Active Efflux of Norfloxacin by *Bacteroides fragilis*

SHIN MIYAMAE,^{1,2} HIROSHI NIKAIIDO,^{3*} YOSHINOBU TANAKA,² AND FUMINOBU YOSHIMURA¹

Department of Microbiology¹ and the First Department of Prosthodontics,² School of Dentistry,
Aichi-Gakuin University, Nagoya 464-8650, Japan, and Department of Molecular and
Cell Biology, University of California, Berkeley, California 94720³

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Norfloxacin was actively pumped out by *Bacteroides fragilis*, which is intrinsically resistant to most fluoroquinolones. Reserpine moderately inhibited the efflux. A one-step spontaneous mutant had increased resistance to norfloxacin, ethidium bromide, and puromycin, a result suggesting that the efflux is catalyzed by a multidrug pump with specificity similar to that of NorA/Bmr.

In about 90% of the cases of intra-abdominal infections following a perforated appendix or surgery on the gastrointestinal tract, both aerobic and anaerobic species of bacteria are found (7). *Bacteroides* species are involved in more than 60% of these cases, with *Bacteroides fragilis* being found in 30 to 60% of the specimens (15). *B. fragilis* and its relatives (*B. fragilis* group) often present serious problems in therapy, as they are intrinsically resistant to many antibiotics, including aminoglycosides, most of the penicillins and cephalosporins (except carbapenems), and fluoroquinolones (except a few recently developed compounds such as trovafloxacin and clinafloxacin) (6). Aminoglycoside resistance is thought to be caused by a low membrane potential, a common feature among obligate anaerobes. β -Lactam resistance is usually explained by the combination of low permeability of the outer membrane (24) and the presence of highly active β -lactamase of the Bush 2e class (4). However, the remarkable fluoroquinolone resistance is unexplained (21); for example, norfloxacin having MIC₅₀s of 16 to 32 μ g/ml for *B. fragilis* in comparison with those of 0.06 to 0.12 μ g/ml for *Escherichia coli* (22).

During recent years, intrinsic resistance of gram-negative bacteria to various agents, formerly ascribed to the outer membrane permeability barrier alone, was found to be the result of synergy between the outer membrane barrier and active drug efflux, often catalyzed by broad-specificity, multidrug efflux pumps (9, 17, 19). We reasoned that the fluoroquinolone resistance of *B. fragilis* may be in part due to an active efflux process and examined the intracellular accumulation of radiolabeled norfloxacin in three strains of *B. fragilis*, the type strain ATCC 25285 and two typical clinical isolates, YCH11 and YCH12 (obtained from S. Akimoto, Wakayama Medical University), whose fluoroquinolone susceptibility levels were not known until this study was undertaken.

The strains were grown overnight in general anaerobic GAM broth (Nissui, Tokyo, Japan) at 37°C in an anaerobic chamber. In the morning, the cultures were diluted 50-fold into fresh GAM broth and incubation was continued usually for 3 h in order to obtain populations maximally enriched in exponentially growing cells. The cultures were harvested by centrifugation at 1,500 $\times g$ for 10 min at room temperature, and the cells were washed once with a solution containing 20 mM potassium phosphate buffer (pH 7.0), 0.2% glucose, and 1 mM dithio-

threitol. After resuspension in the same buffer at the density of 1.0 mg (dry weight) per ml, as judged by optical density at 600 nm, the suspension was immediately warmed to 37°C in a water bath and [¹⁴C]norfloxacin (a gift from Merck & Co., Rahway, N.J.) (specific activity, 14.9 mCi/mmol) was added to a final 12 μ M concentration. (As shown below, this concentration is far below the MIC of norfloxacin for these strains.) Fifty-microliter portions of the suspension were removed at intervals and diluted into 1 ml of ice-cold 0.1 M LiCl, and the diluted suspensions were immediately filtered through Millipore type HA membrane filters (0.45- μ m pore size), which were then washed quickly with 5 ml of ice-cold 0.1 M LiCl. Ten minutes after the addition of the drug, a portion of the suspension was added to a prewarmed tube containing enough carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to make its final concentration 200 μ M, and sampling was done from this tube as well. The filters were dried, and the radioactivity was determined by liquid scintillation counting.

We found that the presence of proton conductor CCCP produced large increases (between 2.6- and 13-fold) in the amount of intracellular norfloxacin accumulated in all of the 10 experiments performed (Fig. 1), suggesting that the energized *B. fragilis* cells actively pump out norfloxacin and that the drug accumulation increases when the pump(s) ceases to function through the deenergization of the membrane by the addition of CCCP. Although the Δ pH across the cytoplasmic membrane may produce unfavorable passive distribution of norfloxacin in the cytoplasm, such an effect is negligible at an external pH of 7.0 (see Fig. 3B of reference 18), used in these experiments. Even in the experiment in which the difference in accumulation level was the smallest, the size of the difference (2.6-fold) could not be explained by a passive distribution effect and indicated active efflux. Furthermore, the CCCP-induced stimulation obtained in several experiments (about 10-fold or more) was extraordinarily large and suggests an exceptionally efficient efflux process, when we consider that the difference in norfloxacin accumulation between CCCP-exposed and nonexposed cells was only about fivefold even in a *nalB*-type mutant strain of *Pseudomonas aeruginosa*, overproducing the MexAB-OprM efflux pump (10).

Interestingly, in most experiments norfloxacin accumulated rapidly to a high level and then was apparently pumped out to attain a low, steady-state level. In some cases (Fig. 1A), the peak of the initial accumulation occurred already in the first sample collected soon after the addition of norfloxacin (about 10 s), and in others the accumulation peak occurred somewhat later (Fig. 1B). The reasons for this initial overshoot are not clear at present.

* Corresponding author. Mailing address: Department of Molecular and Cell Biology, 229 Stanley Hall, University of California, Berkeley, CA 94720-3206. Phone: (510) 642-2027. Fax: (510) 643-9290. E-mail: nhiroshi@uclink4.berkeley.edu.

FIG. 1. Accumulation of [^{14}C]norfloxacin by intact cells of YCH12. Accumulation was monitored as described in the text (●). At 10 min, CCCP was added to a final concentration of 200 μM to one-half of the incubation mixture, and the accumulation in the presence of this proton conductor was also monitored (■). (A and B) Results obtained on different days. These two types of accumulation kinetics were also seen with ATCC 25285 and YCH11 (data not shown).

All three strains were susceptible to chloramphenicol and erythromycin, and at least the type strain, ATCC 25285, was quite susceptible to tetracycline as determined by disk diffusion assays carried out with Tridisk antibiotic disks (Eiken Chemicals Ltd., Tokyo, Japan) at three different concentrations. This result suggested that the pump contributing to fluoroquinolone resistance might be of a different type from the well-known gram-negative multidrug efflux pumps such as AcrAB of *E. coli* and MexAB-OprM of *P. aeruginosa*, which pump out a wide range of compounds including fluoroquinolones, chloramphenicol, and tetracycline (and often β -lactams and erythromycin) (17). Rather, the phenotype involved in the fluoroquinolone efflux process in *B. fragilis* resembled that produced by the NorA/Bmr-type transporter in gram-positive bacteria (13, 14, 23), a phenotype including strong resistance to fluoroquinolones, cationic dyes, and puromycin but no increase in resistance to β -lactams, erythromycin, or tetracycline.

Since the NorA/Bmr quinolone efflux pump is known to be inhibited by the alkaloids reserpine and rescinnamine (1, 13), we examined the effect of these compounds on the norfloxacin susceptibility of *B. fragilis*. MICs of norfloxacin were determined by microbroth dilution assay with both GAM broth and supplemented Trypticase soy broth (8). The inoculum was 10^6 cells/ml, and the results were read after 1 or 2 days of anaerobic incubation at 37°C. With all three strains used, the MIC was 16 $\mu\text{g}/\text{ml}$ in the absence of alkaloids, and it was decreased to 8 $\mu\text{g}/\text{ml}$ in the presence of 20 μg of reserpine or rescinnamine per ml. Although the differences were small, exactly the same results were obtained when the assay was repeated twice more. Furthermore, verapamil (100 $\mu\text{g}/\text{ml}$), another inhibitor of the NorA/Bmr pump (13), also decreased the norfloxacin MIC for all three strains to 8 $\mu\text{g}/\text{ml}$. When the steady-state accumulation levels of [^{14}C]norfloxacin were determined in YCH11 cells as described above, by adding reserpine (dissolved at 5 mg/ml in acetone) to a final concentration of 50 $\mu\text{g}/\text{ml}$, accumulation in the presence of reserpine was $32\% \pm 5\%$ higher than that in the control cell suspension that had received the same amount of acetone, to compensate for the effect of this solvent (four data sets). These differences in MICs and the accumulation were rather small but possibly reflect the fact that the inhibitors, large lipophilic molecules of about 500

to 600 Da, penetrate only slowly both through porin channels (16) and through bilayer regions of the outer membrane (20). In fact, we are not aware of any previous report that the NorA/Bmr-type transporter activity could be significantly inhibited by reserpine or verapamil in gram-negative bacterial cells, with their outer membrane barrier.

Several spontaneous mutants with increased resistance to norfloxacin were isolated by plating about 10^8 cells of ATCC 25285 on GAM agar plates containing 16 μg of norfloxacin per ml. One of these single-step mutants, NFX06, appeared to be more resistant to other agents and was examined in detail by determining precise MICs with gradient plates (5). As seen in Table 1, it became more resistant to ethidium bromide and puromycin, known substrates of the NorA/Bmr multidrug efflux pump (13), but the MICs of cefoxitin, tetracycline, and erythromycin remained essentially unchanged. Among fluoroquinolones, practically no change was seen with the more lipophilic sparflaxacin, again as reported for the NorA pump (23), and the MIC of cetyltrimethylammonium bromide, a substrate of NorA/Bmr (13), showed a modest increase. These results are consistent with those of the inhibitor studies and suggest that at least some of the fluoroquinolone efflux in *B. fragilis* occurs through a pump with a specificity and inhibitor profile resembling that of NorA/Bmr, an efflux pump with a more limited specificity than the AcrAB/MexAB-type pumps. This conclusion is also consistent with the observation that many *B. fragilis* strains are susceptible, in spite of their poorly permeable outer membrane (see reference 24), to erythromycin, chloramphenicol, and tetracycline, all excellent substrates of the pumps of the latter type. We are currently trying to identify the gene(s) responsible for the drug efflux activity in *B. fragilis* through transposon mutagenesis.

As far as we are aware, this is the first report of a drug efflux process in an obligate anaerobe, although such processes have been known for aerotolerant organisms with essentially anaerobic metabolism, such as wild-type strains of *Enterococcus hirae* (12) and *Enterococcus faecalis* (11) and drug-resistant mutants of *Lactococcus lactis* (3) and *Streptococcus pneumoniae* (2). The possible efflux of other drugs should be examined for *B. fragilis* and other obligate anaerobes, many of which are known to be resistant, either intrinsically or in an acquired manner, to a wide range of antibiotics (7, 21).

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TABLE 1. MICs for ATCC 25285 (parent) and its single-step mutant NFX06, determined by the gradient plate method^a

Drug	MIC ($\mu\text{g}/\text{ml}$)	
	ATCC 25285	NFX06
Norfloxacin	8.3	25.8
Ofloxacin	0.6	1.0
Sparflaxacin	0.4	0.5
Ethidium bromide	22	60
Puromycin	15	44
Cetyltrimethylammonium bromide	7.5	10.9
Cefoxitin	5.4	6.5
Chloramphenicol	1.9	2.1
Tetracycline	0.15	0.15
Erythromycin	0.2	0.2

^a Antibacterial agents were purchased from Sigma, except sparflaxacin, which was a gift from Dainippon Pharmaceuticals Co., Ltd.

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