

Comparison of Recalcitrance to Ciprofloxacin and Levofloxacin Exhibited by *Pseudomonas aeruginosa* Biofilms Displaying Rapid-Transport Characteristics

JULIA D. VRANY,^{1,2†} PHILIP S. STEWART,^{1,2} AND PETER A. SUCI^{1*}

Center for Biofilm Engineering¹ and Chemical Engineering Department,²
Montana State University, Bozeman, Montana 59717

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Attenuated total reflection Fourier transform infrared spectroscopy was used to measure transport of the fluoroquinolones (FQs) ciprofloxacin and levofloxacin into *Pseudomonas aeruginosa* biofilms. Biofilms were exposed to each FQ at dose levels of 100, 250, and 500 $\mu\text{g/ml}$ for 30 min. A mathematical transport model was used to extract the diffusion coefficient, binding site density, and adsorption and desorption rates for each experiment. Recalcitrance of the biofilms toward each FQ was evaluated by comparison of efficacies with planktonic bacteria. By this criterion, biofilms were found to exhibit more recalcitrance toward levofloxacin than ciprofloxacin under the experimental conditions. These results cannot be explained by the more hindered transport of levofloxacin, implicating the domination of physiological factors.

Biofilm recalcitrance toward components of the host immune system and antimicrobial agents has been implicated as a factor contributing to the persistence of infections associated with a number of medical conditions. These include periodontal disease (30), osteomyelitis (22), lung obstruction accompanying cystic fibrosis (12), biliary system gallstone formation (41), and biomaterial-centered bacteremias (5, 10, 43). Recalcitrance of bacterial biofilms to antimicrobial agents has been demonstrated repeatedly in *in vitro* studies (1–3, 6, 15, 28, 35, 37). However, the underlying reasons for biofilm recalcitrance remain obscure. Relevant hypotheses can be categorized as transport or physiology related. Biofilm bacteria are typically enclosed in a matrix of biopolymers (extracellular polymeric substances [EPS]) which may prevent the bactericidal dose from reaching certain regions (3, 20, 23, 24, 34). Alternatively, biofilm recalcitrance may originate primarily from special physiological attributes which bacteria assume during biofilm life (17).

A broad objective of the research described here is to develop relationships between chemical properties of antimicrobial agents and their efficacies, specifically against biofilm organisms. The fluoroquinolones (FQs) have inspired this type of study (8, 13, 18, 25, 26), although there has not been a focus on biofilms. In this study, ciprofloxacin and levofloxacin were compared with respect to transport characteristics within the biofilms and with respect to the recalcitrance exhibited by the biofilms to each FQ. The kinetics of transport of the antimicrobial agents to the base of the biofilms was measured directly by attenuated total reflection Fourier transform infrared (ATR/FT-IR) spectroscopy.

MATERIALS AND METHODS

Organism, culture conditions, and antimicrobial agents. *Pseudomonas aeruginosa* ERC-1 was obtained from the Center for Biofilm Engineering (Montana State University—Bozeman). The culture medium contained 0.28 g of D-glucose

per liter, 0.017 g of CaCl_2 per liter, 0.24 g of NH_4Cl per liter, 0.051 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per liter, 0.1 g of K_2HPO_4 (anhydrous) per liter, and 0.6 ml of Wolfe's mineral salts solution (pH was adjusted to 7.1 to 7.2 with HCl). Water was deionized, carbon filtered, and subjected to reverse osmosis. Concentrated solutions of glucose, CaCl_2 , and Wolfe's mineral salts solution were filter sterilized and added to the balance of medium, which had been sterilized by autoclaving. The composition of the Wolfe's mineral salts was 1.5 g of nitrolic acid per liter, 0.5 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ per liter, 1.0 g of NaCl per liter, 0.1 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ per liter, 0.1 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ per liter, 0.1 g of CaCl_2 per liter, 0.1 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ per liter, 0.01 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per liter, 0.01 g of $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ per liter, 0.01 g of H_3BO_3 per liter, and 0.01 g of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ per liter. Ciprofloxacin powder was obtained from Bayer (Levokusen, Germany), ciprofloxacin aqueous stock solutions (10 mg/ml) were obtained from Miles Canada (Etobicoke, Canada), and levofloxacin powder was supplied by the R. W. Johnson Pharmaceutical Research Institute (Spring House, Pa.). Ciprofloxacin powder was less available than the aqueous solution. It was used to verify the FQ content (mass per volume) of the aqueous solution (since the powder could be weighed precisely) with a calibration curve obtained by UV-visible spectroscopy. It was also used in the biofilm experiments. The IR spectra of equimolar preparations made from the aqueous stock solution and an aqueous solution of the powder were identical.

Flow system, biofilm culturing, and FQ dosing. A schematic diagram of the flow system is shown in Fig. 1. For each experiment, biofilms were cultured on internal reflection elements (IREs) in each of the two flow chambers. Tubing was silicone Masterflex tubing (Cole-Parmer Instruments, Niles, Ill.), except for noreprene Masterflex tubing, which preceded and followed bifurcating connections for 2 cm, and Teflon tubing, which was used as a 3-cm-long leader to the cylindrical flow chamber. Masterflex peristaltic pumps (Cole-Parmer Instruments) controlled the flow rates, which were measured by counting drops in glass break tubes. A continuous culture (dilution rate, 0.1 h^{-1}) provided the inoculum to each flow chamber through a T-shaped connection (0.5 ml/min) to the main lines. The flow rate through the chambers was 3.2 ml/min. After 20 h, the inoculum was turned off and fresh medium continued to be supplied to each flow chamber for an additional 30 h. At 50 h, the FQs were introduced into their respective flow chambers at a rate of 3.2 ml/min. An FQ was added to each reservoir immediately before dosing. Preceding this step, culture medium was transferred aseptically from the main reservoir to the FQ reservoir to ensure that the medium containing the FQ was identical (except for the presence of FQ) to the culture medium. For dosing, medium containing FQ was channeled to the flow chambers by removing and relocating a clamp. For each experiment, one flow chamber was dosed with ciprofloxacin and the other was dosed with levofloxacin. Biofilms were dosed for 30 min and then rinsed for 60 min with fresh, sterile medium containing no FQ. For the control experiments, the same procedure was followed but FQs were not used.

ATR/FT-IR spectroscopy. Technical details of the ATR/FT-IR technique for measuring transport of an antimicrobial agent to the base of a biofilm have been described previously (40). Significant features of the methodology are illustrated in Fig. 2. IR spectra of bacterial colonization and FQ transport were collected with a Perkin-Elmer model 1800 FT-IR spectrophotometer. This double-beam instrument enabled a direct, simultaneous comparison of biofilms cultured in two flow chambers consisting of Micro-Circle cells designed to enclose a cylindrical

* Corresponding author. Mailing address: Center for Biofilm Engineering, 409 Cobleigh Hall, Montana State University, Bozeman, MT 59717. Phone: (406) 994-1732. Fax: (406) 994-6098.

† Present address: Menlo Care Inc., Johnson & Johnson Co., Menlo Park, CA 94025.

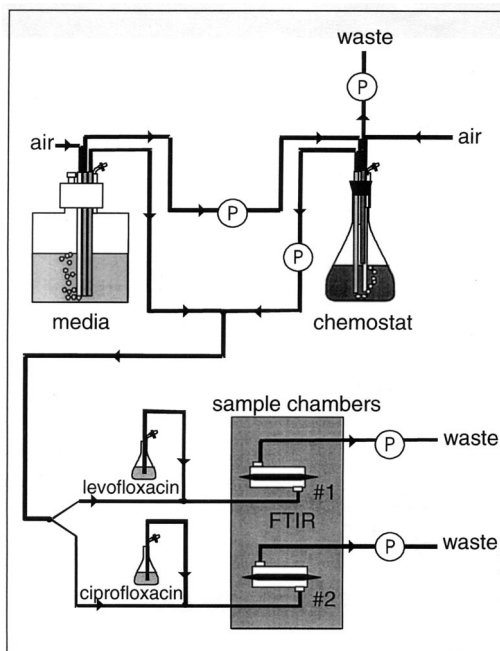


FIG. 1. Experimental setup for culturing two biofilms and measuring FQ transport by ATR/FT-IR spectroscopy. P, peristaltic pump.

germanium (Ge) IRE (Spectra Tech, Stamford, Conn.). Dimensions and hydrodynamic characteristics of this flow chamber have been reported previously (40).

IREs were cleaned before each experiment by sonication in a base bath of isopropyl alcohol saturated with potassium hydroxide (10 min) followed by sonication in a water bath (10 min). All water for cleaning was ultrafiltered (Nanopure system; Barnstead-ThermoLyne, Dubuque, Iowa). IREs were then gently scrubbed with Micro (International Products Corporation, Burlington, N.J.) with cotton swabs, rinsed in a stream of water, and sonicated three times in water baths (10 min each) followed by sonication in a 95% high-pressure liquid chromatography-grade ethanol bath (10 min). The flow chamber was cleaned by sonication in the base bath (10 min). It was then rinsed under tap water for 3 min and sonicated in a 95% ethanol bath (10 min). All tubing and flasks were cleaned with 5% nitric acid and sterilized by autoclaving at 121°C for 30 min. The flow chamber containing the IRE was sterilized immediately before beginning the experiment by filling the chamber with 70% ethanol and soaking it for 3 h.

IR spectra of ciprofloxacin and levofloxacin are shown in Fig. 3. Bands used to monitor FQ transport are indicated. These bands were chosen on the basis of two criteria: they yielded a linear correspondence between concentration and band

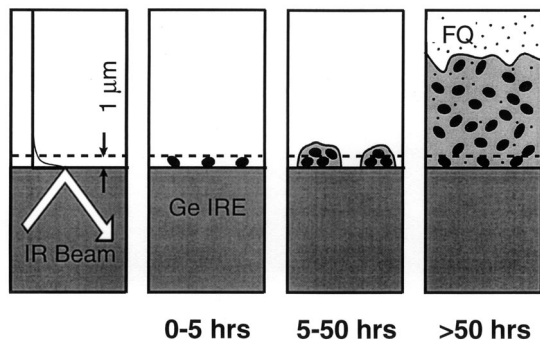


FIG. 2. ATR/FT-IR methodology (40). IR absorbance is measured in a region within the aqueous medium confined to a distance of approximately 1 μm from the Ge substratum. Cell attachment and lateral colonization of the substratum can be monitored with the bacterial IR signal (lateral growth curve). The unique IR spectrum of an FQ which appears in the region adjacent to the substratum (base of the biofilm) can be detected if the bacterial (background) spectrum does not change too drastically. Egg-shaped symbols, dots, and dark and light shaded areas represent cells, FQ, IRE, and EPS matrix, respectively.

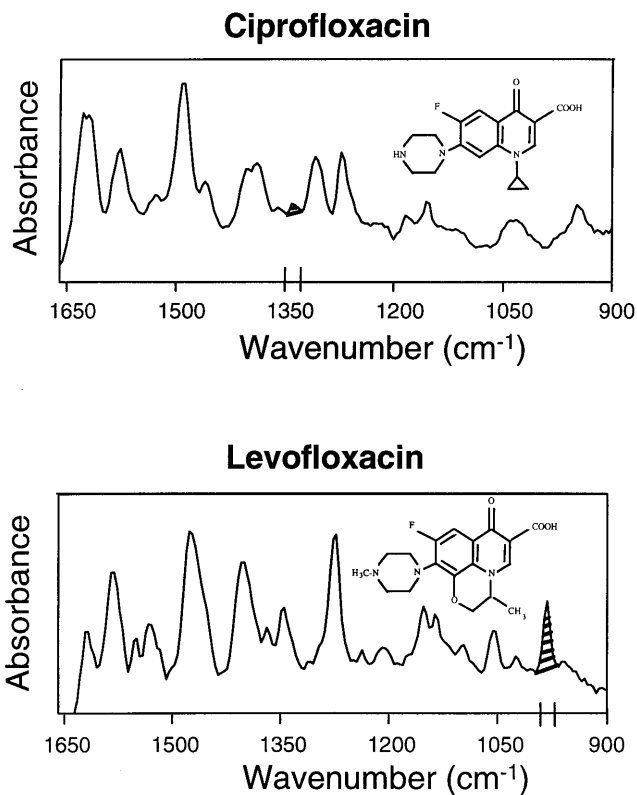


FIG. 3. IR spectra of ciprofloxacin and levofloxacin indicating bands used to monitor FQ transport. FQ structures are shown.

area for concentrations in the range of 0 to 500 $\mu\text{g/ml}$ in the culture medium and they appeared in spectral regions which were relatively free of interference from the bacterial background spectrum. These two criteria excluded many of the most prominent bands.

FT-IR spectra were acquired each hour, beginning at the time of the flow chamber inoculum. Immediately before dosing, an FT-IR spectrum was acquired from each flow chamber. This was used as the background to compute difference spectra during dosing. Spectra were acquired every 36.6 s during dosing and the subsequent rinse. Features of the dosed FQ appeared clearly in difference spectra. Areas of bands indicated in Fig. 3 were used to construct transport data curves. Experiments were performed at room temperature (21°C).

Evaluation of efficacy. Efficacies were assessed for both planktonic and biofilm bacteria by comparing log reductions, i.e., the negative logs of the ratios of viable cells to total cells [$-\log(v/t)$ values]. Viability was determined by counting CFU on R2A agar (catalog no. 1826-17-1; Difco Laboratories, Detroit, Mich.). Total cell counts were made on cells fixed with 2% glutaraldehyde, stained with 0.05% acridine orange, and filtered onto 25-mm-diameter polycarbonate membranes (pore size, 0.2 μm ; Nucleopore). Total cells were enumerated by epifluorescence microscopy.

Biofilms were removed from the IRE by scraping the entire surface with a sterile, disposable cell scraper (Fisher Scientific) into 5 ml of the dilution broth (0.1 g of K_2HPO_4 per liter, pH 7.1 to 7.2). The sample was sonicated for 6 min to break up any cell aggregates before the dilution series was made. Reported $-\log(v/t)$ values are the means of duplicate experiments.

For planktonic efficacy measurements, 10-ml samples containing approximately 5×10^7 cells/ml were drawn from a batch culture near the end of exponential growth phase and exposed to FQs at 100, 250, or 500 $\mu\text{g/ml}$ for 30 min. The culture medium was identical to that used for biofilm experiments. Samples were then spun down for 10 min in a Sorvall Instruments model RC-3B refrigerated centrifuge at 20°C at $5,000 \times g$ and resuspended in fresh medium. This process was repeated for a total of four rinses in culture medium without an FQ, requiring 60 min, to simulate the rinse in the biofilm experiment. Each centrifugation and resuspension resulted in an approximately 1:20 dilution of the FQ. The subsequent protocol, used to evaluate efficacy of the final cell pellet, was identical to that for biofilms after scraping. Reported $-\log(v/t)$ values are the means of triplicate experiments.

Standard errors of the mean were pooled for the total of five (three planktonic and two biofilm) experiments performed at each concentration with each FQ. These pooled standard errors were used to estimate P values by the two-sample t test.

MIC and MBC tests were performed by standard methods (29). Aliquots of a continuous bacterial culture in glucose minimal salts medium containing 10^5 CFU/ml were treated with concentrations of FQs ranging from 0.06 to 32 $\mu\text{g/ml}$ for 28 h. For the MIC, visual turbidity was noted and the optical density was recorded at 600 nm with a spectrophotometer. Nonturbid aliquots were then plated to check for viable cells.

Examination of biofilm morphology. The morphologies of thin sections of intact biofilm were examined by sampling Ge coupons placed in the effluent tubing approximately 30 cm downstream of the flow chamber. Biofilm from these coupons was cryosectioned or examined by confocal scanning laser microscopy (CSLM) with the Bio-Rad MRC600 system. For cryoembedding, the colonized IRE was coated in Tissue-Tek OCT compound (catalog no. 4583; Sukura Fine-tek, Torrance, Calif.) on a slab of dry ice. Sections 5 μm thick were made with a cryostat (Reichert-Jung Cryocut 1800; Leica) and fixed in a solution of 10% formaldehyde, 5% glacial acetic acid, and 85% ethanol. The sections were then stained with a 0.0004% solution of acridine orange (Sigma, St. Louis, Mo.) and examined by epifluorescence microscopy. For the CSLM examination, the Ge coupons were fixed with a 2% glutaraldehyde solution, rinsed with a trizma base solution (0.12% trizma base, 0.9% NaCl [pH 7.4]), and stained with propidium iodide. Images were taken at various planes within the biofilm, and Z sections were constructed by the software package to provide images of biofilm cross sections.

Extraction of parameters by modeling. Models of transport data were constructed with Aquasim (38). Processes included were diffusion, adsorption, and desorption. The biofilm was assumed to be a thin sheet which was partitioned into lamellae parallel to the substratum. The following coupled differential equations were solved (numerically) progressively through time for each lamella:

$$\frac{\partial C_A}{\partial t} = D \frac{\partial^2 C_A}{\partial z^2} - K_S C_S C_A + K_D C_{AS} \quad (1)$$

$$\frac{\partial C_{AS}}{\partial t} = K_S C_S C_A - K_D C_{AS} \quad (2)$$

$$\frac{\partial C_S}{\partial t} = -\frac{\partial C_{AS}}{\partial t} \quad (3)$$

where the variables are C_A (the concentration of unbound FQ), C_S (the free binding site density), C_{AS} (the concentration of the bound FQ), t (time), and z (the distance perpendicular to the interface). Parameters which were optimized by a least-squares fit to the data curves were D (the diffusion coefficient), K_S and K_D (rate constants for adsorption and desorption, respectively), and S_O (binding site density [see equation 4]). No weighting scheme was used (homoscedasticity). FQ concentration was set at zero initially ($t = 0$) within a region estimated to be the diffusion boundary layer (approximately 100 μm from the substratum) for the laminar flow conditions of the experiment and at the dosing level outside this region. Binding site density was constrained to be saturable at S_O by the formula

$$C_S + C_{AS} = S_O \quad (4)$$

RESULTS

Biofilm characterization. Lateral growth curves obtained for each biofilm by ATR/FT-IR spectroscopy are shown in Fig. 4 (see Fig. 2 for details of the methodology). Curves obtained for experiments performed simultaneously in the two flow chambers of the FT-IR instrument are, in general, more similar than those obtained in different experiments. This is the case even though conditions were maintained as identical as possible between experiments. This greater similarity between biofilms cultured simultaneously is also reflected in the data for total cell counts, which are slightly correlated for experiments performed in the two flow chambers in parallel ($r^2 = 0.715$; slope of orthogonal least-squares line, 1.32). Cryosections revealed that bacteria were fairly homogeneously distributed in a sheet approximately 16 μm thick (16.3 ± 9.5 μm for 100 measurements). Biofilm morphology as revealed by sections of CSLM images was consistent with that obtained by the cryosectioning technique, though the former images were less resolved. Differences in thickness could not be correlated with exposure to FQ (i.e., differences in morphology were greater within than between biofilms).

Transport of FQ in biofilms. Figure 5 shows transport curves for ciprofloxacin and levofloxacin to the base of *P. aeruginosa* biofilms. For each FQ the dosing level was 250 $\mu\text{g/ml}$. The curves exhibit some characteristics which are typical of all the

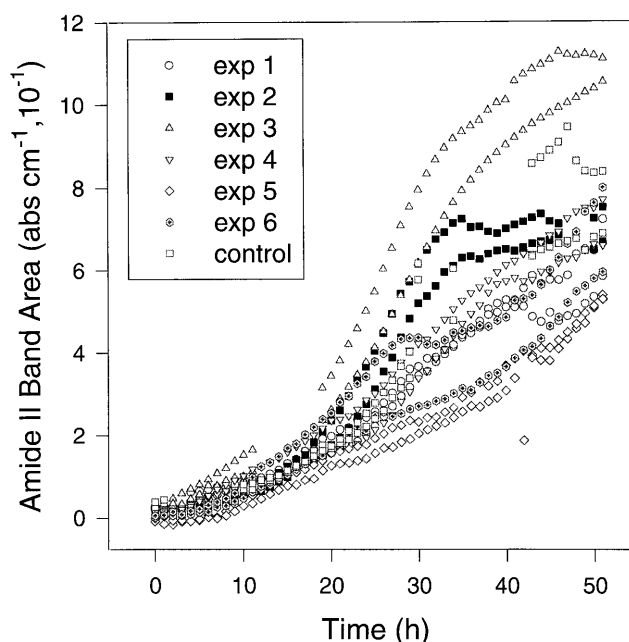


FIG. 4. Lateral growth curves obtained by plotting the area of the amide II band of the bacterial spectrum versus time during biofilm colonization of the substratum. Curves obtained from biofilms cultured simultaneously are indicated with the same symbol. abs, absorbance; exp, experiment.

transport data curves obtained with these biofilms under the experimental conditions described. There is an initial, almost immediate rise to the dosing level (indicated on the figure) followed by a slower increase which exceeds the dosing level. The initial decrease in concentration at the base of the film, beginning with the rinse period, is also fairly rapid, followed by a more gradual decrease at later times.

The rate of approach to the dosing level attained at the base of the biofilm has been quantified in Table 1 for experiments performed with ciprofloxacin and levofloxacin in duplicate at dosing levels of 100, 250 and 500 $\mu\text{g/ml}$. Within the first 5 min, concentrations for all the experiments except one attain over 60% of the dosing level.

The transport data curves were fit by using a transport model as described above. The biofilm was assumed to be 16 μm thick. Figure 5 illustrates that the process of diffusion alone cannot account for the shape of the data curve. The curve is more accurately fit when the processes of adsorption and desorption are incorporated. Table 1 gives parameter values for the best fits of the data curves for each experiment. In some experiments, the IR absorbances from the bacterial background signal overwhelmed the FQ IR spectral absorbances to such an extent that the transport data curves appeared physically unrealistic; for example, curves had negative values or large inflection points (i.e., were wavy). These curves produced fits which were obviously inadequate in some sense, and parameter fits for these experiments are not included in Table 1.

In order to obtain a statistical assessment of these data, the parameter values for all experiments were grouped for each FQ (Table 1). This grouping is meaningful, since the value of each parameter is, in principle, independent of the FQ dosing level used in a particular experiment. The standard errors are quite high, and a t test reveals that at a 0.05 level, none of the parameter value means differ significantly for the two FQs.

Antimicrobial efficacy. In order to obtain a measure of recalcitrance exhibited by biofilms toward the two FQs, plank-

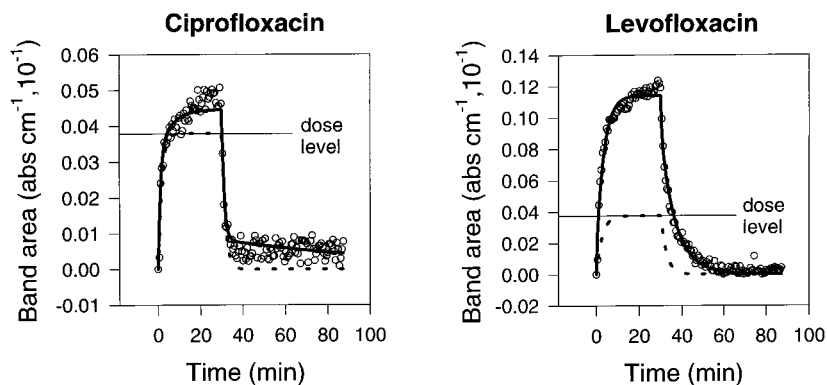


FIG. 5. Data curves for ciprofloxacin and levofloxacin transport into *P. aeruginosa* biofilms. The solid lines indicate best fits with equations 1 to 4. The broken lines indicate best fits by the process of diffusion only. Band areas estimated for dosing levels are indicated. abs, absorbance.

tonic bacteria were exposed to approximately the same doses of FQs for the same periods as the biofilms. The density of planktonic bacteria was chosen so that the total cell/FQ mass ratios were equivalent for the biofilm and planktonic experiments. This did not ensure the equivalence of the kinetics of the adsorption and desorption reactions of each FQ with the biofilm and planktonic bacteria but did increase the possibility that equilibrium-adsorbed amounts would be equivalent.

The mean $-\log(v/t)$ values for FQ treatments of planktonic and biofilm bacteria are presented in Fig. 6. Mean $-\log(v/t)$ values for both ciprofloxacin and levofloxacin are greater for planktonic bacteria than for biofilms at all dose levels. For levofloxacin, this difference between the mean $-\log(v/t)$ for planktonic bacteria and that for biofilms is significant at the 0.01 level for all dose levels. For ciprofloxacin, this difference is significant at the 0.05 level only for the 500- $\mu\text{g}/\text{ml}$ dose.

For biofilm experiments with both FQs and for the planktonic experiment with levofloxacin, the $-\log(v/t)$ means exhibit an increase as the FQ dose level is increased. This trend is significant only for the levofloxacin planktonic experiments

($P = 0.002$, by one-way analysis of variance). For biofilm experiments, the mean difference in $-\log(v/t)$ for each experiment done in parallel in the two flow chambers is 0.291 (ciprofloxacin minus levofloxacin). A paired t test indicates that this difference, which suggests that ciprofloxacin is slightly more efficacious than levofloxacin against the biofilms, is significant at the 0.05 level (0.046).

Results of standard tests for MICs and MBCs are as follows: the MICs of ciprofloxacin and levofloxacin were 0.25 and 0.50 $\mu\text{g}/\text{ml}$, respectively, and their MBCs were 2.0 and 4.0 $\mu\text{g}/\text{ml}$, respectively.

DISCUSSION

The recalcitrance of biofilms toward antimicrobial agents has conventionally been assessed by a comparison: efficacy against the sessile form has been referenced to efficacy against the planktonic form for a given species (1–3, 14, 15, 28, 35, 37). Thus, greater recalcitrance is associated with a lesser extent of relative killing of biofilm organisms compared to their plank-

TABLE 1. Parameter fits^a to transport data curves

FQ	Expt ^b	Dose ($\mu\text{g}/\text{ml}$)	% Bulk (5 min) ^c	D ($\text{cm}^2 \text{s}^{-1}$) (10^{-6})	K_S/K_D (M^{-1}) (10^4)	$K_S S_O$ (s^{-1}) (10^{-4})	S_O (M) (10^{-4}) ^d
Ciprofloxacin	1	100	76	1.18	1.21	3.89	4.35
	2	100	74	4.43	2.42	4.09	5.65
	3	250	115	0.521	1.74	6.29	1.91
	4	250	102	1.14	2.22	16.0	3.63
	5	500	65	0.415	28.8	0.349	0.286
	6	500	48	— ^e	—	—	—
Mean (SE)				1.54 (0.74)	7.27 (5.38)	6.13 (2.60)	3.17 (0.94)
Levofloxacin	1	100	79	6.92	162	3.42	10.3
	2	100	79	0.498	10.1	4.75	14.6
	3	250	281	1.58	0.146	160	34.9
	4	250	78	—	—	—	—
	5	500	140	1.32	0.020	15.0	16.1
	6	500	100	1.10	0.015	7.30	55.5
Mean (SE)				2.28 (1.20)	34.5 (31.9)	38.3 (31.0)	26.3 (8.4)

^a Some parameters are reported as combinations for comparison with data from other sources (see Discussion). P values for the data are as follows: 0.61 (D), 0.45 (K_S/K_D), 0.36 (K_S/S_O), and 0.053 (S_O). The two-sample t test was used for equal means.

^b Experiments with the same number were performed in parallel.

^c Percentage of bulk fluid concentration measured at the base of the biofilm at 5 min after FQ inoculation.

^d All concentrations are referenced to the FQ; therefore, this molar concentration assumes implicitly a 1:1 binding (FQ-binding site).

^e The data curve indicated interference from the bacterial background spectrum.

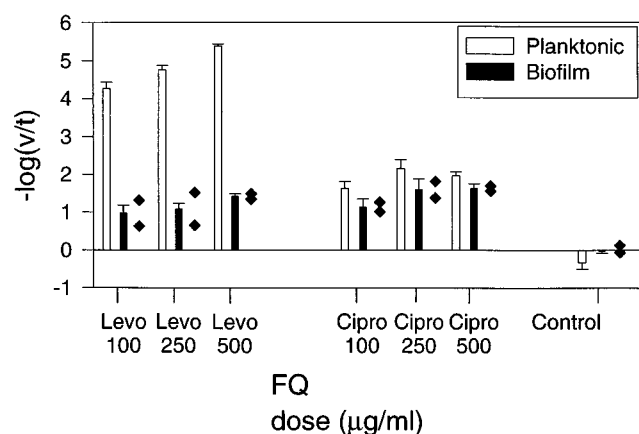


FIG. 6. FQ efficacies indicated as mean $-\log(v/t)$ values for planktonic cultures and biofilms of *P. aeruginosa*. Error bars are the standard errors of the means, which were pooled for planktonic and biofilm experiments performed for each concentration of each FQ. For biofilm experiments results of duplicate experiments are indicated (diamonds). Levo, levofloxacin; Cipro, ciprofloxacin.

tonic counterparts. By this criterion, biofilms tested here exhibited less recalcitrance toward ciprofloxacin than toward levofloxacin. This finding suggests that substitution of a number of chemical groups into the FQ basic structure (Fig. 3) can significantly influence the recalcitrance exhibited by a bacterial biofilm. One caveat is that in order to use ATR/FT-IR spectroscopy to characterize transport, relatively high concentrations of the FQ had to be used. This factor may have induced a paradoxical response (9). In addition, the length of the exposure period was constrained to be relatively short (30 min), because changes in the large bacterial absorbances obscure the FQ IR bands and the magnitude of this background interference increases with time.

ATR/FT-IR spectroscopic measurements indicated that the *P. aeruginosa* biofilms did not pose a significant transport barrier to either ciprofloxacin or levofloxacin. Not only was the dosing level attained or exceeded within the 30-min dosing period in all experiments, but the rate of transport to the base of the biofilms was rapid. These results suggest that differences in levels of recalcitrance to the two FQs originated primarily from interactions involving biofilm characteristics unrelated to transport. Other studies support the conclusion that hindered transport plays only a minor role in biofilm recalcitrance (33, 39). It has been proposed that special physiological conditions inherent to biofilms, for example, reduced growth rates resulting from nutrient limitation (17), are responsible for recalcitrance. It is unlikely that bacteria in the biofilms in this study were nutrient limited considering their slight thickness, the rich composition of the medium, and the residence time of the flow chamber (approximately 1 s). It may be that bacteria within biofilms which are not nutrient limited grow at a reduced rate compared to their planktonic counterparts due to the relatively high density of cells.

There is evidence that for some biofilms, the replication rate is reduced while the metabolic rates for specific pathways are maintained at high levels (36). FQs disrupt transcription as well as DNA replication (44). Competition between these two processes has been proposed as a mechanism underlying the paradoxical response for the quinolone nalidixic acid (9). The differential responses of biofilm and planktonic bacteria to ciprofloxacin and levofloxacin may originate from differences in the relative rates of the FQs' interference with transcription and DNA replication.

Results obtained previously indicated that *P. aeruginosa* biofilms impede the transport of ciprofloxacin significantly (40). Cryosections revealed that biofilms cultured on Ge fragments for experiments described here were less thick than those obtained previously. Both lateral growth curves and total cell counts indicated that the biofilms were also less dense. Experimental conditions which may account for differences in biofilm characteristics are (i) a 10-times-lower phosphate concentration in the culture medium (0.1 g/liter) and (ii) a different Ge cleaning protocol (no alumina paste was used). These previously reported results suggest that for some biofilms, hindered transport may play a role in biofilm recalcitrance. The possible extent of organizational complexity of some bacterial biofilms has only recently been recognized. For example, biofilm architecture may be structured to allow efficient perfusion of nutrients (11, 45) or exclusion of antagonists (27). This complexity and capacity for self-organization implies an adaptive capability which may alter hypotheses about the exclusive role of either transport or physiology in determining biofilm recalcitrance.

Parameter values presented in Table 1 have large standard errors. Thus, there is no statistically significant difference between any of the transport parameters measured for the two FQs presented in Table 1 at the 0.05 level, even though the means are quite disparate. The large standard errors result from applying a semirealistic quantitative model to a biofilm system. For a continuous planktonic culture, growth conditions can be reproduced extremely accurately. The added complexity which originates from association with a surface makes it more difficult to obtain uniform, reproducible growth conditions within a biofilm. The variance in measurements originating from biofilm complexity is amplified by an attempt to apply a semirealistic quantitative model to the data. There is a trade-off between making the model realistic and limiting the number of floating parameters.

Although the parameter values presented in Table 1 have large standard errors, the parameter value means are reasonable quantities, indicating that the means may converge near values listed in the table with enough repetitions of the experiment. The mean diffusion coefficients for ciprofloxacin ($1.54 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$) and levofloxacin ($2.28 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$) are 31 and 45%, respectively, of those for diffusion in water ($4.91 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for ciprofloxacin and $5.01 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for levofloxacin). This result is within the range expected for reduction of the diffusion coefficients of antimicrobial agents of comparable sizes in an EPS matrix (19, 20, 34). The K_s/K_D ratio is an affinity coefficient for binding of the FQ to biofilm components. These coefficients have been reported for specific binding of small molecules (ligands) to their binding sites in proteins. The means for ciprofloxacin ($7.27 \times 10^4 \text{ M}^{-1}$) and levofloxacin ($34.5 \times 10^4 \text{ M}^{-1}$) are on the low end of the range for specific protein-ligand interactions (21, 42), which might be expected for relatively high-affinity, nonspecific binding.

The accumulation of FQs in the biofilm above the dosing levels may be a result of adsorption to the EPS matrix. However, accumulation of FQs in whole cells to concentrations above the external dosing level is common for planktonic cultures, with the rate of penetration being proportional to the external concentration (4, 7, 16, 31, 32). The assumption that adsorption can be accounted for exclusively by penetration into, and accumulation within, whole cells leads to the interpretation of $K_s S_O$ (Table 1) as the ratio of the rate of penetration to external FQ concentration. For planktonic cells, these data have been reported for penetration of pefloxacin into *Staphylococcus aureus* (16). The rate of penetration into cells is expressed in units of mass of pefloxacin per mass of cells

per time. After converting this to concentration (mass/aqueous volume) per time by using the average cell density in the biofilms (0.1 g/ml at $3 \times 10^{11} \text{ cells/ml}$), one obtains a proportionality between the rate of penetration and the external antimicrobial agent concentration of 0.255 s^{-1} . The $K_S S_O$ parameter means for ciprofloxacin ($6.13 \times 10^{-4} \text{ s}^{-1}$) and levofloxacin ($38.3 \times 10^{-4} \text{ s}^{-1}$) are 3 to 2 orders of magnitude lower. This comparison suggests that the adsorption behavior of the FQs to the biofilm components can be accounted for exclusively by relatively slow penetration and accumulation within cells.

In conclusion, a model of the transport data indicates that levofloxacin may penetrate into the biofilm bacteria more rapidly, and to a greater extent, than ciprofloxacin. These superior transport characteristics did not enhance levofloxacin's efficacy against biofilms used in this study. However, the efficacy of levofloxacin against biofilms in which transport is significantly hindered may be exceptional. Since transport into biofilms was rapid for both FQs, hypotheses to account for differences in levels of recalcitrance observed for this biofilm system can be limited to those based on physiological differences between biofilm bacteria and their planktonic counterparts. This may provide the basis for future studies targeted to investigate physiological causes of biofilm recalcitrance.

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