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Active Efflux as a Mechanism of Resistance to Ciprofloxacin in *Streptococcus pneumoniae*

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The accumulation of fluoroquinolones (FQs) was studied in a FQ-susceptible laboratory strain of *Streptococcus pneumoniae* (strain R6). Uptake of FQs was not saturable, was rapidly reversible, and appeared to occur by passive diffusion. In the presence of glucose, which energizes bacteria, the uptake of FQs decreased. Inhibitors of the proton motive force and ATP synthesis increased the uptake of FQs in previously energized bacteria. Similar results were observed with the various FQs tested and may be explained to be a consequence simply of the pH gradient that exists across the cytoplasmic membrane. From a clinical susceptible strain (strain SPn5907) we isolated in vitro on ciprofloxacin an FQ-resistant mutant (strain SPn5929) for which the MICs of hydrophilic molecules were greater than those of hydrophobic molecules, and the mutant was resistant to acriflavine, cetrimide, and ethidium bromide. Strain SPn5929 showed a significantly decreased uptake of ciprofloxacin, and its determinant of resistance to ciprofloxacin was transferred by transformation to susceptible laboratory strain R6 (strain R6tr5929). No mutations in the quinolone resistance-determining regions of the *gyrA* and *parC* genes were found. In the presence of arsenate or carbonyl cyanide *m*-chlorophenylhydrazone, the levels of uptake of ciprofloxacin by the two resistant strains, SPn5929 and R6tr5929, reached the levels of uptake of their susceptible parents. These results suggest an active efflux of ciprofloxacin in strain SPn5929.

Streptococcus pneumoniae is an important pathogen responsible for upper and lower respiratory tract infections, acute otitis, and meningitis. By now, 30 to 50% of the strains are resistant to penicillin (MICs, ≥0.15 μg/ml) (14, 29), and effective alternative molecules are therefore needed.

Fluoroquinolones (FQs) are synthetic broad-spectrum antibiotics. Initially moderately active against streptococci, the newer FQs, especially sparfloxacin (SPX) and trovafloxacin, exhibit good in vitro activity against these pathogens (3, 8, 22). However, therapeutic use of FQs has resulted in the rapid emergence of resistance in gram-positive organisms, in particular in *Staphylococcus aureus* (2, 35). Moreover, in *S. pneumoniae*, selection of FQ resistance in vitro (16, 20, 31) as well as in vivo during treatment with FQs has already been reported (5).

Mutational alterations of the targets of FQs, topoisomerase IV and DNA gyrase, have recently been described as the main mechanism of resistance to FQs in *S. pneumoniae* (16, 23, 30, 31). However, a second mechanism of resistance to FQs, namely, active efflux of the drug, might be expected, as already described in *S. aureus* (18, 19, 25, 37). In this latter species, the active drug export system, mediated by the transmembrane protein NorA, is dependent on the proton motive force and is therefore sensitive to protonophores such as 2,4-dinitrophenol (DNP) or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP).

The only study on the uptake and efflux of antibiotics in *S. pneumoniae* was recently described for macrolides in isolates with a particular phenotype of resistance (phenotype M), namely, resistance to macrolides with susceptibility to lincosamide and streptogramin B (33). Since we were interested in determining whether an efflux mechanism could account for FQ resistance in *S. pneumoniae*, we first investigated the accumulation of FQs in FQ-susceptible strain R6. We then studied an in vitro-selected FQ-resistant strain which presented a particular phenotype of resistance to FQs and to several other structurally diverse substances such as cetrimide (CET), ethidium bromide (ETB), and acriflavine (ACR), a resistance profile similar to that conferred by NorA in *S. aureus* (18, 19) or Bmr in *Bacillus subtilis* (24), and which might therefore present an active efflux of ciprofloxacin chlorhydrate (CIP).

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MATERIALS AND METHODS

Antibiotics and chemicals. Norfloxacin (NFX) was obtained from Merck-Sharp & Dohme-Chibret, Paris, France; CIP and Bayer Y3118 (BAY) were obtained from Bayer, Sens, France; ofloxacin (OFX), levofloxacin (LVFX), D-ofloxacin (D-OFX), tetracycline (TET), and chloramphenicol were obtained from Roussel-Uclaf, Romainville, France; pefloxacin mesylate dihydrate (PEF), SPX, ^{14}C-SPX, josamycin, and pristinamycin were obtained from Rhône-Poulenc Rohrer, Vitry-sur-Seine, France; flumequine (FLU) was obtained from Rikker, Malakoff, France; erythromycin was obtained from Abbott, Rungis, France; penicillin (PEN) was obtained from Specia, Paris, France; clindamycin was obtained from Upjohn, Paris La Défense, France; and azithromycin was obtained from Pfizer, Orsay, France.

CCCP, DNP, nigericin, valinomycin, lactic acid, novobiocin, ACR, benzalkonium chloride, and CET were from Sigma; disodium hydrogen arsenate was from Merck; ETB was from Bioprobe, Montreuil-sous-Bois, France; and silicone oil was from Fluka.

Bacterial strains. *S. pneumoniae* SPn5907 is a clinical wild-type FQ-susceptible (FQ^s) strain. *S. pneumoniae* SPn5929 is an FQ-resistant (FQ^r) strain derived from SPn5907 by in vitro selection on 4 μg of CIP per ml. R6 is a noncapsulated laboratory FQ^s strain (28). R6tr5929 was obtained from *S. pneumoniae* R6 after transformation with the DNA of strain SPn5929 at a frequency of 10^4/ml and isolated on CIP at 2 μg/ml. R6p16b1b4 is a resistant mutant derived from strain R6. It was selected stepwise on PEF at 16 μg/ml, BAY at 1 μg/ml, and then BAY at 4 μg/ml (16, 17). This strain shows two mutations on the quinolone resistance-determining region (QRDR) of *gyrA* (Ser84Tyr and Glu88Lys) and two others on the QRDR of *parC* (Ser79Tyr and Asp83Tyr).

Strains were grown without agitation at 37°C in a medium containing Biocase "low salt" (bioMérieux, Marcy l'Etoile, France) at 10 g/liter, tryptone (Difco) at 5 g/liter, yeast extract at 4 g/liter, NaCl at 5 g/liter, K_2HPO_4 at 17 mM, and glucose at 0.2% (wt/vol) (adjusted to pH 7.5). It was called CAT medium (34).

Isolation of quinolone-resistant mutants. The colonies of the susceptible strain *S. pneumoniae* SPn5907 from two heavily inoculated agar plates were

suspended in 20 ml of buffered glucose broth (peptone, 20 g/liter; meat extract, 2 g/liter; NaCl, 2.5 g/liter; KH_2PO_4, 0.7 g/liter; Na_2HPO_4, 8.3 g/liter; glucose, 4 g/liter;) Diagnostic Pasteur, Marnes-La-Coquette, France) supplemented with 20% horse serum (bioMérieux), the mixture was incubated for 2 h at 37°C, and the organisms were harvested by centrifugation. The pellet was resuspended in buffered glucose broth and spread onto Mueller-Hinton agar plates (Difco) supplemented with 4% horse blood (bioMérieux) containing CIP (4 μg/ml), and the plates were incubated at 30°C for 3 days. Colonies appearing after 2 to 3 days were restreaked onto agar plates containing the same amount of antibiotic as the initial plates.

MIC determinations. MICs were determined by the standard method of twofold antibiotic dilution in agar. Inocula of 10^4 CFU were spotted onto Mueller-Hinton agar plates supplemented with 4% horse blood and containing the different compounds to be tested. MICs were read after 18 h of incubation at 37°C.

Preparation of chromosomal DNA, transformation experiments, PCR experiments, and DNA sequencing. Preparation of chromosomal DNA and transformation experiments were done as described previously (16, 28). Amplification by PCR and sequencing of a 251-bp fragment of *gyrA* and a 346-bp fragment of *parC*, encompassing the QRDRs of these genes, were done with primer pair PNC6 and PNC7 and primer pair PNC10 and PNC11, respectively (16).

Uptake of FQs by whole cells. Cells were grown at 37°C in CAT medium to an optical density at 550 nm of 0.4 to 0.5, washed once with the uptake medium (NaCl, 110 mM; NH_4Cl, 50 mM; KCl, 7 mM; Na_2HPO_4, 0.4 mM; Tris base, 52 mM; HCl, 50 mM [pH 7.5]), and concentrated 20-fold in this medium to approximately 2×10^9 cells/ml. The cell suspension was held at 4°C until use. Before uptake measurements, the suspension was supplemented with 0.2% glucose (11 mM) and allowed to glycolyze at 37°C for 5 min (except for the experiments in which the absence of glucose or a different temperature was specified). FQs were added at a final concentration of 20 μg/ml. Uptake was then performed by the silicone oil assay as described previously (10), and the concentration of FQs in the supernatant was determined by fluorescence. The accumulation of SPX was carried out with ^{14}C-SPX at 5 μCi and 20 μg/ml. The results were expressed as nanograms of product per 10^9 bacteria, with bacterial counts being estimated before the addition of FQs by spreading dilutions of the concentrated culture on Columbia agar plates (bioMérieux) supplemented with 4% horse blood and incubating the plates for 18 h at 37°C before counting.

When used, an energy inhibitor, CCCP (100 μM), DNP (2 mM), nigericin (5.3 μM), or valinomycin (3.5 μM), was added to the suspension of energized cells. Because *S. pneumoniae* relies upon glycolysis as a source of energy, sodium arsenate, an analog of phosphate, was used to deplete the cells of ATP (34). Sodium arsenate instead of Na_2HPO_4 was added at a concentration of 20 mM to the uptake medium, and the cells were incubated for 15 min at 20°C before initiating the uptake. When necessary, the pH of the uptake medium was modified with either NaOH (1 N) or HCl (1 N).

Each assay was repeated two or three times, and the results are the means of at least two experiments; the error falls within 10 to 15%.

RESULTS

Antimicrobial susceptibilities of FQ-susceptible and -resistant strains. The MICs of various compounds for the different strains used in this study are listed in Table 1. Compared to the parent susceptible strain, strain SPn5907, the MICs of the hydrophilic FQs (CIP and OFX) for the resistant strain, strain SPn5929 (fourfold increase) increased more than the MICs of the hydrophobic ones (PEF and SPX; twofold increase). This dissociated resistance profile was more pronounced when susceptible strain R6 was compared to its resistant transformant, R6tr5929: the MICs of CIP and OFX increased 16- and 8-fold, respectively, and those of PEF and SPX increased twofold. The MIC of BAY was not affected. By comparison, the resistant mutant R6p16b1b4, which is derived from R6 and which harbors mutations in *gyrA* and *parC*, showed no such dissociated resistance profile.

With regard to other families of antibiotics, the susceptibility to neither PEN nor to macrolides (erythromycin, azithromycin, and josamycin; MICs, 0.06 μg/ml), clindamycin (MICs, 0.06 μg/ml), pristinamycin (MICs, 0.25 μg/ml), and chloramphenicol was significantly modified. Only the MIC of TET was increased fourfold for strain R6tr5929 compared to the MIC for strain R6; this was not apparent for the pair of strains SPn5907 and SPn5929, since the MIC of TET was already high for the FQ-susceptible strain, presumably because of the presence of another mechanism of resistance. Among the dyes ACR and ETB and the antiseptic CET, two- to fourfold and four- to eightfold increased MICs of ACR, CET, and ETB were observed for resistant strains SPn5929 and R6tr5929, respectively. Thus, the mechanism of resistance present in SPn5929 and its R6 transformant R6tr5929 affects several basic dyes, TET, and hydrophilic FQs to a greater extent than it affects hydrophobic FQs. This pleiotropic resistance profile resembles that of the efflux-mediated resistance conferred by NorA in *S. aureus* (18, 19) or Bmr in *B. subtilis* (24).

TABLE 1. Susceptibilities of the different strains to fluoroquinolones, other antibiotics, and antiseptics

Compound[a]	MIC (μg/ml)				
	SPn5907	SPn5929	R6	R6tr5929	R6p16b1b14
CIP	2	8	0.5	8	64
OFX	2	8	1	8	256
LVFX	1	2	0.5	1	128
PEF	8	16	4	8	128
SPX	0.25	0.5	0.125	0.25	64
BAY	0.06	0.06	0.03	0.06	16
PEN	2	2	0.015	0.015	0.015
CHL	8	8	2	2	2
TET	16	16	0.25	1	0.5
NOV	2	1	0.5	1	0.25
ACR	8	16	4	16	4
CET	1	4	0.5	4	0.5
ETB	8	32	4	32	4
BCL	16	16	16	16	4

[a] CHL, chloramphenicol; NOV, novobiocin; BCL, benzalkonium chloride. The other abbreviations are defined in the text.

To ensure that the mechanism of resistance present in SPn5929 was neither due to nor associated with mutations in *gyrA* or *parC*, the QRDRs of these genes were sequenced. No mutational alteration was found.

Uptake of FQs by FQ-susceptible strain R6. (i) Mechanism of uptake and influence of energizing agents and metabolic inhibitors of the proton motive force and of ATP synthesis. Uptake of OFX occurs at 4°C, although more slowly than uptake at 37°C, in the absence of glucose (Fig. 1). In order to reveal the activity of a putative pump, glucose-energized bacteria were used for all subsequent uptake measurements unless otherwise mentioned. To determine the saturability of accumulation, energized cells were exposed to increasing concentrations of OFX, LVFX, and PEF for 10 min. The concentrations of cell-associated FQs were proportional to the external concentrations and were unsaturable over a range of 10 to 400 μg/ml (data not shown). These results suggest that FQs enter

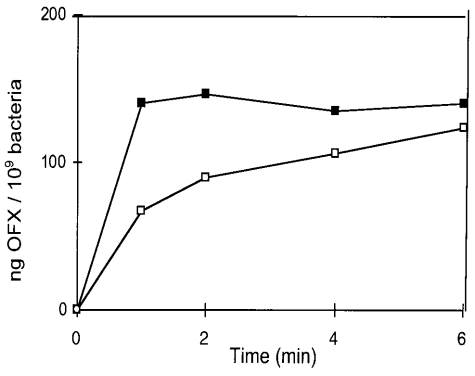

FIG. 1. Uptake of OFX by whole cells of *S. pneumoniae* R6 in the absence of glucose at 37°C (■) and 4°C (□) as a function of time.

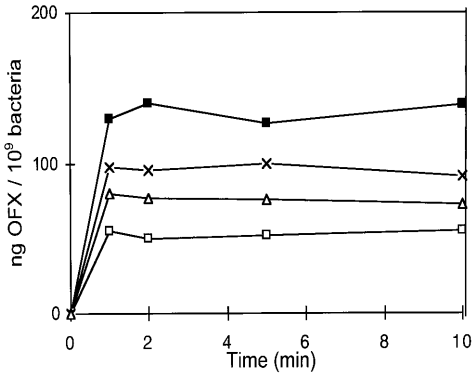

FIG. 2. Uptake of OFX by whole cells of *S. pneumoniae* R6 in the absence of an energizer (■) and in the presence of glucose at 11 mM (□) and lactic acid at 20 mM (×) and 100 mM (△).

S. pneumoniae by passive diffusion through the cytoplasmic membrane.

The addition of glucose or lactic acid to the cell suspension resulted in a decrease in the amount of cell-associated OFX (Fig. 2). Because glucose was active at lower concentrations, it was subsequently used in all experiments. The amounts of cell-associated NFX, CIP, OFX, PEF, SPX, and FLU with and without glucose are indicated in Fig. 3. The values were obtained from samples taken 10 min after the addition of the antibiotic. There was a decrease in uptake upon the addition of glucose for all the FQs assayed. The results obtained with LVFX and D-OFX were the same as those obtained with OFX (data not shown). While SPX accumulation into *S. pneumoniae* was twice as high as that of the other FQs, FLU did not appear to enter *S. pneumoniae* at all.

Since in the absence of glucose the amounts of cell-associated FQs were higher, the influence of energy deprivation was examined in more detail with OFX, which was used as a reference FQ. After a 10-min contact, the protonophore CCCP (100 μM) increased the amount of cell-associated OFX in energized bacteria nearly to the level in nonenergized cells. In contrast, no effect of CCCP was noted at 4°C or in the absence of glucose. A similar effect of CCCP was observed with several FQs: OFX, LVFX, D-OFX, NFX, CIP, PEF, and SPX (data not shown). Other inhibitors of the proton motive force such as DNP or more selective agents such as valinomycin, which collapses the electrical gradient, or nigericin, which collapses the pH gradient, had the same effect as CCCP on the uptake of OFX. Incubation of the cells with arsenate, an analog of phosphate which causes ATP deprivation (34), also increased cell-associated OFX. Starting from a value of 85 ng of OFX/10^9 bacteria, the accumulation levels were 125, 124, 138, 128, and 130 ng of OFX/10^9 bacteria in the presence of CCCP (100 μM), DNP (2 mM), valinomycin (3.5 μM), nigericin (5.3 μM), and arsenate (20 mM), respectively.

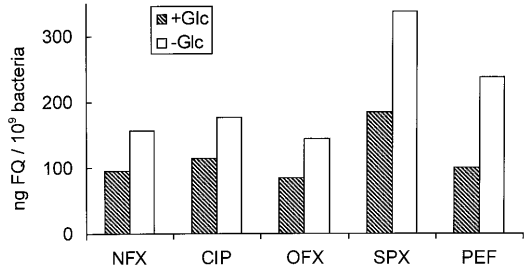

FIG. 3. Amount of cell-associated FQ in *S. pneumoniae* R6 in the presence (columns with slashes) and in the absence (white columns) of glucose (Glc). The samples were taken at 10 min.

These results suggest that in energized cells an active process takes place. This process decreases the uptake of FQs and is reversed by inhibitors of the proton motive force and ATP synthesis. This process is not specific, since it was shared by all the molecules assayed. Although such a phenomenon could result from activation by glucose of an efflux machinery which would be inhibited by the energy inhibitors, it could also result from the collapse by the metabolic inhibitors of the energy-dependent pH gradient, which modifies the equilibrium concentration of FQs across the membrane (26).

(ii) Influence of pH on FQ uptake and on the effect of CCCP. Uptake of CIP, OFX, PEF, and SPX by *S. pneumoniae* R6 with and without CCCP was measured at pH 6.5, 7, 7.5, and 8. Higher or lower pH values could not be evaluated because of the possibility of cell lysis. Only the results of the experiments carried out at pH 6.5 and 8 are presented (Fig. 4). The level of uptake at pH 7 and 7.5 was between those at pH 6.5 and 8. No differences in uptake or in the effect of CCCP were noted between pH 6.5 and 8 for CIP. By contrast, the uptake of PEF was more important at pH 8 than at pH 6.5, and the effect of CCCP was abolished at pH 8. The results obtained with SPX were similar to those obtained with PEF (data not shown), while with OFX, the results were between those obtained with CIP and PEF. The complex behavior of the assayed FQs, whose physicochemical properties varied (32) (Table 2), can be explained to be the result of simple, passive diffusion through the cytoplasmic membrane and their equilibrium distribution dictated by the Donnan potential and the pH gradient, which depend on their respective physicochemical properties, in agreement with the scheme of Nikaido and Thanassi (26): when the external pH is 8 or when the pH gradient is collapsed by CCCP and assuming an internal pH of 7.8, an influx of FQs takes place and the total concentration of free FQs will become equal on both sides of the membrane. This effect is particularly obvious for PEF (Fig. 4c).

Accumulation of CIP and PEF by the isogenic FQ-susceptible and FQ-resistant strains and by a resistant transformant. We first measured the uptake of the hydrophilic molecule CIP and the hydrophobic molecule PEF by an FQ-susceptible clinical isolate (strain SPn5907) and its FQ-resistant derivative (strain SPn5929), which was selected in vitro on CIP (4 μg/ml). These strains were selected from among 49 strains of *S. pneumoniae* that we tested for their quinolone uptake properties. As indicated in Fig. 5a, the uptake of CIP in *S. pneumoniae* SPn5929 was reduced by 33% compared to that in susceptible strain SPn5907. The difference in uptake was more pronounced during the first 5 min, since the amount of CIP decreased slightly in the susceptible strain after 5 min. Such a decrease in the uptake levels of an FQ in

TABLE 2. Physicochemical properties of some FQs[a]

Quinolone	pK_{a1}	pK_{a2}	pI	HQ^- (%)	HQ^0 (%)	Log D_{app} (pH 7)
NFX	6.3	8.38	7.34	83.7	0.06	0.25
PEF	6.27	7.55	6.91	54.5	0.06	0.25
OFX	6.05	8.22	7.14	86.9	0.06	0.25
CIP	6.09	8.74	7.42	95.1	0.06	0.25
SPX	6.23	8.57	7.4	87.7	0.06	0.25

[a] Data are from previous reports (11, 32). HQ^{+-}, zwitterionic form; HQ^0, neutral form; log D_{app}, apparent partition coefficient (octanol/water).

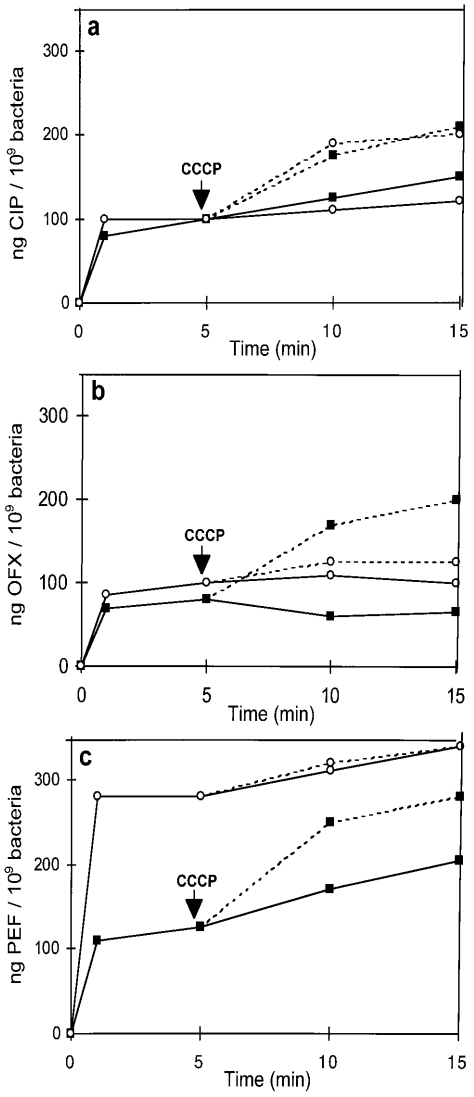

FIG. 4. Uptake of CIP (a), OFX (b), and PEF (c) by whole cells of *S. pneumoniae* R6 at pH 6.5 (■) and 8 (○). CCCP (100 μM) (dashed line) was added at the time indicated by the arrow.

a susceptible isolate was previously observed by Li et al. (21). The level of accumulation showed a dramatic increase upon the addition of arsenate, an inhibitor of ATP synthesis. Thus, the final levels of CIP uptake after incubation with arsenate were similar in the susceptible strain and in the resistant strain. The effect of the protonophore CCCP on the uptake of CIP by these strains was less obvious than the effect of arsenate (from 140 to 238 ng/10^9 bacteria for SPn5907 and from 78 to 187 ng/10^9 bacteria for SPn5927). The uptake of the hydrophobic molecule PEF was slightly but reproducibly higher in the resistant strain than in the susceptible strain (Fig. 5b), and arsenate had no obvious effect.

The uptake assays were also carried out with the FQ-sensitive strain *S. pneumoniae* R6 and with the resistant transformant R6tr5929. Figure 6 indicates that CIP uptake was reduced by 60% in R6tr5929 and that the addition of the protonophore CCCP increased the level of CIP uptake to the same level in both strains, indicating that the strains behave similarly once the putative efflux system is inactivated because

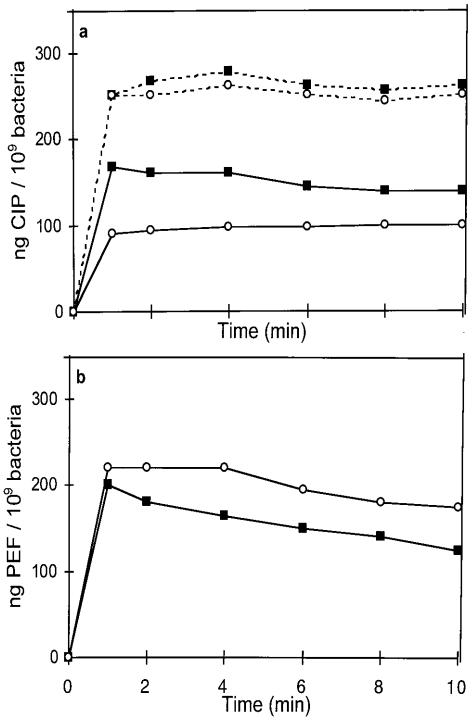

FIG. 5. Uptake of CIP (a) in the absence (—) and presence (. . . .) of 20 mM arsenate, and uptake of PEF (b) by whole cells of *S. pneumoniae* SPn5907 (susceptible strain) (■) and SPn5929 (resistant strain) (○).

of the deprivation of energy. Arsenate had an effect similar to that of CCCP (from 123 to 194 ng/10^9 bacteria for R6 and from 50 to 193 ng/10^9 bacteria for R6tr5929). As a control, we checked that high-level-resistant strain R6p16b1b4 (MIC of CIP, 64 μg/ml), which harbored several mutations in its topoisomerase genes, did not have lower uptake levels of CIP than strain R6 (125 ng/10^9 bacteria in both cases). Similar results were obtained with the hydrophilic molecule NFX (96 ng/10^9 bacteria for R6 and 20 ng/10^9 bacteria for R6tr5929). Such differences were not observed for the hydrophobic molecules PEF (130 ng/10^9 bacteria for R6 and 150 ng/10^9 bacteria for R6tr5929) and SPX (225 ng/10^9 bacteria for R6 and 260 ng/10^9 bacteria for R6tr5929). Finally, the particular phenotype of resistance of strains SPn5929 and R6tr5929 (resistance to hy-

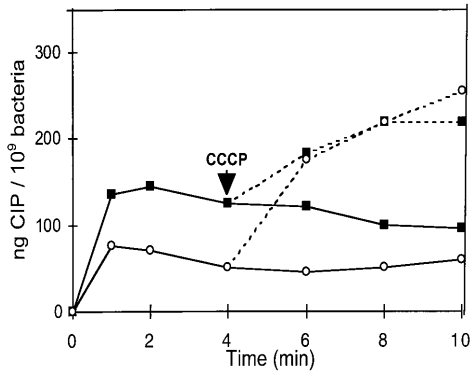

FIG. 6. Uptake of CIP by whole cells of *S. pneumoniae* R6 (susceptible strain) (■) and R6tr5929 (resistant transformant) (○) in the absence (—) and presence (. . . .) of 100 μM CCCP, added at the time indicated by the arrow.

drophilic FQs and several antiseptics) associated with a decrease in CIP accumulation, reversed by arsenate and CCCP, are consistent with an active efflux of CIP as the mechanism of resistance in these strains.

DISCUSSION

Active efflux as a mechanism of resistance to various antibiotics has been found in an increasing number of gram-negative bacteria and, more recently, gram-positive bacteria (9, 12, 27, 33). We were interested in looking for this possible mechanism of resistance to FQs in *S. pneumoniae*. The mode of penetration of quinolones in this organism has not been described before; thus, we investigated some aspects of this problem, and the first step of this work consisted of studying the uptake into a susceptible strain (*S. pneumoniae* R6). Uptake of FQ in *S. pneumoniae* appears to occur by simple diffusion, as observed with other bacterial species (9–11, 26, 38). Among the different molecules tested, SPX accumulated at twice the amount that the more hydrophilic compounds such as CIP, NFX, and OFX accumulated, and this favorable equilibrium distribution across the membrane might play a role in the better in vitro activity of SPX against *S. pneumoniae*, although its activity on the topoisomerase targets is probably the major factor, as discussed by others (26). Similar results were described previously with another gram-positive coccus, *S. aureus* (4, 11, 38). The modification of CCCP of the uptake of quinolones into susceptible strains is still a subject of controversy in the literature. Some investigators suggest that this indicates the presence of an endogenous efflux (19, 37), others propose a chemical explanation on the basis of the interaction of the transmembrane electrochemical proton potential, $\Delta\mu_{H^+}$, with the FQs, which have complex physical and chemical properties (11, 26, 32). Our studies with susceptible strain *S. pneumoniae* R6, indicating that CCCP has the same nonspecific effect affecting hydrophilic and hydrophobic FQs, suggest that this effect probably results from the collapse of the energy-dependent pH gradient. Thus, CCCP affects an active process, as was also shown by the differences in accumulation between glucose-energized and glucose-nonenergized cells, but it does not likely involve a specific transporter, although the association of both processes cannot be excluded.

The multidrug resistance phenotype of the in vitro-selected low-level CIP-resistant strain (SPn5929), which is resistant not only to hydrophilic quinolones but also to other structurally unrelated agents such as ETB, CET, and ACR, resembles that conferred by NorA in *S. aureus* (18, 19) or Bmr in *B. subtilis* (24) and suggests the presence of an efflux pump. Our results are consistent with those of Pan et al. (31), who described a first-step mutant that was selected on CIP and that displayed low-level resistance to this drug (MIC, 3 μg/ml). No mutations in the topoisomerases genes of this mutant were detected. Among the three possibilities discussed by the investigators to explain this low-level resistance, active efflux of CIP was considered the most likely.

The results of accumulation of CIP into strain SPn5929, for which the MICs of the hydrophilic FQs increased more than those of the hydrophobic molecules, are consistent with an active efflux of CIP. This is supported by the fact that no mutational alterations of the QRDRs of the two FQ targets (gyrase and topoisomerase IV) were found in this strain. Furthermore, the resistance to CIP, associated with increased active efflux, was transferable to susceptible, nonencapsulated strain R6 by one round of transformation with the DNA of *S. pneumoniae* SPn5929. The efflux of CIP from this transformant strain (R6tr5929) was even more pronounced than that from the original strain, strain SPn5929. This difference could be due to the absence of a capsule in R6 and its transformant. For strains SPn5907 and SPn5929, the presence of a capsule could generate a higher degree of nonspecific binding of the antibiotics to surface components and therefore create an important background, which would decrease the apparent differences in uptake of CIP between the susceptible and resistant strains. This point is supported by the fact that all the FQ-resistant derivatives obtained from R6 that we studied (10 derivatives [this study] [16], unpublished data]) had similar levels of cell-associated FQs. In contrast, all clinical encapsulated strains (46 strains) had higher amounts of cell-associated antibiotics (data not shown).

In this study, both arsenate and CCCP inhibited the efflux of CIP specifically. Thus, the drug export system in *S. pneumoniae* could be mediated directly by ATP hydrolysis or via the proton motive force, but our data are insufficient to settle this question. Several transport systems already described in *S. pneumoniae* are members of the ATP-binding cassette transporter family (1, 15, 34). In a review of the transport of cations in *Enterococcus faecalis*, Heefner (13) concluded, first, that all cation pumps in *E. faecalis* are energized directly by ATP hydrolysis and, second, that the $\Delta\mu_{H^+}$ in these bacteria is much inferior to that in respiring organisms. Heefner suggests that the ATP-dependent cation pumps have developed in response to an inadequate $\Delta\mu_{H^+}$. These hypotheses could also concern *S. pneumoniae*, in which anaerobic metabolism is close to the metabolism in *E. faecalis* (13). In *Lactococcus lactis*, two types of multidrug resistance transporters with similar substrate profiles have been found; one is energized by the proton motive force (LmrP) and the other is energized by ATP (LmrA) (6, 7, 36).

With regard to the MICs of several antiseptics, we postulate that the simultaneously acquired resistance to ACR, CET, and ETB could be mediated by the same efflux system. No cross-resistance to other families of antibiotics except to TET, which is also possibly a substrate of the pump, was observed. To determine fully the specificity of the putative pump and its multidrug resistance, further accumulation assays with other compounds will be necessary.

The level of resistance to hydrophilic FQs conferred by active efflux in *S. pneumoniae*, for which the CIP MIC was up to 8 μg/ml, appears sufficient to consider a change in therapy if this type of resistance were to occur in a clinical strain. Fortunately, the activities of the more hydrophobic molecules against gram-positive bacteria remain practically unchanged by this efflux system. Finally, starting from the high-level-resistant strain R6p16b1b4 (MICs of CIP, 64 μg/ml; ETB, MICs of 4 μg/ml), which harbored several mutations in its topoisomerase genes, we selected on 128 μg of CIP per ml and then 8 μg of ETB per ml a strain (strain R6VZ2) with a level of uptake of CIP that was reduced by 38% compared to that of the parent strain (strain R6p16b1b4). This defect of CIP uptake was abolished by arsenate, and the uptake of PEF into R6p16b1b4 was the same as that into R6VZ2 (data not shown), as was the case for the two other pairs of strains with an active efflux that we studied. This indicates that in vivo several mechanisms might simultaneously contribute to an increase in the level of resistance of *S. pneumoniae.*

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