Accumulation of Norfloxacin by *Mycobacterium aurum* and *Mycobacterium smegmatis*

KERSTIN J. WILLIAMS, GAVIN A. C. CHUNG, AND LAURA J. V. PIDDOCK

Antimicrobial Agents Research Group, Department of Infection, The Medical School, University of Birmingham, Birmingham B15 2TT, United Kingdom

Received 18 August 1997/Returned for modification 24 October 1997/Accepted 22 December 1997

Although several hundred antimicrobial agents are available worldwide, very few are effective against *Mycobacterium tuberculosis* and even fewer are effective against atypical mycobacteria, such as *Mycobacterium avium* complex. It has been widely proposed that the mycobacterial cell wall has low permeability and that this reduced permeability plays a major role in the intrinsic resistance of mycobacteria to most antibiotics (4, 14, 15). However, few studies of the accumulation or transport of antituberculosis agents by mycobacteria or the role that permeability plays in mycobacterial drug resistance have been performed.

The fluoroquinolones are broad-spectrum, bactericidal antimicrobial agents that were developed in the 1980s and that are active against gram-positive bacteria, gram-negative bacteria, and some mycobacteria (6, 29, 32). Norfloxacin, ciprofloxacin, and, in particular, sparfloxacin show good in vitro activity against *Escherichia coli* and *Staphylococcus aureus* (3, 16, 18). The fluoroquinolones act by inhibiting the topoisomerase II enzyme DNA gyrase, which catalyzes the unwinding or negative supercoiling of double-stranded DNA during replication (12). Topoisomerase IV, which is encoded by parC and parE (grlA and grlB in *Staphylococcus aureus*), has been proposed as a secondary target of fluoroquinolones in *Escherichia coli* when a susceptible DNA gyrase is lacking (13, 38). However, for some gram-positive bacteria such as *S. aureus* and *Streptococcus pneumoniae*, topoisomerase IV is thought to be the primary target and DNA gyrase is thought to be the secondary target of fluoroquinolones (25, 26). Both enzymes are intracellular; therefore, in order to exert their antibacterial effect, the fluoroquinolones must cross the bacterial cell envelope.

Fluoroquinolone resistance arises due to mutations in *gyrA* and, less frequently, *gyrB*, which encode the A and B subunits of DNA gyrase, respectively (30). Although other mechanisms of resistance to fluoroquinolones have been documented for other bacteria, e.g., *parC* mutations, reduced intracellular accumulation, and enhanced drug efflux (30), only *gyrA* mutations have been reported to give rise to fluoroquinolone resistance in clinical *M. tuberculosis* isolates (1, 39). Due to the moderate in vivo activity of fluoroquinolones and increasing resistance to these drugs, the fluoroquinolones are generally considered second-line agents against tuberculosis (5). However, with the increasing incidence of multiple-drug-resistant *M. tuberculosis* and the increased use of fluoroquinolones in combination with other agents for the treatment of tuberculosis, much attention has focused upon the therapeutic value of the fluoroquinolones for the treatment of tuberculosis and other mycobacterial diseases (42).

Although it is thought that the hydrophobic mycobacterial cell wall acts as an efficient barrier to hydrophilic molecules (4, 14, 15), hydrophilic nutrients must be taken up by the cell, and some hydrophilic drugs, such as isoniazid and ethambutol, are extremely effective against *M. tuberculosis* (23). Mycobacterial porins have recently been characterized in *Mycobacterium chelonae* (33, 34) and *Mycobacterium smegmatis* (35), and it is thought that similar porins are widely distributed among mycobacteria (4). However, these porins are thought to be both less abundant and less efficient than porins of other bacterial species, such as OmpF in *E. coli* and OprD in *Pseudomonas aeruginosa* (33, 34). For example, studies have shown that the cell wall permeability of *M. chelonae* is about 10-fold lower than that of *P. aeruginosa* to cephalosporins (14). However, there does appear to be significant differences in cell wall permeability between various mycobacterial species. For ex-
ample, *M. smegmatis* and *M. tuberculosis* have been shown to be more permeable to β-lactams than *M. chelonae* (7, 35). Furthermore, hydrophilic drugs have a wide range of MICs for mycobacteria and the low levels of cell wall permeability to these drugs may play a major role in this intrinsic drug resistance (15).

The accumulation of norfloxacin by *E. coli* and *S. aureus* is well documented (22, 24), and recently, studies on the accumulation of norfloxacin by *M. smegmatis* CNCM 7326 and *M. tuberculosis* H37Ra have been reported (10, 17). Mycobacterium *aurum* and *M. smegmatis* are nonpathogenic, fast-growing, saprophytic mycobacteria and give homogeneous cell suspensions without the use of detergents, such as Tween 80. *M. aurum* A – possesses a MIC profile similar to that of *M. tuberculosis* for the first-line antituberculous drugs and is used in high-throughput screenings for the detection of novel antituberculous agents (8). *M. smegmatis* mc²155 is the strain of *M. smegmatis* most commonly used in laboratory experiments and genetic manipulations. Therefore, in order to gain further insight into the mechanisms of fluoroquinolone accumulation by saprophytic mycobacteria and, by extrapolation, by *M. tuberculosis*, this study sought to determine and compare the accumulation of norfloxacin by *M. aurum* and *M. smegmatis* and to characterize the mechanism of norfloxacin accumulation by *M. aurum*.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *M. aurum* A – (Pasteur Institute, Paris, France) and *M. smegmatis* mc²155 (W. R. Jacobs, Albert Einstein College of Medicine, New York, N.Y.) were maintained on Lownstein-Jensen slopes and were cultured on Middlebrook 7H11 agar (Difco, West Molesey, United Kingdom) supplemented with 10% (vol/vol) OADC (oleic acid, albumin fraction V, dextrose, and catalase). Cultures were grown aerobically in Middlebrook 7H9 broth (Difco) supplemented with 10% (vol/vol) ADC (albumin fraction V, dextrose, and catalase) at 37°C.

**Growth kinetics.** A total of 250 ml of 7H9 broth was inoculated with a 3-day-old culture (25 ml) of *M. aurum* followed by incubation at 37°C with a 1-min episode of shaking (180 rpm) every 4 h. For *M. smegmatis*, 250 ml of 7H9 broth was inoculated with an overnight culture (25 ml), followed by incubation at 37°C with shaking at 180 rpm. Growth was evaluated by measuring the optical density (OD) of the growing culture at 550 nm. The viable cell counts of the growing cultures were determined by diluting culture samples in sterile distilled water and spreading 100 μl of each dilution over the surfaces of 7H11 agar plates. The plates were incubated at 37°C, and the colonies were counted after 48 and 72 h. The numbers of CFU per milliliter were calculated by counting the number of colonies on the agar plate and assuming that one viable organism gives rise to one colony. Cell dry weights for *M. aurum* and *M. smegmatis* were determined by removing 20-ml aliquots of the growing culture at selected OD measurements. The cells were immediately centrifuged at 12,000 g for 15 min at 4°C. The cell pellets were washed with 10 ml of sterile distilled water and centrifuged as described above. The cell pellets were dried overnight in a 60°C hot-air oven and weighed. The tubes were kept under desiccative conditions and were reweighed until a stable weight was obtained.

**Chemicals.** Norfloxacin and ethambutol were obtained from Sigma Chemical Co., Poole, United Kingdom, and were made up according to the manufacturer’s instructions. 2,4-Dinitrophenol (DNP; 50 mM; Sigma) was dissolved in 3 parts dimethyl sulfoxide to 2 parts distilled water, and the solution was stored at 4°C. 2,4-Dinitrophenol (DNP; 50 mM; Sigma) was dissolved in 3 parts dimethyl sulfoxide to 2 parts distilled water, and the solution was stored at 4°C. Antibiotic susceptibility testing. MICs were determined in 7H9 broth by a standard microdilution technique with an inoculum of 10³ CFU/ml. The plates were incubated at 37°C, and the results were read after 48 and 72 h. The MIC was defined as the lowest concentration of drug at which no visible growth was observed.

**Measurement of norfloxacin accumulation.** The modified fluorometric method of Mortimer and Piddock (24) was altered slightly to accommodate the growth characteristics of the mycobacteria. Cells were grown to the mid-exponential phase in 7H9 broth (A₅₅₀ of 0.1 to 0.12 for *M. aurum* and A₅₅₀ of 0.4 to 0.5 for *M. smegmatis*) and were harvested by centrifugation in a Mistral centrifuge (MSE) at 3,000 × g for 20 min at 15°C. The cells were washed in 10 ml of 50 mM sodium phosphate buffer (pH 7) and were concentrated with the same buffer to give the bacterial suspension of *M. aurum* an A₅₅₀ of 5 and that of *M. smegmatis* an A₅₅₀ of 20. The bacterial suspension was placed in a stirring 37°C water bath and was left for 10 min to equilibrate. Norfloxacin was added to the required final concentration (10 μg/ml), and 1-ml samples were removed at timed intervals. The cells were immediately centrifuged at 12,000 × g (Jouan MR1812) for 3 min at 4°C. The cell pellets were washed once with ice-cold sodium phosphate buffer (50 mM; pH 7) and were resuspended in 1 ml of 0.1 M glycine hydrochloride (pH 3). The samples were left overnight at room temperature with agitation to lyse the cells. The efficacy of this lysis procedure has been assessed previously by transmission electron microscopy (10). On the following day, the samples were centrifuged at 12,000 × g (Jouan MR1812), and the fluorescence of the supernatants was determined at an excitation wavelength of 281 nm and an emission wavelength of 440 nm. Background fluorescence (i.e., that of norfloxacin-free controls) possibly due to the porphyrins present in mycobacterial cells (10) was subtracted from the fluorescence for all samples taken. A standard curve of norfloxacin fluorescence in the presence of 0.1 M glycine hydrochloride (pH 3) was constructed, and the results are expressed as nanograms of norfloxacin per milligram (dry weight) of cells.

To test for the possible saturation of transport, the accumulation of norfloxacin over a concentration range of 0 to 100 μg/ml was studied with *M. aurum*. The cells were incubated at 37°C for 5 min in the presence of each norfloxacin concentration. To study the effect of the pH of the wash buffer on norfloxacin adsorption to the cell wall, accumulation experiments were performed, as described above, except that the cell pellets were washed with either pH 7 or pH 9 sodium phosphate buffer. The pH 9 buffer was prepared by adding a few drops of 0.1 M sodium hydroxide to the pH 7 buffer.

To study the effect of DNP on norfloxacin accumulation by mycobacteria, cells were treated with 1 or 2 mM DNP either 10 min before or 5 min after the addition of norfloxacin to the cell culture.

The silicon oil method was performed essentially as described by Li et al. (19). The cells were prepared as described above. Samples (0.5 ml) were removed at timed intervals and were placed on 0.5-ml aliquots of silicon oil (a 6:5 [vol/vol] mixture; Dow Corning silicon oils) in Eppendorf tubes. The tubes were immediately centrifuged at 13,249 × g (Microcentur; MSE), and the top aqueous layer was removed. The tubes were then snap frozen in liquid nitrogen for 1 s and the oil was removed by careful pipetting. The cell pellet was resuspended in 1 ml of 0.1 M glycine hydrochloride (pH 3) and left overnight with agitation to lyse cells. The accumulated norfloxacin was quantified as described above.

**Statistical analysis.** The differences in the accumulation data obtained by the modified fluorescence method and the silicon oil method were analyzed by Student’s t test. A P value of <0.05 was considered significant.

**RESULTS**

**Mycobacterial growth curves.** An example of a growth curve obtained for *M. aurum* is shown in Fig. 1. For *M. aurum*, no lag phase was observed and the logarithmic phase lasted about 3 days (72 h); the generation time was 21 h. For *M. smegmatis*, a lag phase of less than 2 h was observed and the logarithmic phase lasted 12 h; the generation time was 2 h.

**Accumulation of norfloxacin.** The MIC of norfloxacin for both *M. aurum* and *M. smegmatis* was 2 μg/ml. The accumulation of norfloxacin (10 μg/ml) by *M. aurum* resulted in a steady-state concentration (SSC) of 160 to 180 ng of norfloxacin/mg of cells (Fig. 2). Accumulation was rapid; approximately 80% of the final concentration of norfloxacin was accumulated within the first minute of drug exposure. A further 30 ng of...
norfloxacin/mg of cells was accumulated at between 1 and 5 min. The accumulation of norfloxacin by *M. smegmatis* resulted in an SSC of 120 to 140 ng of norfloxacin/mg of cells (Fig. 2). Accumulation kinetics very similar to those for *M. smegmatis* were observed for *M. aurum*. By the modified fluorescence method (24), the cell cultures are concentrated to an OD of 20. However, due to the low density of *M. aurum* cells, the cells were concentrated to an OD of 5, because an OD of 20 could not be achieved. This ensured both a sufficient volume of culture for performance of an experiment and sufficient numbers of cells for intracellular norfloxacin detection.

The accumulation of norfloxacin by *M. smegmatis* by the silicon oil method described by Li et al. (19) was studied at 0 and 37°C (Table 1). Higher readings were obtained by the silicon oil method, but these were subsequently determined to be due to high levels of norfloxacin adsorption onto the cell. Intracellular accumulation values, i.e., accumulation at 37°C minus binding at 0°C, were similar by both methods (Table 1) (19) and were not found to be statistically significantly different (*P* > 0.05 for all comparisons).

**Effect of exogenous drug concentration on norfloxacin accumulation by *M. aurum***. To determine if norfloxacin transport into *M. aurum* was saturable, the effect of the exogenous norfloxacin concentration on accumulation was studied over a concentration range of 0 to 100 μg/ml (Fig. 3). It was found that norfloxacin accumulation by *M. aurum* was not saturated. The concentration of norfloxacin accumulated increased at a rate proportional to the increase in the exogenous norfloxacin concentration. An increase in the exogenous norfloxacin concentration of 20 μg/ml resulted in an increase of the accumulated norfloxacin concentration of 190 ng of norfloxacin/mg of cells.

**Effect of the pH of the wash buffer on norfloxacin accumulation by *M. aurum***. According to Corti et al. (10), an increase in the pH of the cell washing buffer from 7 to 9 reduces the amount of norfloxacin adsorption to the cell wall of *M. smegmatis* during an uptake experiment. Therefore, the effect of an increase in the wash buffer pH from 7 to 9 on the accumulation of norfloxacin by *M. aurum* was studied. Experiments were performed at 0°C with either pH 7 or pH 9 wash buffer to determine the level of adsorption of norfloxacin to *M. aurum* cells. The concentration of norfloxacin absorbed to the *M. aurum* cell wall at 0°C was greater when a pH 7 wash buffer was used than when a pH 9 wash buffer was used (data not shown). However, at 37°C the pH of the wash buffer did not significantly affect the final concentration of norfloxacin accumulated by *M. aurum* (Fig. 4).

**Effect of metabolic inhibitors on norfloxacin accumulation by *M. aurum***. To determine whether the accumulation of norfloxacin by *M. aurum* is energy independent or whether norfloxacin is actively effluxed from *M. aurum*, cells were treated with 1 or 2 mM DNP either 10 min before, 1 h before, or 5 min after the addition of norfloxacin (10 μg/ml) to the cell culture. DNP at 2 mM has previously been used to study the effect of metabolic inhibitors on quinolone accumulation by bacteria (9, 20). Although 10 min should be sufficient to dissipate the proton motive force in *M. aurum*, because of the longer generation time of *M. aurum* (21 h), DNP was also added 1 h prior to the addition of norfloxacin. Norfloxacin accumulation by *M. aurum* was unaffected by the presence of 1 and 2 mM DNP. In all experiments (*n* = 3), whether the metabolic inhibitor was added before or after the addition of norfloxacin, an SSC of 160 to 180 ng of norfloxacin/mg of cells was obtained (data not shown).

To determine if norfloxacin is actively effluxed from *M. smegmatis*, 2 mM DNP was added 5 min after the addition of norfloxacin and the effect on accumulation was monitored. As with *M. aurum*, norfloxacin accumulation in *M. smegmatis* was unaffected by the addition of DNP; an SSC of 120 to 140 ng of norfloxacin/mg of cells was obtained (data not shown).

### TABLE 1. Comparison of silicon oil and centrifugation methods

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Silicon oil separation method</th>
<th>Centrifugation separation method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level (ng/mg) of binding at 0°C</td>
<td>Level (ng/mg) of accumulation at 37°C</td>
</tr>
<tr>
<td>5</td>
<td>241</td>
<td>330</td>
</tr>
<tr>
<td>10</td>
<td>154</td>
<td>259</td>
</tr>
</tbody>
</table>
Effect of ethambutol on norfloxacin accumulation by *M. aurum*. To study the effect of ethambutol on norfloxacin accumulation, ethambutol (0.5 or 1 µg/ml) was added to the growing *M. aurum* culture 12, 24, or 48 h prior to cell harvesting and was added 10 min prior to the addition of norfloxacin to ensure the presence of ethambutol throughout the experiment. These times should produce an *M. aurum* culture that had undergone one, two, or three replication cycles in the presence of ethambutol. In all experiments, an SSC of approximately 180 ng of norfloxacin/mg of cells (data not shown) was obtained. The activity of norfloxacin, with or without ethambutol (0.5 or 1 µg/ml), was studied to determine if ethambutol affected the MIC of norfloxacin for *M. aurum*: the MIC was unaltered.

Effect of Tween 80 on norfloxacin accumulation by *M. aurum*. To determine the effect of Tween 80 in the growth medium on norfloxacin accumulation, cells were grown either in 7H9 alone or in 7H9 supplemented with 0.05% Tween 80. In all experiments, an SSC of approximately 180 ng of norfloxacin/mg of cells (data not shown) was obtained (data not shown) whether Tween 80 was present or not. Furthermore, the MIC of norfloxacin for *M. aurum* was unaltered in the presence of Tween 80.

DISCUSSION

A modified fluorescence method was used to determine the level of accumulation of norfloxacin by *M. aurum* A+ and *M. smegmatis* mc²155 and to study the mechanism of norfloxacin accumulation by *M. aurum*. The fluorescence method exploits the natural fluorescence of the quinolone nucleus and has been used extensively for measuring the level of quinolone accumulation by bacteria (11, 22, 24).

We have already reported that for ¹⁴C-rifampin accumulation by *S. aureus* and *E. coli*, the silicon oil method gave rise to SSCs higher than those obtained by a chilled centrifugation method similar to that used for fluoroquinolones (40). Recently, Lopez-Hernandez et al. (21) also reported higher accumulation values when using the silicon oil method compared with those obtained by the fluorescence method to measure the level of accumulation of fluoroquinolones in *Acinetobacter baumannii*. Therefore, the silicon oil method described by Li et al. (19) was used to study the level of accumulation of norfloxacin by *M. smegmatis*. The silicon oil method gave much greater values at 37 and 0°C than the fluorescence method. Therefore, although the two methods give similar results for intracellular accumulation, cell adsorption (values at 0°C) must be calculated when the silicon oil method is used. Although the silicon oil method has the advantage of quick separation of cells from drug, the high SSCs obtained as a result of not including a cell washing stage can be misleading.

Norfloxacin accumulation by mycobacteria showed kinetics similar to those reported for other bacterial species, such as *E. coli* and *S. aureus* (28). Furthermore, the concentration of norfloxacin accumulated by both species of mycobacteria falls within the range of concentrations previously reported for gram-positive and gram-negative bacteria (2, 22, 24).

The permeability of the mycobacterial cell wall, at least to hydrophilic agents, appears to vary greatly. For example, the permeability of *M. chelonae* to β-lactams is about 3 orders of magnitude lower than that of the *E. coli* outer membrane and 10 times lower than the permeability of *P. aeruginosa* (4). However, this study shows that the level of accumulation of norfloxacin by *M. aurum* and *M. smegmatis* is similar to or even slightly higher than that by *E. coli* and *S. aureus*. The SSCs of norfloxacin in *M. smegmatis* mc²155 reported by Liu et al. (20) were similar to those obtained in the current study. However, Cortí et al. (10) reported an SSC of 35 ng of norfloxacin/mg of cells for a different strain of *M. smegmatis* (strain CNMC 7326; MIC, 4 or 5 µg/ml). Kocagoz et al. (17) reported an SSC of 110 pmol of norfloxacin/mg of cells (approximately 35 ng of norfloxacin/mg of cells) for *M. tuberculosis* H37Rv, suggesting that *M. tuberculosis* is less permeable to norfloxacin than *M. aurum* and *M. smegmatis*. Clearly, the permeability of mycobacteria to norfloxacin, and presumably other agents, varies not only between mycobacterial species but also between different strains of the same species.

The uptake of quinolones by gram-negative bacteria is proposed to occur by passive diffusion through porin channels or the lipid bilayer, depending on the hydrophobicity of the quinolone (28), and the uptake of quinolones by gram-positive bacteria is thought to involve simple diffusion across the cytoplasmic membrane (11). Norfloxacin is a very hydrophilic quinolone, with a partition coefficient in *n*-octanol–phosphate buffer (pH 7.2) of 0.022 (2). Norfloxacin accumulation by *M. aurum* was nonsaturable over the concentration range studied (0 to 100 µg/ml), and transport was apparently unaffected by the presence of DNP. Therefore, it is proposed that norfloxacin enters *M. aurum* by an energy-independent, nonsaturable porin pathway. Mycobacterial porins have been characterized in *M. chelonae* and *M. smegmatis* and are thought to exist in all mycobacterial species, but they are proposed to be both less abundant and less efficient than other bacterial porins (33, 34). However, at least for norfloxacin, the mycobacterial cell wall does not act as a significant barrier to drug accumulation.

Although the major form of quinolone resistance found in bacteria is altered DNA gyrase and/or topoisomerase IV (30), energy-dependent quinolone efflux has been shown to be a major contributory factor in quinolone resistance in *S. aureus* (22). In *S. aureus* the gene encoding this system, *norA*, has been cloned (36). The NorA protein is a multidrug efflux pump that confers an 8- to 64-fold increase in the MICs of hydrophilic quinolones and a 2-fold increase in the MICs of hydrophobic quinolones. Recently, active efflux of norfloxacin has been reported in a quinolone-resistant strain of *M. smegmatis* mc²155, and the gene encoding this system, *lfrA*, has been cloned (20). The LfrA efflux pump is homologous to QacA from *S. aureus* but not to NorA. QacA confers resistance to ethidium and other organic cations, such as chlorhexidine, via proton motive force-dependent efflux (27). However, NorA for NorA, hydrophilic quinolones are preferentially pumped out by LfrA. In the present study, DNP had no effect on norfloxacin accumulation by *M. aurum* or *M. smegmatis*, and therefore, a quinolone efflux system is not suspected of having significant activity in wild-type mycobacteria.
Quinolones are zwitterionic and may exist in several forms. Cotti et al. (10) suggest that the cationic form of norfloxacin may bind to negatively charged compounds in the mycobacterial cell wall, such as the phospholipids, and this could contribute to imprecise measurements of intracellular norfloxacin. Those investigators showed that by increasing the pH of the wash buffer from 7 to 9, the amount of norfloxacin adsorbed by the M. smegmatis cell wall during an uptake experiment was reduced. In this study, the amount of norfloxacin adsorbed by the M. aurum cell wall at 0°C was reduced when a pH 9 wash buffer was used. However, at 37°C the concentration of accumulated norfloxacin was slightly higher with a pH 9 wash buffer compared to that with a pH 7 wash buffer (Fig. 4). If the pH 9 buffer was reducing the amount of norfloxacin adsorption to the mycobacterial cell wall, the opposite effect would be expected. Therefore, pH 7 wash buffer was used throughout.

It is thought that ethambutol may increase the activities of some antimycobacterial drugs by increasing the permeability of the mycobacterial cell wall to these agents (31). Indeed, the MICs of rifampin for M. aurum and M. smegmatis were reduced in the presence of sub-MICs of ethambutol (41). Therefore, the effect of ethambutol on norfloxacin accumulation was studied. However, no difference in the final concentration of norfloxacin by M. aurum cell wall at 0°C was reduced when a pH 9 wash buffer was used. However, at 37°C the concentration of accumulated norfloxacin was slightly higher with a pH 9 wash buffer compared to that with a pH 7 wash buffer (Fig. 4). If the pH 9 buffer was reducing the amount of norfloxacin adsorption to the mycobacterial cell wall, the opposite effect would be expected. Therefore, pH 7 wash buffer was used throughout.

In the presence of ethambutol or Tween 80, those investigators showed that by increasing the pH of the wash buffer from 7 to 9, the amount of norfloxacin adsorbed by the M. smegmatis cell wall during an uptake experiment was reduced. In this study, the amount of norfloxacin adsorbed by the M. aurum cell wall at 0°C was reduced when a pH 9 wash buffer was used. However, at 37°C the concentration of accumulated norfloxacin was slightly higher with a pH 9 wash buffer compared to that with a pH 7 wash buffer (Fig. 4). If the pH 9 buffer was reducing the amount of norfloxacin adsorption to the mycobacterial cell wall, the opposite effect would be expected. Therefore, pH 7 wash buffer was used throughout.

ACKNOWLEDGMENT

K.J.W. was funded by a studentship from the Glaxo Wellcome Action TB initiative.

REFERENCES