, as the accumulation level of [^{14}C]norfloxacin was even higher than that seen with an equal concentration of unlabeled norfloxacin. Enoxacin showed weak competition with norfloxacin. The more hydrophobic quinolone analog nalidixic acid had no effect on norfloxacin accumulation.

Demonstration of the active efflux process. On the basis of measurement of steady-state accumulation levels of [^{14}C]norfloxacin, we had so far inferred that norfloxacin was pumped out actively but there was no direct evidence of efflux. More recently, however, we obtained such direct evidence of the active efflux process. In this experiment, cells of strain mc^2-155/pMN1 were first treated with 0.1 mM CCCP for 1 h at 37°C to deplete endogenous energy sources and then CCCP was removed by repeated washing. When [^{14}C]norfloxacin was added to these cells, it accumulated at the maximal level, since the efflux pump could not function owing to the lack of energy. After 20 min, that is, after equilibrium had been attained, the cells were energized by adding an energy source (0.2% glycerol). Under these conditions, the pump was indeed turned on and we observed rapid, active efflux of [^{14}C]norfloxacin out of the cells (Fig. 3).

We attempted to characterize the kinetic parameters of the process by varying the external concentration of [^{14}C]norfloxacin. The initial rates of efflux were different, depending on the initial external concentration, and consequently the intracellular concentration, of the drug; this observation makes it unlikely that the exit rates were limited by the rate of energization of the membrane. Importantly, there was evidence that the process is a saturable one; this was most clearly shown by analyzing the data by Lineweaver-Burke plotting (Fig. 3B). We can calculate the apparent K_m of the efflux pump if we assume that the internal concentration of free norfloxacin is approximately equal to the external concentration (see Fig. 4B of reference 26). However, because of the uncertainty in the estimation of the intracellular concentration of free norfloxacin (see Discussion), these values may not be precise.

DISCUSSION

Active efflux as a resistance mechanism has been shown increasingly in many species of bacteria, such as the gram-negative bacteria *E. coli* and *P. aeruginosa* and the gram-positive bacteria *Bacillus subtilis* and *S. aureus* (for reviews, see references 15 and 25). We report here the first biochemical study of a mycobacterial multidrug efflux pump, which was genetically characterized previously by one of us (32). As mentioned above, the extremely low permeability of the mycobacterial cell wall is thought to be the major mechanism of its intrinsic resistance to many antimicrobial agents; it is thus somewhat unexpected to see the existence of an active drug efflux pump. On the other hand, even the low permeability of the mycobacterial cell wall is expected to allow half-equilibration of small drug molecules across this barrier in several minutes (see Fig. 2 of reference 14). Indeed, norfloxacin accumulated in the cells and reached a steady-state concentration within approximately 10 min in several fast-growth mycobacterial species, including *M. smegmatis*, *M. chelonei*, *M. vaccae*, and *M. aurum*, as well as in the slow growers *M. tuberculosis* and *M. avium* (Fig. 1 and reference 18). Thus, the accumula-

tion occurs in a time frame shorter than the doubling time of these bacteria and the permeability barrier of the cell wall alone may not be able to provide an effective resistance mechanism. For these reasons, additional mechanisms, such as active efflux, are needed for significant levels of resistance, as already shown for *P. aeruginosa* (16).

We demonstrated here that the presence of the cloned *lfrA* gene produces energy-dependent efflux of several related fluoroquinolones in *M. smegmatis*. It is most likely that LfrA functions as an efflux transporter, especially because the *lfrA* gene sequence is homologous to those of genes encoding other known efflux transporters of fluoroquinolones (32). The active efflux was seen both in strains carrying plasmids (mc^2-155/pMN1 and mc^2-155/pSB51) and in a plasmid-free, ciprofloxacin-resistant, mutant strain (mc^2-552). The plasmid-containing strains presumably express LfrA at higher levels because of the presence of a more effective promoter (32) and also the gene dosage effect. In fact, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the cell membrane fractions indicated that a protein of approximately 50 kDa was overexpressed in mc^2-155/pMN1 and mc^2-155/pSB51 (17).

Compared with mc^2-155, for which the ciprofloxacin MIC is 0.25 μg/ml, the MICs for mc^2-155/pSB51 and mc^2-155/pMN1 were increased 16- and 32-fold, respectively (32). The observed differences in the steady-state accumulation levels of norfloxacin, however, were only twofold, smaller than the MIC differences. This discrepancy between the MICs and accumulation levels has been observed previously (16, 20). Li et al. (16) suggested that the discrepancy presumably arises from the fact that we measure the sum of accumulation in all cellular compartments, only some of which are relevant to the action of the drug and are affected by the efflux process. The very high resistance of mc^2-552 (for which the ciprofloxacin MIC is 64-fold higher than that for mc^2-155 [32]) must be caused partly by the mutation found in its *gyrA* gene.

Our competition experiments and a previous study (32) indicated that hydrophilic fluoroquinolones such as ciprofloxacin, norfloxacin, and enoxacin can be pumped out by LfrA, but hydrophobic ones such as sparfloxacin and nalidixic acid cannot. Similar specificity was also observed in the NorA efflux pump of *S. aureus* (24, 36) and in an endogenous active efflux system in *E. coli* (5), probably AcrAB. It is of interest that the predicted protein sequence showed that LfrA is homologous to QacA from *S. aureus* but not to NorA (32). LfrA also showed cross-specificity to ethidium bromide, acridine, and some quaternary ammonium compounds (32), just as QacA did (29).

We attempted to characterize the kinetic constants of norfloxacin efflux by LfrA. Three problems make their accurate measurement difficult. First, the total norfloxacin accumulation level at steady state was unexpectedly high. If we assume the cell volume to be 3 μl for 1 mg (dry weight) of cells (21, 34), the total norfloxacin concentration inside the cells was much higher (about eightfold in this case) than the external concentration; this is similar to what has been observed in other bacteria (see the references cited in reference 26). Theoretical calculation based on passive distribution in equilibrium with the ΔpH across the membrane indicated that under our experimental condition (pH 7.0), the internal norfloxacin concentration should be approximately equal to the external concentration (26). Binding to the DNA gyrase complex and DNA (30) cannot explain all of the difference. It therefore seems likely that norfloxacin also binds to an unknown macromolecule(s). The intracellular concentration of free norfloxacin is thus uncertain. Second, reenergization did not decrease the norfloxacin level to the 50% expected from Fig. 1A, presumably because some of the drug existed as irreversible complexes with DNA gyrase (30). This increases the uncertainty of the exact free norfloxacin concentration inside the cell. Third, if we analyze the kinetic data in the Lineweaver-Burke form assuming that the internal concentration of free norfloxacin is equal to the external concentration, we could calculate the V_{max}. The value we obtained was approximately 0.9 nmol min^{-1} mg^{-1} (dry weight), which is rather low in comparison with, for example, the V_{max} of the Tet-catalyzed tetracycline efflux in *E. coli*, which was estimated to be 20 nmol min^{-1} mg^{-1} (dry weight) (34). This is probably due to the low permeability of the mycobacterial cell wall. LfrA belongs to the family of drug pumps that includes Tet (34) and Smr (8), in which a single polypeptide, located in the cytoplasmic membrane, catalyzes the efflux process. Like Tet, LfrA is thus likely to pump drugs into the periplasm rather than directly into the medium (34). (Although a periplasm is not commonly thought to exist in gram-positive bacteria, mycobacterial cells are more similar to gram-negative cells, given the presence of a cell wall and an outer membrane, both constructed as lipid bilayers [1].) It is possible that the rates with which the drugs are lost from the cells, measured in the experiment of Fig. 3, are significantly influenced by the rate of drug diffusion from the periplasm to the external medium through the low-permeability mycobacterial cell wall. If so, the V_{max} determined in our experiment may be a serious underestimation. Nevertheless, the experiment of Fig. 3 showed that the norfloxacin accumulated by deenergized cells was indeed pumped out when the energy source, glycerol, was added and suggested that efflux is a saturable process. Both of these findings strongly support the conclusion that LfrA indeed performs a catalyzed, active efflux process.

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REFERENCES

1. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64:**29–63.
2. **Bryan, L. E., and J. Bedard.** 1991. Impermeability to quinolones in gram-positive and gram-negative bacteria. Eur. J. Clin. Microbiol. Infect. Dis. **10:**232–239.
3. **Cambau, E., W. Sougakoff, M. Besson, C. Truffot-Perot, J. Grosset, and V. Jarlier.** 1994. Selection of a *gyrA* mutant of *Mycobacterim tuberculosis* resistant to fluoroquinolones during treatment with ofloxacin. J. Infect. Dis. **170:**479–483.
4. **Chamberland, S., A. S. Bayer, T. Schollardt, S. A. Wong, and L. E. Bryan.** 1989. Characterization of mechanisms of quinolone resistance in *Pseudomonas aeruginosa* strains isolated in vitro and in vivo during experimental endocarditis. Antimicrob. Agents Chemother. **33:**624–634.
5. **Cohen, S. P., D. C. Hooper, J. S. Wolfson, K. S. Souza, L. M. McMurry, and S. B. Levy.** 1988. Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. Antimicrob. Agents Chemother. **32:**1182–1191.
6. **Frieden, T. R., T. Sterling, A. Pablos-Mendez, J. O. Kilburn, G. M. Cauthen, and S. W. Dooley.** 1993. The emergence of drug-resistant tuberculosis in New York City. N. Engl. J. Med. **328:**521–526.
7. **Goble, M., M. D. Iseman, L. A. Madsen, D. Waite, L. Ackerson, and C. R. Horsburgh.** 1993. Treatment of 171 patients with pulmonary tuberculosis resistant to isoniazid and rifampin. N. Engl. J. Med. **328:**527–532.
8. **Grinius, L. L., and E. R. Goldberg.** 1994. Bacterial multidrug resistance is due to a single membrane protein which functions as a drug pump. J. Biol. Chem. **269:**29998–30004.
9. **Hashmi, Z., and J. M. B. Smith.** 1991. Outer membrane changes in quinolone resistant *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **28:**465–470.
10. **Hooper, D. C., J. S. Wolfson, K. S. Souza, C. Tang, G. L. McHugh, and M. N. Swartz.** 1986. Genetic and biochemical characterization of norfloxacin resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **29:**639–644.
11. **Ito, H., H. Yoshida, M. Bogaki-Shonai, T. Niga, H. Hattori, and S. Nakamura.** 1994. Quinolone resistance mutations in the DNA gyrase *gyrA* and *gyrB* genes of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **38:** 2014–2023.
12. **Jacobs, W. R., Jr., G. V. Kalpana, J. D. Cirillo, L. Pascopella, S. B. Snapper,**

R. A. Udani, W. Jones, R. G. Barletta, and B. R. Bloom. 1991. Genetic systems for mycobacteria. Methods Enzymol. **204:**537–555.
13. **Jarlier, V., and H. Nikaido.** 1990. Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. J. Bacteriol. **172:**1418–1423.
14. **Jarlier, V., and H. Nikaido.** 1994. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol. Lett. **123:**11–18.
15. **Levy, S. B.** 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. **36:**695–703.
16. **Li, X.-Z., D. M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob. Agents Chemother. **38:**1732–1741.
17. **Liu, J., and H. Nikaido.** Unpublished results.
18. **Liu, J., E. Y. Rosenberg, and H. Nikaido.** 1995. Fluidity of the lipid domain of cell wall from *Mycobacterium chelonei*. Proc. Natl. Acad. Sci. USA **92:**11254–11258.
19. **Masuda, N., E. Sakagawa, and S. Ohya.** 1995. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**645–649.
20. **McMurry, L. M., R. E. Petrucci, Jr., and S. B. Levy.** 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77:**3974–3977.
21. **Mühlradt, P. F., J. Menzel, J. R. Golecki, and V. Speth.** 1974. Lateral mobility and surface density of lipopolysaccharide in the outer membrane of *Salmonella typhimurium*. Eur. J. Biochem. **43:**533–539.
22. **Nakamura, S., M. Nakamura, T. Kojima, and H. Yoshida.** 1989. *gyrA* and *gyrB* mutations in quinolone-resistant strains of *Escherichia coli*. Antimicrob. Agents Chemother. **33:**254–255.
23. **Neyfakh, A. A., C. M. Borsch, and G. W. Kaatz.** 1993. Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter. Antimicrob. Agents Chemother. **37:**128–129.
24. **Ng, E.-Y., M. Trucksis, and D. C. Hooper.** 1994. Quinolone resistance mediated by *norA*: physiological characterization and relationship to *flqB*, a quinolone resistance locus on the *Staphylococcus aureus* chromosome. Antimicrob. Agents Chemother. **38:**1345–1355.
25. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: role of permeability barriers and active efflux. Science **264:**382–388.
26. **Nikaido, H., and D. G. Thanassi.** 1993. Penetration of lipophilic agents with multiple protonation sites into bacterial cells: tetracyclines and fluoroquinolones as examples. Antimicrob. Agents Chemother. **37:**1393–1399.
27. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for an involvement of an efflux operon. J. Bacteriol. **175:**7363–7372.
28. **Robillard, N. J., and A. L. Scarpa.** 1988. Genetic and physiological characterization of ciprofloxacin resistance in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents Chemother. **32:**535–539.
29. **Rounch, D. A., D. S. Cram, D. DiBeraradino, T. G. Littlejohn, and R. A. Skurray.** 1990. Efflux-mediated antiseptic resistance *qacA* gene from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. Mol. Microbiol. **4:**2051–2062.
30. **Shen, L. L., L. A. Mitsher, P. N. Sharma, T. J. O'Donnell, W. T. Chu, C. S. Cooper, T. Rosen, and A. G. Pernet.** 1989. Mechanism of inhibition of DNA gyrase by quinolone antibacterial. A cooperative drug-DNA binding model. Biochemistry **28:**3886–3894.
31. **Sullivan, E. A., B. N. Kreiswirth, L. Palumbo, V. Kapur, J. M. Musser, A. Ebrahimzadeh, and T. R. Friden.** 1995. Emergence of fluoroquinolone-resistant tuberculosis in New York City. Lancet **345:**1148–1150.
32. **Takiff, H. E., M. Cimino, M. C. Musso, T. Weisbrod, R. Martinez, M. B. Delgado, L. Salazar, B. R. Bloom, and W. R. Jacbos, Jr.** 1996. Efflux pumps of the proton antiporter family confers low level fluoroquinolone resistance in *M. smegmatis*. Proc. Natl. Acad. Sci. USA **93:**362–366.
33. **Takiff, H. E., L. Salazar, C. Guerrero, W. Philip, W. Huang, B. Kreiswirth, S. T. Cole, W. R. Jacobs, Jr., and A. Telenti.** 1994. Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. Antimicrob. Agents Chemother. **38:**773–780.
34. **Thanassi, D. G., G. S. B. Suh, and H. Nikaido.** 1995. Role of outer membrane barrier in efflux-mediated tetracycline resistance of *Escherichia coli*. J. Bacteriol. **177:**998–1007.
35. **Trias, J., and R. Benz.** 1994. Permeability of the cell wall of *Mycobacterium smegmatis*. Mol. Microbiol. **14:**283–290.
36. **Yoshida, H., M. Bogaki, S. Nakamura, K. Vbukata, and M. Konno.** 1990. Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. J. Bacteriol. **172:**6942–6944.