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Transport of Pefloxacin across the Bacterial Cytoplasmic Membrane in Quinolone-Susceptible *Staphylococcus aureus*

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Binding to phospholipids, uptake by simple diffusion, and an energy-dependent, carrier-mediated efflux are thought to characterize interactions between fluoroquinolones and bacterial cytoplasmic membranes. Here, we have found that an endogenous active efflux is unlikely in quinolone-susceptible *Staphylococcus aureus*. The protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), increased pefloxacin uptake in different membrane systems under conditions which excluded carrier-mediated transport, i.e., in bacterial cells at 4°C and in protein-free phosphatidylglycerol liposomes. When plotted as a function of outer pH, the CCCP effect, both in *S. aureus* cells and in phosphatidylglycerol liposomes, correlated with pefloxacin labeling of everted *S. aureus* membrane vesicles, with all three profiles showing maximal effect at an acidic pH. So the CCCP effect may result not from inhibition of the proton motive force, as previously thought, but rather from acidification of the intramembrane space by the protonophore, leading to enhanced binding of the positive pefloxacin species to the inner leaflet of the bilayer. Moreover, antistaphylococcal potency and uptake profiles of pefloxacin in *S. aureus* and phosphatidylglycerol liposomes, assayed as a function of outer pH, peaked at a neutral pH. These observations suggest that zwitterionic and positive quinolone species are responsible for diffusion through and binding to the cytoplasmic membrane, respectively.

To reach their primary target, i.e., DNA gyrase (7), quinolone antibiotics must cross the cytoplasmic membrane. The initiating step of transmembrane passage is probably binding to phospholipids, as suggested from previous liposome experiments (1). Uptake of quinolones is thought to be by simple diffusion, since the incorporation of enoxacin is neither saturable nor reduced by inhibitors of electron transport or glycolytically derived energy (2). Uptake is decreased by acidification (10), and the rate of uptake is more rapid at 37 than at 4°C (2). The existence of an endogenous active efflux has been suggested previously (3–6, 22), notably because quinolone uptake was increased by protonophores like carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and dinitrophenol. This was interpreted to be the result of the inhibition of an efflux generated by a proton motive force. However, no quinolone carrier proteins have yet been found. Here, we have studied uptake of radiolabeled pefloxacin, a fluoroquinolone, by whole bacterial cells, everted membrane vesicles, and protein-free liposomes composed of zwitterionic or negatively charged phospholipids. Particular attention was given to *Staphylococcus aureus*, for which interference from an outer membrane can be excluded. In contrast to their precursor, nalidixic acid, most of the modern fluoroquinolones display two proton-binding sites (Fig. 1) which result in four protonation species: H_2Q^+, Q^-, the zwitterionic species HQ^{+-}, and the uncharged species HQ^0. These species were given special attention, since extracellular pH plays a crucial role in steady-state accumulation of quinolones.

MATERIALS AND METHODS

Strains and chemicals. We used the quinolone-susceptible strain *S. aureus* NCTC 8325-4. Antibiotics were kindly provided by their respective manufacturers. [^{14}C]pefloxacin (specific activity, 23.6 mCi/mmol) was a gift from Rhône-Poulenc, Vitry, France. Lysostaphine, DNase, CCCP, ammonium salts of phosphatidylglycerol, and phosphatidylcholine, the last two originating from egg yolk, were purchased from Sigma Chemicals, St. Louis, Mo.

pK_a values and molecular species of quinolones. Acid base titrations were performed with a digital Metrohm 654 pH meter (Metrohm, Herisau, Switzerland). Aliquots (50 ml) of 0.5 mM quinolone–2 mM HCl solutions were titrated by 50 mM NaOH at 25°C. pK_a values were determined as the means of three experiments. The relative concentrations of the four quinolone species have been calculated by using the following equations:

$$HQ^{+-} = \frac{[H^+]\,K_1}{([H^+] + K_1)([H^+] + K_2)}$$

$$HQ^0 = \frac{[H^+]\,K_2}{([H^+] + K_1)([H^+] + K_2)}$$

$$H_2Q^+ = \frac{[H^+]^2}{([H^+] + K_1)([H^+] + K_2)}$$

$$Q^- = \frac{K_1\,K_2}{([H^+] + K_1)([H^+] + K_2)}$$

where H^+ equals 10^{-pH}, K_1 equals $10^{-pK_{a1}}$, and K_2 equals $10^{-pK_{a2}}$.

pH-dependent susceptibility testing. MICs of pefloxacin for *S. aureus* were determined at 37°C and after 24 h by a microdilution method with Mueller-Hinton broth supplemented with 200 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)–200 mM NaCl buffered to pH 5.2, 5.9, or 6.6 or 200 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–200 mM NaCl buffered to pH 7.0, 7.4, or 8.5. Final inocula of 5×10^5 CFU/ml were used.

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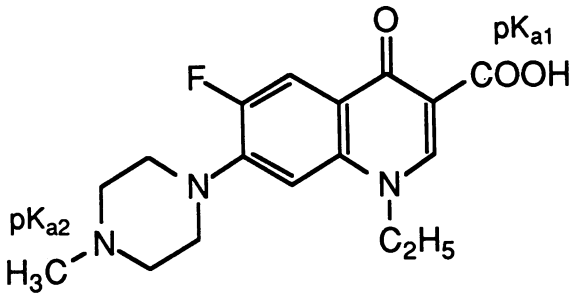

PEFLOXACIN

FIG. 1. Chemical structure of pefloxacin. The two protonation sites are indicated by pK_{a1} and pK_{a2}.

Preparation of everted membrane vesicles from *S. aureus*. Everted membranes are inside-out vesicles derived from the cytoplasmic membrane that create an acidic interior pH gradient by an influx of protons when an energy source is supplied. Mid-log-phase cells of *S. aureus* in 4.1 M sodium chloride were treated with lysostaphine (36 U/ml) for 1 h at 37°C (12). Protoplasts were collected by centrifugation for 12 min at 23,000 × *g* and resuspended in 10 mM HEPES, pH 7.5. All subsequent steps were conducted at 4°C. After 50 μg of DNase per ml was added, the solution was gently homogenized with a glass rod for 5 min. Everted membrane vesicles were obtained with a French pressure cell at 10,000 lb/in^2. Unbroken cells and protoplasts were removed by centrifugation for 20 min at 15,000 × *g*. Vesicles were collected by centrifugation for 1 h at 100,000 × *g* and then were washed and stored at −70°C at a protein concentration of 10 mg/ml. To determine the orientation of vesicles, ATPase activity was assayed as described previously (22). P_i was measured by a photometric assay (22). The vesicles showed 96% of the total ATPase to be orientated outside the everted membrane vesicles. The protein concentration was determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.). Bovine serum albumin was used as the standard.

Preparation of liposomes. Portions (20 mg) of commercially available chloroform solutions of phosphatidylglycerol (10 mg/ml) and phosphatidylcholine (100 mg/ml) were dried under a nitrogen gas stream in previously acid-washed conical tubes. Traces of solvent were eliminated under vacuum. For fixing the interior pH of the liposomes, pellets were suspended at 10 mg of phospholipid per ml in 50 mM HEPES–50 mM NaCl (pH 7.8) to simulate the internal pH of the cells. Liposomes were treated by 40 sonication pulses of 15 s (output, 6 to 7; duty cycle, 50%) (Branson Sonifier B 30; Branson Instruments, Danbury, Conn.) separated by intervals of 30 s. After being incubated for 2 h at room temperature, which permitted the annealing process to come to completion, liposomes were diluted to 0.2 mg of phospholipid per ml in 50 mM MES–50 mM NaCl (pH 5.2, 5.9, or 6.6) or 50 mM HEPES–50 mM NaCl (pH 7.0, 7.4, or 8.5), and the determination of [^{14}C]pefloxacin uptake was immediately performed.

Pefloxacin uptake by whole cells. Bacterial cells were grown at 37°C in Luria broth to an optical density at 600 nm of 0.65. Cells were harvested by centrifugation at 5,000 × *g* for 30 min, resuspended in 50 mM MES–50 mM NaCl (pH 5.2, 5.9, or 6.6) or 50 mM HEPES–50 mM NaCl (pH 7.0, 7.4, or 8.5) at a ratio of 30 ml of buffer per 1 g of drained pellet, and equilibrated for 10 min at 30°C. [^{14}C]pefloxacin was added at a final concentration of 2.5 μg/ml at time zero. Aliquots were filtered at the times indicated in Results through 0.45-μm-pore-size glass microfiber filters (Whatman, Ltd., Maidstone, Kent, United Kingdom) and were washed twice with 2 ml of 0.9% NaCl. Filtration and washings lasted less than 10 s. Filters were dried for 30 min at 80°C, and radioactivity was determined with a Beckman LS-7500 counter in 2 ml of Lumagel SB (Lumac/3M bv, Schaesberg, The Netherlands). The amount of [^{14}C]pefloxacin retained by the filters when cells were omitted was determined and subtracted for each pH value. The dry weight of cells was determined as previously described (13). Uptake was expressed as nanograms of [^{14}C]pefloxacin per milligram (dry weight) of bacteria.

The time courses of uptake at 30 and 4°C were determined with cells suspended in 50 mM MES–50 mM NaCl, pH 6.6. For kinetic studies performed with *S. aureus* at 30°C, appropriate amounts of unlabeled pefloxacin were premixed with [^{14}C]pefloxacin, and samples were taken 5 s after the addition of pefloxacin. Kinetics were determined in the absence or presence of CCCP (when added, CCCP was added 3 min before time zero). To assess the pH dependency of whole-cell uptake of *S. aureus*, plateau values were determined at 30°C after 3 min of incubation with [^{14}C]pefloxacin. In some experiments, CCCP (0.1 mM) in an alcoholic solution was added 3 min before [^{14}C]pefloxacin. Controls were run in the presence of the same amount of ethanol (1% final concentration). The differences between the plateau values in the presence and absence of CCCP were calculated for each pH as CCCP induced increases in [^{14}C]pefloxacin uptake. The CCCP effect was determined at both 30 and 4°C and at pH 6.6.

Pefloxacin uptake by everted membrane vesicles. Everted membrane vesicles were diluted in 50 mM MES–50 mM NaCl (pH 5.2, 5.9, or 6.6) or 50 mM HEPES–50 mM NaCl (pH 7.0, 7.4, or 8.5) to a final concentration of 0.2 mg of protein per ml. pH-dependent [^{14}C]pefloxacin binding was determined at 30°C as described above for whole cells.

Pefloxacin uptake by liposomes. The pH dependence of [^{14}C]pefloxacin accumulation and the CCCP effect in liposomes were determined at 30°C as described above for whole cells.

RESULTS

pK_a values and molecular species of quinolones. Proton dissociation constants allowed the calculation of relative amounts of the zwitterionic (HQ^{+-}) and uncharged (HQ^0) species at pH 7.4 (Table 1). In all cases, more than 50% of the total was represented by the zwitterionic species, i.e., variations were less than twofold when compounds were compared. In contrast, marked differences were found for the uncharged species, with higher and lower values varying up to 3 orders of magnitude.

[^{14}C]pefloxacin uptake and CCCP effect. Preliminary experiments with dinitrophenol uncoupler were performed, and changes in pefloxacin uptake similar to those with CCCP were observed. However, because of the high dinitrophenol concentrations needed (millimolar range), we preferred to use CCCP. A CCCP-induced increase in [^{14}C]pefloxacin uptake by intact *S. aureus* cells was observed at pH 6.6 but not at pH 7.4 (Fig. 4A). Therefore, we determined pefloxacin accumulation as a function of time at pH 6.6 when comparing the effect of CCCP at 30°C with that of CCCP at 4°C.

TABLE 1. pK_a values and percentages of zwitterionic and uncharged quinolone species at pH 7.4

Quinolone	pK_{a1}	pK_{a2}	% HQ^{+-}	% HQ^0
Pefloxacin[a]	6.27	7.55	54.5	2.86
Difloxacin	6.06	7.63	60.2	1.62
Fleroxacin	5.46	8.10	82.4	0.19
Norfloxacin	6.30	8.38	83.7	0.70
Ofloxacin	6.05	8.22	86.9	0.56
Sparfloxacin[a]	6.23	8.57	87.7	0.40
A 56620[a]	6.19	8.49	92.5	0.44
Temafloxacin	5.61	8.75	94.2	0.24
Enoxacin	6.31	8.69	95.1	0.37
Ciprofloxacin	6.09	8.74	95.6	0.20
Lomefloxacin	5.82	9.30	96.2	0.03
PD 117558[a]	5.92	10.54	99.9	0.002
Nalidixic acid	5.95	NA[b]	NA	3.43
WIN-57273[a]	6.15	NA	NA	5.32

[a] pK_a values were determined in this laboratory. pK_a values for the other quinolones were adopted from reference 17.
[b] NA, not applicable.

When CCCP was added, [^{14}C]pefloxacin binding increased to nearly the same extent at both temperatures (Fig. 2).

Uptake kinetic studies at 30°C and pH 6.6 showed that quinolone accumulation increased linearly in *S. aureus* cells with increasing concentrations of [^{14}C]pefloxacin of up to 1 mM, both in the presence and in the absence of CCCP (Fig. 3A). This is a strong indication of nonsaturable uptake within this concentration range. However, the lowest concentration of pefloxacin used (10 μM) may have been already above the saturation threshold. The predicted kinetics for either carrier-mediated influx or free diffusion combined with mediated efflux are shown in Fig. 3B.

Influence of pH on MICs and uptake. We determined the accumulation of [^{14}C]pefloxacin by *S. aureus* cells after 3 min of incubation (i.e., when an equilibrium was achieved, as shown in Fig. 2) at various pHs ranging from 5.2 to 8.5

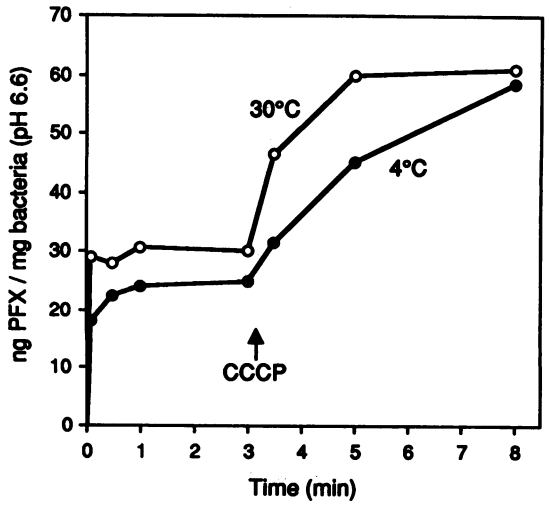

FIG. 2. Time course of pefloxacin (PFX) accumulation by whole cells of *S. aureus* at pH 6.6. [^{14}C]pefloxacin was present at 2.5 μg/ml (23.6 mCi/mmol). Uptake was assayed at the temperatures indicated. CCCP (0.1 mM) was added at the time indicated by the arrow. Each point represents the mean of seven determinations. Student's *t* test comparing results at 30 and 4°C indicated *P* values of <0.05 at 5 s and 3.5 min. Other differences were not statistically significant.

(Fig. 4A). This pH-dependent uptake was compared in parallel with the pH profiles of pefloxacin MICs (Fig. 4A), [^{14}C]pefloxacin uptake by phosphatidylglycerol liposomes (Fig. 4B), and the relative proportions of zwitterionic and uncharged pefloxacin species (Fig. 4C). In spite of some minor differences, all curves plotted in Fig. 4 displayed the same general profile. A maximum was achieved at pH values close to neutrality, while marked decreases were observed at more acidic or basic conditions. The correlation between uptake and MIC in Fig. 4A suggests that most of the radioactive material was actually incorporated and not bound to cellular outer structures, since intracellular pH and anti-DNA gyrase activity were not supposed to change under our experimental conditions.

Influence of pH on CCCP effect. Acidification of the medium enhanced [^{14}C]pefloxacin labeling of everted membrane vesicles and the CCCP effect on labeling of intact *S. aureus* cells (Fig. 5A). Surprisingly, phosphatidylglycerol liposomes devoid of protein also showed a CCCP effect with a comparable pH profile (Fig. 5B). Phosphatidylcholine liposomes tended to bind [^{14}C]pefloxacin at an acidic but not at a neutral pH (Fig. 5B), and CCCP had no effect on this labeling (data not shown). The relative proportion of the positively charged pefloxacin species was enhanced at a lower pH (Fig. 5C).

DISCUSSION

Linear Michaelis-Menten kinetics confirmed that quinolones cross the cytoplasmic membrane by means of simple diffusion. In *S. aureus*, the antibacterial activity and uptake of pefloxacin peak at pH 7.0 to 7.4, suggesting that quinolone species with no net charge (HQ^{+-}, HQ^0) have a predominant role in penetrating the cytoplasmic membrane. If one assumes that quinolone species with net charges (H_2Q^+, Q^-) are not soluble in membrane structures, then accumulation would depend solely on the partition of the neutral forms. Since inner cellular pH is stable, the penetrating activities of quinolones in this model become simply a function of outer pH. The uncharged species HQ^0 is the best candidate for mediating passive diffusion for acidic quinolones (lacking HQ^{+-}), such as nalidixic acid and WIN-57273, a newly developed quinolone with potent antistaphylococcal activity (11), but the zwitterionic species is very likely to be implicated in transmembrane diffusion as well. In a quinolone solution at pH 7.4, the concentration of the predominant HQ^{+-} species remains within the same range whatever the compound, whereas HQ^0 varies by more than 3 logs. The good correlation (21) or even good conformity (20) observed in vitro between MICs and DNA gyrase inhibitory activity indicates comparable transmembrane permeabilities of quinolones in *S. aureus*. This would be in agreement with a predominant role for HQ^{+-} in passive diffusion. Thus, we postulate that both the zwitterionic and the uncharged species of quinolones diffuse across the cytoplasmic membrane.

pH profiles for pefloxacin uptake by phosphatidylglycerol liposomes similar to those for MICs for and whole-cell uptake by *S. aureus* suggest that phospholipids with net negative charges (phosphatidylglycerol is fully negatively ionized above pH 3) facilitate quinolone diffusion across the bilayer structure. CCCP experiments showed the CCCP effect to be comparably pH dependent in whole cells and in phosphatidylglycerol liposomes. In contrast, zwitterionic membrane phospholipids are unlikely to be involved in quinolone diffusion, because pefloxacin uptake by phosphatidylcholine liposomes did not show a (relative) maxi-

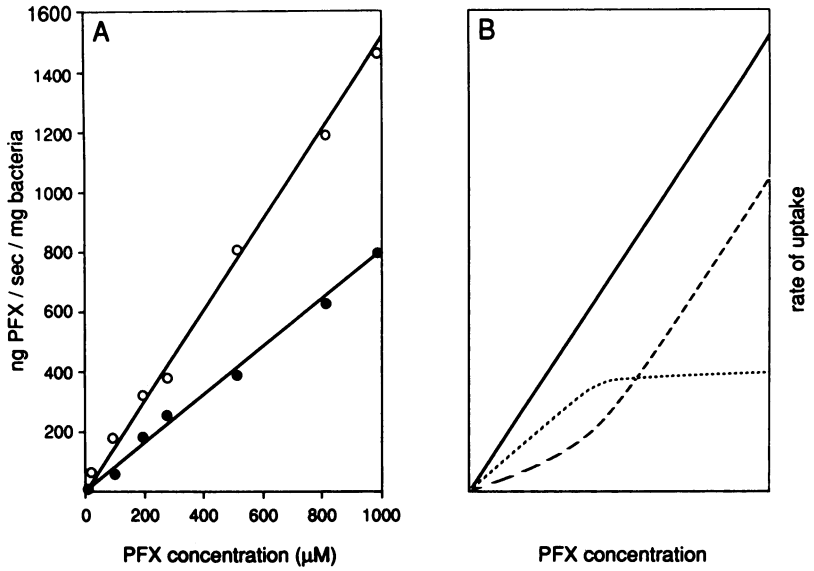

FIG. 3. (A) Observed rate of [^{14}C]pefloxacin (PFX) uptake at pH 6.6 by intact cells of *S. aureus* in the presence (○) and absence (●) of CCCP as a function of the external pefloxacin concentration. (B) Expected curves for the kinetics of simple diffusion (solid line), mediated influx (broken line), and simple diffusion combined with mediated saturable efflux (dotted line). Each point represents the mean of five experiments. Determinations of linear correlation indicated that the CCCP effect was statistically significant ($P < 0.01$).

mum at pH 7.4 and CCCP had no effect on the labeling of these liposomes. Enhanced binding of pefloxacin to both types of liposomes at acidic pHs might be related to the negative charge of the phosphate group in the phospholipid molecule. A study performed with liposomes of heterogeneous constitutions (acidic, zwitterionic, and neutral lipids) showed maximal labeling with ciprofloxacin or enoxacin at neutral and acidic pHs (1), which may correspond to incorporation and binding, respectively. We interpret this decrease in quinolone uptake as the pH falls below 7 (Fig. 4A and B) as follows. Only uncharged and zwitterionic species can penetrate and accumulate in whole cells or liposomes. Positively charged species would only bind to, and not penetrate, the cytoplasmic membrane. At pHs below 7, only the molecules bound to the membrane are measured. Phosphatidylglycerol and the double negatively charged cardiolipin normally represent an important fraction in the bacterial cytoplasmic membrane (9). Extracellular pH influences only weakly the penetration of quinolones into eukaryotic cells (8, 17), where acidic phospholipids are scarce and tend to be distributed in the inner leaflet of the bilayer (16).

A quite surprising observation was the ability of CCCP to increase pefloxacin uptake by phosphatidylglycerol liposomes devoid of proteins and energy production. A similar CCCP effect has been described for bacteria and was interpreted to be the result of an endogenous active efflux of quinolones generated by the proton motive force (3–6, 23), but the liposome data challenge this interpretation for quinolone-susceptible strains of *S. aureus*. In addition, the CCCP effect was observed at 4°C. The CCCP effect was nonsaturable at up to 1 mM of pefloxacin or with a decreasing extracellular pH and was absent at pH 7.4 in *S. aureus*. CCCP is an ionophore which increases the proton permeability of the lipid bilayer in biological and artificial membranes. As a consequence, the membrane proton gradient is short-circuited, the proton motive force is dissipated, and the medium imposes its pH on the bacterial cytoplasm. The CCCP effect on quinolone uptake by *S. aureus* might result

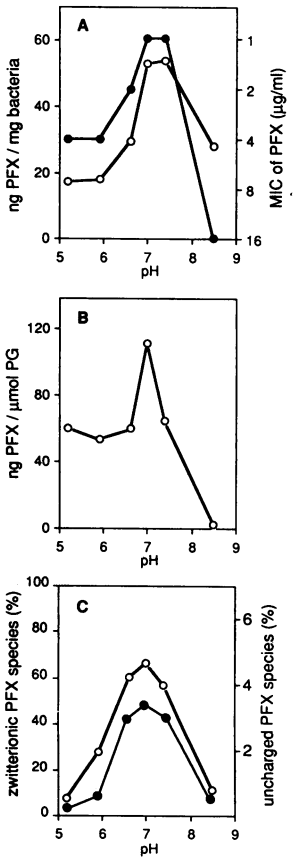

FIG. 4. Influence of pH on MICs of pefloxacin (PFX) for *S. aureus* (●) (A), on [^{14}C]pefloxacin uptake by whole *S. aureus* cells (○) (A) and by phosphatidylglycerol (PG) liposomes (B), and on the relative percentages of the zwitterionic (○) and uncharged (●) pefloxacin species (C).

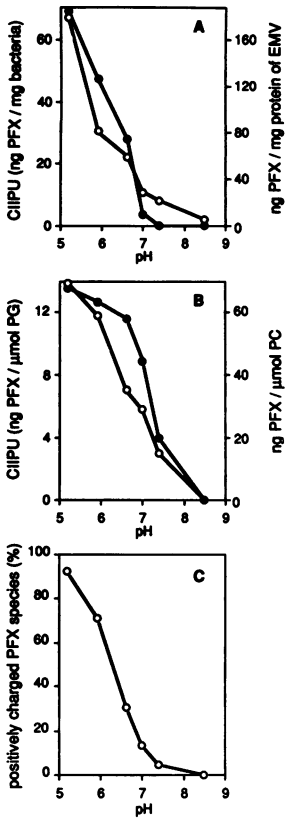

FIG. 5. Influence of pH on CCCP-induced increase of [^{14}C]pefloxacin (PFX) uptake (CIIPU) in whole *S. aureus* cells (●) and pefloxacin labeling of *S. aureus* everted membrane vesicles (EMV) (○) (A), CCCP-induced increase of [^{14}C]pefloxacin uptake in phosphatidylglycerol (PG) liposomes (●) and that in phosphatidylcholine (PC) liposomes (○) (B), and the percentages of the positively charged pefloxacin species (C).

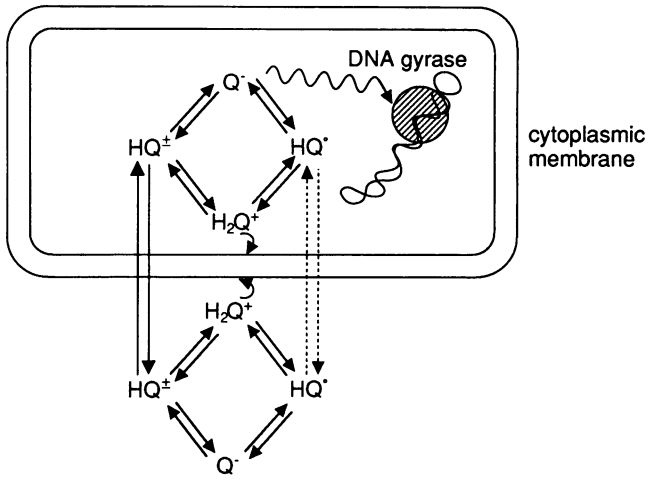

FIG. 6. Proposed model of quinolone action. The zwitterionic and uncharged species are the forms transported across the cytoplasmic membrane. Binding of the positively charged form is responsible for quinolone accumulation. The negatively charged species interacts with DNA gyrase (19).

from acidification of the internal bacterial pH with increased binding of the positive quinolone species to the inner leaflet of the cytoplasmic membrane (negative transmembrane potential within the interior). Supporting this view, an acidic outer pH optimized both the CCCP effect in whole cells and pefloxacin labeling of everted membrane vesicles, in which the outer leaflet corresponds to the inner leaflet of the cytoplasmic membrane.

However, our experiments have been carried out with *S. aureus* NCTC 8325-4, a quinolone-susceptible strain. They cannot rule out the probability of an active endogenous efflux system in *S. aureus* possessing the *norA* gene (23). The deduced NorA polypeptide suggests that this product may constitute a membrane-associated active efflux pump of hydrophilic quinolones (23), leading to a resistance mechanism similar to that recognized for tetracycline (15). Our experiments do not rule out the possibility of an active endogenous efflux in gram-negative rods as well, since there is recent evidence that *S. aureus* possesses quinolone accumulation characteristics different from those in *Escherichia coli* and *Pseudomonas aeruginosa* (14). In particular, washing preloaded cells with quinolone-free buffer decreased accumulation in *E. coli* but not in *S. aureus*.

In conclusion, our results are consistent with the model proposed in Fig. 6. The zwitterionic and uncharged species of pefloxacin would be the forms transported across the cytoplasmic membrane. Binding of the positively charged form to phospholipids located on the inner leaflet of the cytoplasmic membrane would be responsible for quinolone accumulation. Finally, as shown previously (19), the negatively charged pefloxacin species affects DNA gyrase activity.

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