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Contribution of Permeability and Sensitivity to Inhibition of DNA Synthesis in Determining Susceptibilities of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Alcaligenes faecalis* to Ciprofloxacin

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To examine the correlation between bacterial cell susceptibility to ciprofloxacin and the magnitude of uptake and cell target sensitivity, the relative contribution of ciprofloxacin accumulation in intact cells and its ability to inhibit DNA synthesis were investigated among strains of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Alcaligenes faecalis*. Uptake studies of [^{14}C]ciprofloxacin demonstrated diffusion kinetics for *P. aeruginosa* and *E. coli*. Ciprofloxacin was more readily removed from *E. coli* J53 and *A. faecalis* ATCC 19018 by washing than from *P. aeruginosa* PAO503. These results indicate that the process of cell accumulation is different for *P. aeruginosa* in that the drug is firmly bound at an extracellular site. Whatever the washing conditions, *A. faecalis* accumulated less drug than either of the other two bacteria. Magnesium chloride (10 mM) caused a substantial decrease of ciprofloxacin accumulated and an increase in the MIC, depending upon the nature of the medium. The addition of carbonyl cyanide *m*-chlorophenylhydrazone caused a variable increase in drug accumulated, depending on the medium and the bacterial strain. The concentration of ciprofloxacin required to obtain 50% inhibition (ID_{50}) of DNA synthesis for *P. aeruginosa* PAO503 and *A. faecalis* ATCC 19018 did not correlate with their corresponding MICs but did for *E. coli* J53. Treatment with EDTA decreased the ID_{50} of ciprofloxacin for *P. aeruginosa* PAO503 and its *gyrA* derivative by 5- and 2-fold, respectively, and decreased the ID_{50} for *E. coli* JB5R, a strain with a known decrease in OmpF, by 1.4-fold but did not decrease the ID_{50} for the normally susceptible *E. coli* J53. The ID_{50} for *P. aeruginosa* obtained after EDTA treatment or in ether-permeabilized cells was higher than that obtained for the other two strains. The protonophore carbonyl cyanide *m*-chlorophenylhydrazone prevented killing by low ciprofloxacin concentrations, but sodium azide did not. The latter compound did not enhance killing in association with inhibition of a previously described energy-dependent efflux of ciprofloxacin. These results are consistent with the major determinant of ciprofloxacin susceptibility being the susceptibility to inhibition of DNA synthesis in *E. coli*, poor permeability associated with the small pore size of *A. faecalis*, and a combination of low permeability and reduced susceptibility of DNA synthesis to inhibition for *P. aeruginosa*.

Studies have shown that there is a reasonable correlation between concentrations of some of the fluoroquinolone antimicrobial agents needed to inhibit DNA gyrase activity and the MICs for *Escherichia coli* (17) and *Staphylococcus aureus* (33) and for inhibition of early DNA synthesis of *E. coli* (7). However, this is not always so, for example, for *Micrococcus luteus* (39) and for several compounds examined as part of structure-function studies (11, 22). Thus, it is possible for certain fluoroquinolones and bacteria that factors other than the concentration needed to produce a critical degree of inhibition of DNA gyrase activity go into the determination of the MIC. One of these is the accumulation of the quinolone agent.

Several aspects of the process of accumulation of fluoroquinolones in *E. coli* have recently been reported. Uptake appears to be by a nonsaturable process that does not require cellular energy (3, 6, 8) and meets the characteristics of a simple diffusion system. Uptake across the outer membrane involves the porin route, as shown by a combination of reduced rates of diffusion of enoxacin (3), low-level resistance (19), and reduced uptake in porin-deficient strains (2, 14, 15). A second mechanism to cross the outer membrane by the self-promoted uptake pathway (13) has been described for fleroxacin (6). An energy-dependent efflux system in *E. coli* for norfloxacin, enoxacin, ofloxacin, and ciprofloxacin but not nalidixic acid has been reported by Cohen et al. (8). Ciprofloxacin and enoxacin can bind in a pH-dependent fashion to liposomes containing predominantly phospholipids, which could be the first step in diffusion of these compounds across the lipid bilayers of the inner membrane and, where involved, the outer membrane (2a). Thus, there are several possible ways that reduced accumulation could be effected and possibly modify susceptibility of a bacterium.

Bacterial resistance to fluoroquinolones thus far seems to be restricted to a cell target mutation affecting the A subunit of DNA gyrase and to a decrease of cell permeability. In *E. coli* K-12 strains, several investigators have isolated drug-resistant mutants associated with a reduction of drug uptake and with an alteration of the porin pathway of the outer membrane (1, 3, 14, 15). In *Pseudomonas aeruginosa*, a less well-defined mechanism seems to be involved. Norfloxacin-resistant (*nfxB*) and ciprofloxacin-resistant (*cfxB*) mutants associated with a possible alteration in outer membrane permeability have been reported (16, 28). Interestingly, an apparently new 54-kilodalton protein of unknown function was detected in the case of the *nfxB* mutant (16). Only one case of a fluoroquinolone-resistant *P. aeruginosa* isolate

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TABLE 1. Bacterial strains used

Bacterial strain	Source	Characteristics
Escherichia coli J53	B. J. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.	*E. coli* K-12 derivative, F⁻ *met63 pro-23*
E. coli SA1306	K. E. Sanderson, University of Calgary	*E. coli* J53 derivative, F⁻ *gyrA met63 pro-23*
E. coli JB5R	Our laboratory (3)	*E. coli* J53 derivative, F⁻ *gyrA met63 pro-23*, enoxacin resistant
Pseudomonas aeruginosa PAO503	B. W. Holloway, Department of Genetics, Monash University, Clayton, Victoria, Australia	*P. aeruginosa* PAO1 derivative, *met-9011*
P. aeruginosa PAO236	B. W. Holloway	PAO1 derivative, *gyrA hisIV ilvB/C lys-12 met28 proA trpCD*
P. aeruginosa PCC1989	A. J. Godfrey, University of Calgary	PAO503 derivative, *gyrA*, F116 transductant
Alcaligenes faecalis ATCC 19018	American Type Culture Collection, Rockville, Md.	Porin diameter reported smaller than that of *E. coli* porins (21)

involving an OmpF⁻ mutation has been reported (26). This mutant was isolated after antibiotic therapy with enoxacin. Clinical isolates of *P. aeruginosa* have been reported with apparent changes in outer membrane proteins but without examination of drug uptake (10). Determinations of a DNA gyrase mutation by gene mapping with transduction and by use of a DNA supercoiling assay with purified DNA gyrase subunits have been done with both *E. coli* (2, 15, 19, 31) and *P. aeruginosa* (16, 20, 28). Resistance in other bacteria has also been reported due to apparent reduced permeability or modified DNA gyrase or sometimes both (1, 12, 29, 30).

It has become evident that target affinity and cell permeability are important factors in the determination of the susceptibility phenotype of bacteria to fluoroquinolones. We investigated their relative contributions in *P. aeruginosa* and *Alcaligenes faecalis* and contrasted them with the better-characterized situation in *E. coli*. These bacterial species have different fluoroquinolone susceptibilities and different permeabilities and reported sizes of the porin in the outer membrane (21, 25, 37, 38).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are given in Table 1.

Reagents. Radiolabeled [^{14}C]ciprofloxacin (14.8 Ci/mol) was generously provided by A. Dalhoff, Bayer AG, Pharma Research Center, Wuppertal, Federal Republic of Germany. Ciprofloxacin hydrochloride was from Miles Pharmaceuticals, Toronto, Ontario, Canada; carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and EDTA (disodium salt) were from Sigma Chemical Co., St. Louis, Mo., and sodium azide (NaN_3) was from Fisher Scientific Co., Fairlawn, N.J.

Media and MICs. Brain heart infusion broth (BHIB) from Difco Laboratories, Detroit, Mich., was used for all viability counts. Nutrient broth (NB) was from BBL Microbiology Systems, Cockeysville, Md. Either BHIB or NB was used for uptake with or without the addition of 10 mM $MgCl_2$. MICs were determined in NB, BHIB, or Vogel-Bonner medium (35) with grown supplements as required added to a final concentration of 1 mM or in L broth (4) with a final inoculum of 10^5 to 10^6 CFU/ml. Incubation was for 18 h at 35°C. The basic medium used for DNA synthesis studies of ether-permeabilized cells was that described by Rella and Haas (27).

Uptake studies. Uptake studies were performed in NB and BHIB with and without CCCP and with and without 10 mM $MgCl_2$. Cells were grown overnight in the appropriate medium at 35°C with shaking. A sample of 5 (NB) or 1 (BHIB) ml of this culture was used to inoculate 50 ml of fresh medium. Cells were incubated until they reached the log phase of growth (optical density at 600 nm of 0.5). Cells were collected by centrifugation and suspended to produce an optical density of 30 to 40 in the same medium. For uptake measurements in the presence of CCCP, it was added at a final concentration of 20 μM 2 min before the addition of [^{14}C]ciprofloxacin. The latter was used at its original specific activity and added immediately before the zero time sample to a final concentration of 0.154 μg/ml unless otherwise stated. Uptake experiments were performed at 35°C in a block heater. At various times 80-μl samples were taken and treated immediately by one of the following wash procedures with the same broth used for uptake unless otherwise specified. For the minimal wash, the sample was pipetted onto a 2.5-cm Whatman GF/F microfiber glass filter (used for all wash procedures), and 4 ml of broth was pipetted directly onto the filter over the area occupied by the filtered cells. For the 2-ml wash, the sample was pipetted into 2 ml of broth and filtered, and a further 2 ml of broth was pipetted onto the filter as above. For the 10-ml wash, the sample was pipetted into 2 ml of broth and filtered, and a further 10 ml was pipetted onto the filter as above. Filters were dried and counted in Beckman Ready Safe liquid scintillation cocktail in a Beckman LS6800 scintillation counter. Uptake is reported as nanograms of ciprofloxacin per milligram (dry weight) of cells.

DNA inhibition studies. Three methods were used to measure DNA synthesis and its inhibition by ciprofloxacin. Chow et al. (7) recently reported a correlation between fluoroquinolone concentrations needed to cause early inhibition of DNA synthesis and MICs. We followed this approach exactly to determine concentrations of ciprofloxacin needed to inhibit early DNA synthesis of *E. coli* by 50% (ID_{50}), except that L broth was used. EDTA, when used, was added at the appropriate concentration 2 min before the addition of ciprofloxacin in this and the following procedure. The method of Benbrook and Miller (4) was used for *P. aeruginosa*. This organism and *A. faecalis* do not incorporate thymidine into DNA. Cells were grown in Vogel-Bonner medium with additions of the amino acids (1 mM) required by the auxotrophy of the strains. From preparations to which ciprofloxacin was added, samples were taken at 0 to 5 min of DNA synthesis before the addition of the drug. Immediately after the 5-min sample, various concentrations of ciprofloxacin were added, sufficient to produce a wide

range of inhibition of DNA synthesis by 15 min. Samples were taken at 5-min intervals up to 30 min. The extent of inhibition of DNA synthesis was determined at 20 and 25 min from time zero, which was the maximal point of inhibition of DNA synthesis.

In addition, the method of Rella and Haas (27) was used for all organisms to overcome a possible permeability barrier to ciprofloxacin and because toluene treatment has been shown to be unsatisfactory for the assay of DNA synthesis in *P. aeruginosa* (27). The method uses ether-permeabilized cells to allow penetration of [^{3}H]dTTP into bacteria. Ether permeabilization has also been used to measure DNA synthesis in *E. coli* (36). Bacterial cells were grown at 35°C to the log phase in BHIB. The cells were permeabilized by a 45-s ether treatment, which was reported not to affect DNA synthesis (27). The permeabilized cells were suspended in basic medium to a concentration of 2×10^{10}/ml, kept on ice, and used within 45 min of the treatment. The rate of DNA synthesis was determined by a modification of the method of Rella and Haas (27). The permeabilized cells were diluted (2×10^9/ml) in the prewarmed basic medium containing various concentrations of ciprofloxacin and incubated at 35°C. At various times, 0.1 ml of the suspension was added to 0.1 ml of prewarmed basic medium supplemented with 80 μM each dATP, dCTP, and dGTP (Boehringer Mannheim, Dorval, Quebec, Canada) and [^{3}H]dTTP (New England Nuclear Research Products, Lachine, Quebec, Canada; specific activity, 2 Ci/mmol), 0.4 mM NAD (Sigma), and 4 mM ATP (Sigma). After 2 min of pulse-labeling at 35°C, the reaction was terminated by adding 3 ml of cold 10% trichloracetic acid containing 0.05% thymidine (Sigma) and 0.1 M potassium pyrophosphate (Sigma). The samples were kept on ice for 30 min; the precipitates were collected on a Whatman GFC filter and washed three times with 10% trichloracetic acid, once with 0.01 M HCl, and once with 95% ethanol. The filters were dried and placed in 10 ml of toluene containing 4 g of dimethyl-1,4-bis-(5-phenyl-oxazolyl)benzene per liter. Filters from other methods were placed in Beckman Ready Safe liquid scintillation cocktail. The radioactivity was measured with a Beckman LS6800 liquid scintillation system. The rate of DNA synthesis was expressed as a percentage of the initial rate at time zero in the absence of drug. Maximal inhibition of DNA synthesis was about 60% for all three bacteria; this was arbitrarily set at 100% for determination of the ID_{50}.

The ID_{50} was defined as the concentration of drug required to produce 50% of the maximal inhibition of DNA synthesis. Excellent agreement was obtained between the results of different methods.

Cell viability studies. *E. coli* J53 was grown overnight in 10 ml of BHIB at 35°C with shaking. A sample of this culture was used to subculture 100 ml of fresh BHIB to an optical density at 600 nm of 0.1. Cells were grown until they reached the early log phase (optical density at 600 nm of about 0.4). Cultures were divided into four parts: (i) control with no additions, (ii) ciprofloxacin at either 0.05 or 0.1 μg/ml, (iii) either sodium azide at 1 mM or CCCP at 20 μM (final concentrations), and (iv) ciprofloxacin at 0.05 or 0.1 μg/ml plus either sodium azide or CCCP. After incubation periods of 0.5, 1, 1.5, and 2 h (and 4 h in some experiments), a sample of 1 ml of cells was removed, centrifuged at 15,000 × *g* for 4 min, and suspended in the same volume of sterile saline, and dilutions were prepared. Viable cells were counted after incubation for 16 h at 35°C on Trypticase soy agar (BBL). All counts were in triplicate, and each experiment is the average of two separate determinations (a total of six viability counts for each point in Fig. 7).

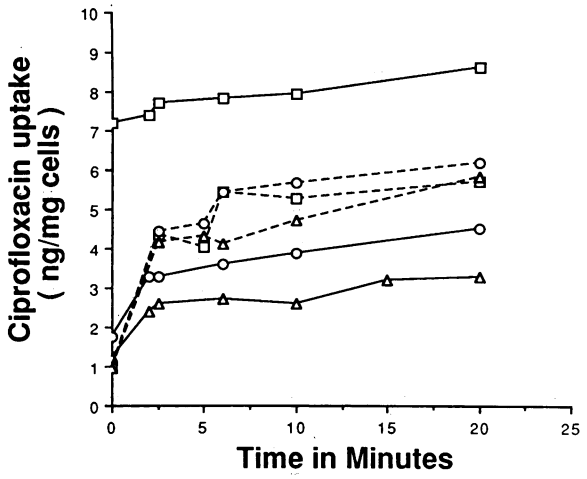

FIG. 1. Accumulation of ciprofloxacin at a concentration of 0.154 μg/ml with time at 35°C in NB by *E. coli* J53 (——) and *P. aeruginosa* PAO503 (– – –) under the following washing conditions: minimal wash (□), 2-ml wash (○), and 10-ml wash (△).

RESULTS

Ciprofloxacin uptake study. Assays of uptake of ciprofloxacin were performed with all of the washing conditions described in Materials and Methods and two different media with and without CCCP to compare the characteristics of the accumulation process for *P. aeruginosa* and *A. faecalis* with that for *E. coli*. All studies were done at least three times, and typical results are presented. The accumulation of ciprofloxacin by *E. coli* J53 and *P. aeruginosa* PAO503 in NB with different washing conditions is shown in Fig. 1. Ciprofloxacin accumulation by *P. aeruginosa* PAO503 was different from that seen with *E. coli* J53 (Fig. 1) or *A. faecalis* (data not shown but very similar to those for *E. coli* J53). The pattern under all conditions for *P. aeruginosa* was that of an obvious influx phase, even with minimal washing. The effect of washing with minimal or 2- or 10-ml amounts was markedly different from that for *E. coli* or *A. faecalis*, causing much less change in the pattern of uptake and a much smaller reduction in cell-associated drug. Increased washing did modestly decrease the amount associated at zero time and the subsequent influx phase, but the effect was much less than that with *E. coli*. Thus, although total accumulation was less for *P. aeruginosa* with minimal washing, accumulation was equal to or greater than that for *E. coli* when more extensive washing was used.

The addition of 10 mM $MgCl_2$ to NB produced a major decrease in the accumulation of ciprofloxacin for *E. coli* J53 (Fig. 2) and the other two bacterial strains (data not shown) under all washing conditions. Accumulation of ciprofloxacin was markedly reduced in BHIB for the three bacteria (data shown in Fig. 2 for *E. coli* J53). The addition of 10 mM $MgCl_2$ to BHIB had little effect (Fig. 2). The quantities of magnesium in NB and BHIB were found by flame photometry to be 0.06 and 0.17 mM, respectively.

Cohen et al. recently reported characteristics of an efflux system with everted membrane vesicles (8), indicating that fluoroquinolones are pumped out from an intracytoplasmic site in an *E. coli* strain. Under the conditions of this study only a small increase in cell-associated ciprofloxacin was

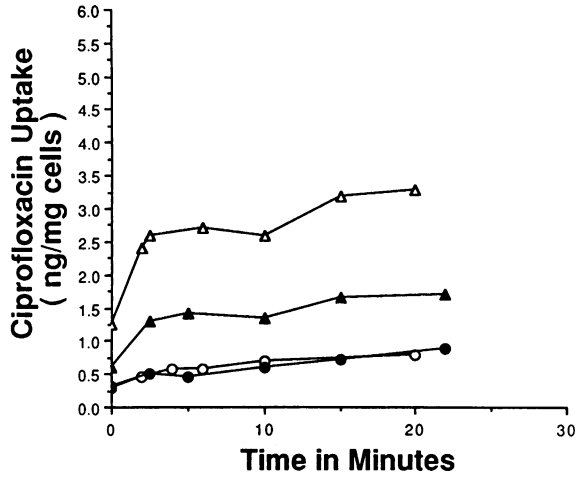

FIG. 2. Accumulation of ciprofloxacin (0.154 μg/ml) with time at 35°C in NB without (△) and with (▲) 10 mM $MgCl_2$ and in BHIB without (○) and with (●) 10 mM $MgCl_2$ by *E. coli* J53 under 10-ml washing conditions.

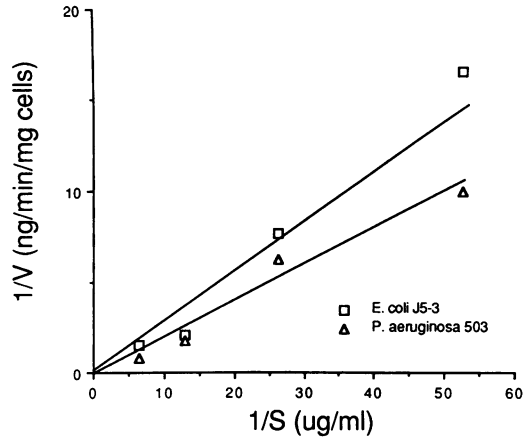

FIG. 4. Lineweaver-Burk plot of 1/*V* (nanograms of ciprofloxacin per minute per milligram of cells) based on uptake in NB at 35°C over the first 2 min versus 1/*S* (concentration of ciprofloxacin) for *E. coli* J53 and *P. aeruginosa* PAO503 with a 2-ml wash. Lines were drawn by using the least-squares method.

seen in BHIB for cells washed with 10 ml and treated with the protonophore CCCP (Fig. 3). For other washing conditions and the other bacteria, the effect of CCCP was to produce either no increase or a small increase in accumulation but never a decrease.

Ciprofloxacin uptake was not saturable in *E. coli* or *P. aeruginosa* (Fig. 4), as shown previously for enoxacin (3). The accumulation rate was somewhat greater for *P. aeruginosa* (note that Fig. 4 is a double-reciprocal plot so that greater uptake is seen as a lower slope) even at concentrations below the MIC for the organism in NB (Table 2). The plateau or equilibrium quantity accumulated after 10 min was also consistently greater for *P. aeruginosa* at 0.019, 0.038, and 0.077 μg/ml. The failure of CCCP, sodium azide, and sodium fluoride to decrease uptake (5) and rather to increase uptake supports a diffusion influx process for ciprofloxacin. The lack of energy requirement for accumulation has also been confirmed for fleroxacin and norfloxacin (6, 8). Since an energy-dependent efflux system has been shown for fluoroquinolones (8), it remains possible that this transport mechanism may work reversibly and cause some energy-dependent accumulation that is not demonstrable because of its low magnitude.

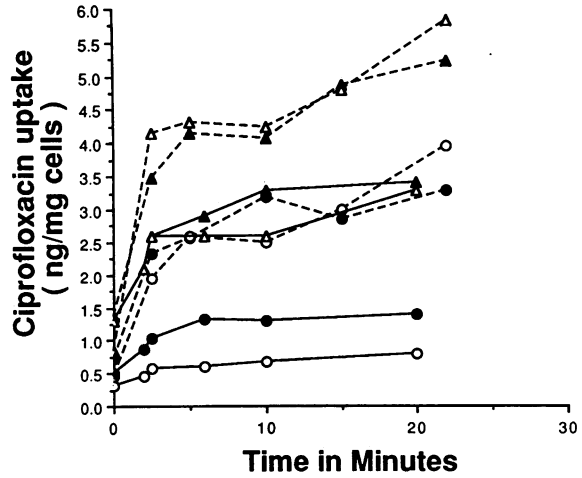

FIG. 3. Accumulation of ciprofloxacin (0.154 μg/ml) with time at 35°C by *E. coli* (——) and *P. aeruginosa* (– – –) under 10-ml washing conditions in NB without (△) and with (▲) CCCP and in BHIB without (○) and with (●) CCCP.

As shown previously for enoxacin (3), a *gyrA* mutation in *E. coli* did not influence the rate of accumulation or total accumulation of ciprofloxacin at equilibrium. Uptake by *E. coli* J53 and its *gyrA* derivative SA1306 was identical at 0.154 μg of ciprofloxacin per ml in NB and BHIB with and without CCCP and with minimal, 2-ml, and 10-ml washing steps (data not shown).

As noted, ciprofloxacin accumulation was also examined in *A. faecalis*, because this organism has been reported to have small outer membrane pores (21). Accumulation by *A. faecalis* was much less than that by *E. coli* J53 in both NB and BHIB; accumulation in the latter was extremely low (Fig. 5). $MgCl_2$ greatly depressed uptake in NB (data not shown) but had little effect on uptake in BHIB.

The MICs of ciprofloxacin in the various media used for uptake studies of the bacterial strains used in this study are shown in Table 2. In general the MICs were higher for *E. coli*, *P. aeruginosa*, and *A. faecalis* in BHIB and NB with 10 mM $MgCl_2$ than in media without $MgCl_2$. MICs were higher in NB for *P. aeruginosa* and *A. faecalis* than in BHIB, whereas the reverse was true for *E. coli*.

DNA synthesis inhibition study. We used three methods to measure early DNA synthesis in response to ciprofloxacin

TABLE 2. Susceptibility of *E. coli*, *P. aeruginosa*, and *A. faecalis* to ciprofloxacin

Bacterial strain	MIC (μg/ml) of ciprofloxacin					
	NB		BHIB		Vogel-Bonner	L broth
	−Mg	+Mg[a]	−Mg	+Mg[a]		
E. coli J53	0.006	0.25	0.025	0.15		0.031
P. aeruginosa PAO503	0.15	3	0.1	1.5	0.125	
A. faecalis ATCC 19018	0.95	3.75	0.25	0.95	0.5	
E. coli SA1306	0.078		0.31			0.125
E. coli JB5R						1

[a] Final concentration, 10 mM $MgCl_2$.

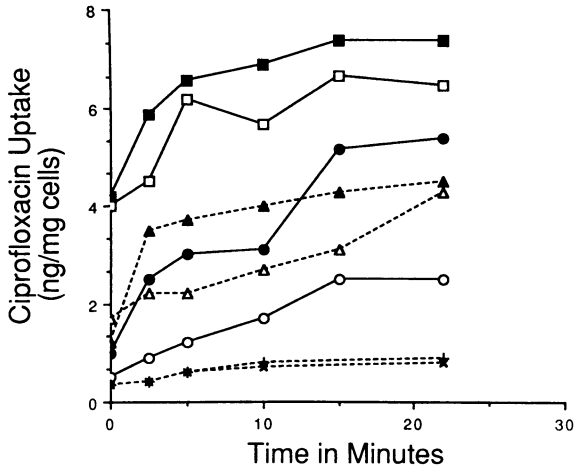

FIG. 5. Accumulation of ciprofloxacin (0.2 μg/ml) with time at 35°C with 2-ml washes in NB by *E. coli* J53 without (□) and with (■) 20 μM CCCP and *A. faecalis* ATCC 19018 without (△) and with (▲) CCCP and in BHIB by *E. coli* J53 without (○) and with (●) CCCP and *A. faecalis* ATCC 19018 without (×) and with (+) CCCP.

for *E. coli* and *P. aeruginosa* strains. A linear relationship existed between the extent of inhibition of DNA synthesis and ciprofloxacin concentration (Fig. 6). The ID_{50}s are given in Table 3. A reasonable correlation between ID_{50} and the MIC existed for *E. coli* J53 and its *gyrA* derivative, SA1306. These findings are in agreement with those reported for different strains by Chow et al. (7). We treated these *E. coli* strains and strain JB5R, a strain having a mutation causing a major reduction in the quantity of the F porin, with 10 mM EDTA. This concentration was selected by rendering cells susceptible to sodium dodecyl sulfate lysis but causing no reduction in the rate of DNA synthesis. No significant reduction of ID_{50} could be achieved for *E. coli* J53 (Fig. 6) or SA1306 (data not shown). Values consistently fell on the

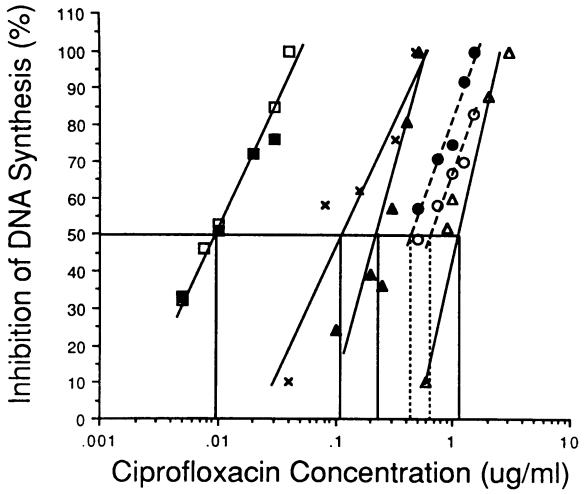

FIG. 6. Inhibition of DNA synthesis with different concentrations of ciprofloxacin by the method of Chow et al. (7; see Materials and Methods) for *E. coli* J53 without (□) or with (■) 10 mM EDTA, SA1306 (×), JB5R without (○) or with (●) 10 mM EDTA, and *P. aeruginosa* PAO503 using the method of Benbrook and Miller (4; see Materials and Methods) without (△) or with (▲) 2.5 mM EDTA. Vertical lines intersect the horizontal axis at the ID_{50}.

TABLE 3. Concentrations of ciprofloxacin required to inhibit DNA synthesis in *E. coli*, *P. aeruginosa*, and *A. faecalis* strains

Bacterial strain	ID_{50} (μg/ml)
E. coli J53	0.01 (0.03)[a]
E. coli J53 (EDTA treated)	0.01
E. coli JB5R	0.65 (0.5–1)
E. coli JB5R (EDTA treated)	0.45
E. coli SA1306	0.1 (0.125)
P. aeruginosa PAO503	1 (0.125)
P. aeruginosa PAO503 (EDTA treated)	0.2
P. aeruginosa PCC1989	2 (1)
P. aeruginosa PCC1989 (EDTA treated)	1
A. faecalis ATCC 19018	0.015 (0.25–0.95)

[a] Numbers within parentheses are MICs in the medium used for DNA synthesis studies except for *E. coli* JB5R and *A. faecalis*, for which they are the MIC ranges in the three media used. ID_{50}s were obtained by the methods of Chow et al. (7) for *E. coli*, Benbrook and Miller (4) for *P. aeruginosa*, and Rella and Haas (27) for *A. faecalis*.

same best-fit line whether or not EDTA was included in the system. In the case of *E. coli* JB5R, with a known defect in outer membrane permeability, a small reduction in ID_{50} was achieved (Fig. 6 and Table 3). However, the value obtained with EDTA was closer to that of *E. coli* SA1306, its parental strain with a known *gyrA* mutation.

The results with *P. aeruginosa* PAO503 demonstrated that the ID_{50} was about eightfold greater than the MIC. Measurement of DNA synthesis was carried out with 2.5 mM EDTA due to the greater sensitivity of *P. aeruginosa* than *E. coli* to EDTA. This EDTA concentration caused no reduction in the rate of DNA synthesis but allowed cell lysis by 0.05% sodium dodecyl sulfate. In the presence of 2.5 mM EDTA there was a fivefold decrease in the ID_{50} (Fig. 6 and Table 3). The value obtained is near the MIC of 0.125 μg/ml. For PCC1989, a *gyrA* transductant of PAO503, a similar set of correlations was observed (Table 3). The ID_{50} for *A. faecalis* was determined by examining DNA synthesis by the method of Rella and Haas (27). A maximum of 60% inhibition of DNA synthesis for *A. faecalis* could be obtained with ciprofloxacin concentrations 10 or more times the ID_{50}; this extent of inhibition was set as 100%. The ID_{50}s obtained by this method correlated with the susceptibility of DNA gyrase in control strains, with the following values obtained: *E. coli* J53, 0.015 μg/ml; *E. coli* SA1306, 0.15 μg/ml; *P. aeruginosa* PAO503, 0.1 μg/ml; and *P. aeruginosa* PAO236, 4.0 μg/ml (MIC, 4 μg/ml). The ID_{50} obtained for *A. faecalis* ATCC 19018 was 0.015 μg/ml. Thus, the susceptibility of DNA gyrase of this organism was far below the MIC in any medium. These studies also confirmed the lower ID_{50} for *P. aeruginosa* in permeabilized cells compared with whole cells untreated with EDTA (Fig. 6 and Table 3) and showed reasonable agreement for the ID_{50}s between the two different methods of permeabilizing cells (0.1 versus 0.2 μg/ml).

Effect of inhibitors of energy generation and transduction on the bactericidal activity of ciprofloxacin. We examined the effect of two inhibitors of energy generation and transduction on the capability of ciprofloxacin to kill *E. coli* at low concentrations of ciprofloxacin. Sodium azide (1 mM), an inhibitor of electron transport (Fig. 7), did not affect the extent of cell killing at the concentrations used. CCCP, a protonophore, on the contrary had a profound effect on cell killing by ciprofloxacin, reducing it by greater than 2 log units (Fig. 7B). The effect of CCCP (20 mM) on the action of ciprofloxacin is consistent with that described previously for nalidixic acid with the protonophore 2,4-dinitrophenol (9). In spite of the inhibition of efflux of ciprofloxacin from *E. coli*

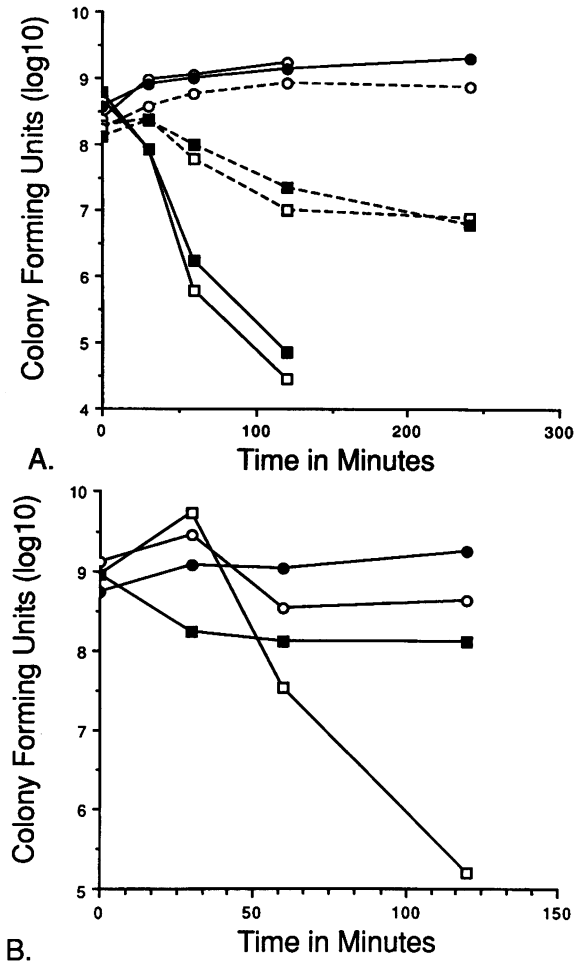

FIG. 7. Viability of *E. coli* J53 in BHIB with time at different ciprofloxacin concentrations in the presence and absence of 20 μM CCCP and 1 mM NaN_3. (A) Symbols: O----O, control, no additions; O——O and ●——●, NaN_3 only added; □----□, ciprofloxacin (0.05 μg/ml); ■----■, ciprofloxacin (0.05 μg/ml) and NaN_3; □——□, ciprofloxacin (0.1 μg/ml); ■——■, ciprofloxacin (0.1 μg/ml) and NaN_3. (B) Symbols: O, control, no additions; ●, CCCP only added; □, ciprofloxacin (0.1 μg/ml); ■, ciprofloxacin (0.1 μg/ml) plus CCCP.

by sodium azide (5), its presence did not enhance killing at 0.05 or 0.1 μg of ciprofloxacin per ml. These findings suggest that the efflux of drug at these concentrations is not a limiting factor in the extent of viability loss produced by ciprofloxacin. A possible but, we feel, improbable alternative explanation is that azide prevents increased killing in some as-yet-unrecognized manner.

DISCUSSION

The inhibitory effect of ciprofloxacin on DNA synthesis was chosen as a measurement of the drug affinity for the target A subunit of the DNA gyrase, since the exact mechanism of the lethal action of fluoroquinolones remains unknown. For *E. coli* J53 and SA1306, there was good correspondence between MICs and ID_{50}s. Thus, for these two related strains the major determinant of cell susceptibility is the affinity of the drug target. However, our study revealed a significant difference between the ID_{50} for inhibition of DNA synthesis and the MICs for *P. aeruginosa* PAO503 and *A. faecalis* ATCC 19018. In the case of *A. faecalis*, a major factor accounting for the discrepancy was the lower accumulation of ciprofloxacin in either of the media used compared with the *E. coli* and *P. aeruginosa* strains studied. This conclusion was supported by the ID_{50}s for DNA synthesis of *A. faecalis* obtained in ether-permeabilized cells; the ID_{50}s were from about 15- to 60-fold lower than the MICs obtained in three different media. Thus, we conclude that the outer membrane of this organism is a barrier to ciprofloxacin entry, a conclusion consistent with the small diameter of the outer membrane pores of *A. faecalis* (21).

P. aeruginosa PAO503 demonstrated a more complex situation; PAO503 had an apparently lower target affinity for ciprofloxacin, in that the ID_{50} for DNA synthesis was higher than those for *E. coli* J53 and *A. faecalis* even after treatment of cells with EDTA (or in ether-permeabilized cells). It is possible that EDTA treatment of *P. aeruginosa* PAO503 did not totally overcome a barrier effect of the outer membrane, but the values obtained with this method and ether-treated cells were in reasonable agreement. EDTA treatment did produce substantial decreases in the ID_{50} of ciprofloxacin for *P. aeruginosa* PAO503 and PCC1989, indicating that the outer membrane is a normal barrier to effective entry of the drug to the target. This conclusion is also supported by the result obtained with ether-permeabilized cells. That interpretation is further supported by the decrease of ID_{50} with EDTA for an OmpF-deficient strain, *E. coli* JB5R. These observations indicate that the higher MIC for *P. aeruginosa* is likely due to a combination of lower permeability and target drug affinity than for the *E. coli* strain and lower target drug affinity than for the *A. faecalis* strain. However, the ID_{50} for the *Pseudomonas* strains was above the MIC in nonpermeabilized cells, suggesting that less than 50% inhibition of DNA synthesis is necessary to produce inhibition of *P. aeruginosa*, in contrast to *E. coli* and *A. faecalis*.

The reason why *P. aeruginosa* accumulates drug effectively in uptake studies but has a permeability barrier, as shown by the EDTA treatment, is not clear at this point, but an explanation is suggested. The accumulation profile with time and after different washing procedures was distinct from that of *E. coli*. Ciprofloxacin accumulation always showed time-dependent kinetics whatever the wash procedure, and the effect of washing was much reduced compared with that in *E. coli*. These observations and the effect of EDTA on ID_{50} suggest that the drug is trapped in an extracytoplasmic compartment, possibly the periplasm or outer membrane. It is possible that ciprofloxacin enters the outer membrane en route to the periplasm by the so-called self-promoted nonporin uptake pathway (6, 13). Once in the periplasm, poor permeability of the porin pathway in *P. aeruginosa* could result in ciprofloxacin being trapped so that washing steps are ineffectual in its removal. Magnesium could act by directly chelating to ciprofloxacin, making the complex a poor substrate for the nonporin pathway, by inhibiting the interaction with the nonporin pathway or by reducing permeation across the cytoplasmic membrane. This explanation is supported by the observations of Chapman and Georgopapadakou (6) that fleroxacin interacts with the outer membrane of *E. coli* by a nonporin pathway as well as by the porin pathway and chelates magnesium. It is likely that ciprofloxacin has enough similarities with fleroxacin that it interacts with the phosphate-rich lipopolysaccharide of *P. aeruginosa* (24). Thus, time-dependent uptake may involve non-porin-mediated accumulation through the outer membrane of all bacteria but more so in those with phosphate-rich lipopolysaccharide, like *P. aeruginosa*, as well as influx

through porins and diffusion through the inner membrane. In the case of *P. aeruginosa*, accumulation was observed well below the MIC, showing that uptake is not effective at reaching the cytoplasmic target at these concentrations. At concentrations closer to the MIC, either the lipopolysaccharide route (self-promoted uptake pathway) (13) or the porin pathway or both allow ciprofloxacin to enter the cell. This view is consistent with our observation that *P. aeruginosa* PAO503 undergoes some lysis (30 to 40% reduction in A_{600}) after 2 h at 35°C in NB with 10 mM $MgCl_2$ and with 0.05% sodium dodecyl sulfate when exposed to 0.2 to 0.4 μg of ciprofloxacin per ml but not at the much lower concentrations of 0.038 and 0.077 μg/ml, at which effective cellular accumulation was shown (Fig. 4), or in the absence of sodium dodecyl sulfate.

Information from this and other studies on the accumulation of fluoroquinolones makes it clear that several factors go into the total accumulation at equilibrium (3, 6, 8, 14). These include cellular binding presumably to components of the outer membrane as well as the inner membrane, diffusion through the outer and inner membranes, and active efflux. Binding to DNA (32, 34) has been proposed; the contribution of this component to the total accumulation is unclear, although no difference was observed between a DNA gyrase mutant and its parent for enoxacin (3) and as shown here for ciprofloxacin. Under most conditions of our accumulation studies, the addition of CCCP and the apparent inhibition of efflux caused small increases in total ciprofloxacin accumulated. The activity of the apparent efflux system seems to vary substantially depending on uptake conditions and the type of bacteria. This observation is consistent with our previous findings that the amount of increase of uptake with CCCP is greater when a lower ratio of cells to medium is used in the uptake procedure (5). Cellular metabolism would be expected to be more active under these conditions, and the effect of a protonophore would be expected to be greater. Under conditions of inhibitor use with *E. coli* J53 (5), in which enhancement of accumulation of ciprofloxacin was most active, we could not show that cells were more susceptible to low concentrations of ciprofloxacin. We feel that the most likely interpretation of this result is that efflux is not the factor limiting susceptibility under our test conditions.

Our results indicate that in the case of *E. coli* J53 nearly all components of accumulated drug can be washed off or out of the cell. One of these components seems to be a relatively weak binding at the cell surface, which is readily removed by slightly more stringent washing conditions, as shown by the effects of the 2-ml wash compared with the minimal wash. With more extensive washing with 10 ml, a small, apparently more sequestrated fraction of the drug was retained which was about 1 ng/mg of cells at an uptake concentration of 0.154 μg/ml in both NB and BHIB. Under these conditions the cellular concentration per milliliter of cell water would be approximately 0.6 μg/ml, or about 4 times the extracellular concentration. Since all characteristics of the uptake process are those of simple diffusion, the intracellular concentration is most likely explained by binding inside the cell or firmly to an extracellular site. As noted above, *P. aeruginosa* is in marked contrast in that washing has a limited effect, whereas *A. faecalis* resembles *E. coli* in this respect.

Total accumulation of ciprofloxacin could be dramatically reduced in all three bacteria by the addition of magnesium to the magnesium-poor medium NB. This reduction in accumulation correlated with an elevation of MICs for all three organisms. One explanation of the increase in MICs is reduced drug binding to and entry into cells. For all the bacteria, the addition of Mg to BHIB caused a substantial increase in MICs, indicating that even a modest reduction of ciprofloxacin accumulation is important or that the effect is at a different level, possibly acting on DNA synthesis. Hooper and colleagues have reported reduced binding of norfloxacin to *E. coli* by 1 to 14 mM magnesium as well as by changing the pH from 7 to 6. These changes correlated with reduced susceptibility to norfloxacin (18). The protonophore CCCP prevented killing of *E. coli* J53 by ciprofloxacin, but an inhibitor of ATPase and electron transport did not. Thus, as in the case of the older quinolone nalidixic acid, a proton gradient seems necessary to allow bacterial killing mediated by a fluoroquinolone. The killing capability of ciprofloxacin has been reported to be not abolished by inhibitors of RNA and protein synthesis in *E. coli*; however, this may not be true in all bacteria, since killing of *S. aureus* has been reported to be affected by such agents (23). Whether the effect of dissipation of the proton gradient is mediated indirectly through effects on protein or RNA synthesis or directly on an as-yet-unidentified mechanism of killing is not clear.

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