Gyrase Mutations in Laboratory-Selected, Fluoroquinolone-Resistant Mutants of *Mycobacterium tuberculosis* H37Ra

TANIL KOCAGÖZ,[1,2] CORINNE J. HACKBARTH,[1] IBRAHIM ÜNSAL,[1,2] EMIKO Y. ROSENBERG,[3] HIROSHI NIKAIDO,[3] AND HENRY F. CHAMBERS[1]*

Department of Medicine, University of California, San Francisco, California 94110[1]; Department of Molecular and Cell Biology, University of California, Berkeley, California 94720[3]; and Department of Microbiology, Hecettepe University, Ankara, Turkey[2]

To characterize mechanisms of resistance to fluoroquinolones by *Mycobacterium tuberculosis*, mutants of strain H37Ra were selected in vitro with ofloxacin. Their quinolone resistance-determining regions of *gyrA* and *gyrB* were amplified and sequenced to identify mutations in gyrase A or B. Three types of mutants were obtained: (i) one mutant (TKp1) had no mutations in *gyrA* or *gyrB*; (ii) mutants that had single missense mutations in *gyrA*, and (iii) mutants that had two missense mutations resulting in either two altered gyrase A residues or an altered residue in both gyrases A and B. The TKp1 mutant had slightly reduced levels of uptake of [14C]norfloxacin, which was associated with two- to fourfold increases in the MICs of ofloxacin, ciprofloxacin, and sparfloxacin. Gyrase mutations caused a much greater increase in the MICs of fluoroquinolones. For mutants with single *gyrA* mutations, the increases in the MICs were 4- to 16-fold, and for mutants with double gyrase mutations, the MICs were increased 32-fold or more compared with those for the parent. A *gyrA* mutation in TKp1 secondary mutants was associated with 32- to 128-fold increases in the MICs of ofloxacin and ciprofloxacin compared with the MICs for H37Ra and an eight-fold increase in the MIC of sparfloxacin. Sparfloxacin was the most active fluoroquinolone tested. No sparfloxacin-resistant single-step mutants were selected at concentrations of >2.5 µg/ml, and high-level resistance (i.e., MIC, ≥5 µg/ml) was associated with two gyrase mutations. Mutations in *gyrB* and possibly altered levels of intracellular accumulation of drug are two additional mechanisms that may be used by *M. tuberculosis* in the development of fluoroquinolone resistance. Because sparfloxacin is more active in vitro and selection of resistance appears to be less likely to occur, it may have important advantages over ofloxacin or ciprofloxacin for the treatment of tuberculosis.

New drugs are needed for the control of tuberculosis because resistance to classical antituberculosis drugs has dramatically increased (39). Fluoroquinolone antibiotics are active in vitro against *Mycobacterium tuberculosis* isolates (7, 20) and are increasingly being used in combination with other agents to treat tuberculosis. As with all antituberculosis agents, *M. tuberculosis* strains can develop resistance to fluoroquinolones (42). Two principal mechanisms of resistance to fluoroquinolones have been identified in other bacterial species: (i) alteration of the target protein, e.g., DNA gyrase or topoisomerase IV (4, 12, 16), and (ii) decreased levels of accumulation of drug within the cell (4, 11, 18, 34, 38).

DNA gyrase is composed of two A and two B subunits, encoded by *gyrA* and *gyrB*, respectively (36). Mutations in *gyrA* have been associated with high-level resistance to fluoroquinolones in several bacterial species (5, 14, 33, 40, 41, 45, 47), including *M. tuberculosis* strains (42). DNA gyrase is the primary target of fluoroquinolones in *Escherichia coli* isolates. Although subunit A is considered to be the principal target of fluoroquinolones, mutations in subunit B have also been identified in resistant mutants of *E. coli* (48). *gyrB* mutations associated with resistance have not been reported for *M. tuberculosis* isolates. In *Staphylococcus aureus* strains, first-step fluoroquinolone-resistant mutants have mutations in *gylA*, which encodes the A subunit of topoisomerase IV, but not in *gyrA*, suggesting that

the primary target of fluoroquinolones in this species is topoisomerase IV and not gyrase (12).

Decreased levels of drug accumulation, associated with a change in outer membrane proteins, have been identified as a second mechanism of fluoroquinolone resistance in gram-negative bacteria (3, 4, 10, 25, 26, 46). An energy-dependent efflux pump in association with altered outer membrane proteins appears to play a critical role in reducing intracellular drug concentrations by pumping drug back across the cytoplasmic membrane and, in some cases, the outer membrane (8, 16, 22, 23, 29, 32). The *S. aureus norA* gene product is an energy-dependent efflux pump whose overexpression has also been associated with fluoroquinolone resistance (27, 49).

The contributions of both of these mechanisms to the development of fluoroquinolone resistance were examined in laboratory-derived fluoroquinolone-resistant mutants of the attenuated strain *M. tuberculosis* H37Ra. The regions of the gyrase genes that are associated with fluoroquinolone resistance were sequenced, and the level of accumulation of norfloxacin by whole cells was assayed.

MATERIALS AND METHODS

Antibiotics and reagents. Ofloxacin was a gift from the R. W. Johnson Pharmaceutical Institute, ciprofloxacin was from Miles Corporation, and sparfloxacin was from Rhône-Poulenc Rorer. [*piperazine*-14C]norfloxacin was a gift from Merck Sharpe & Dohme.

Strains and growth conditions. *M. tuberculosis* H37Ra was grown in Middlebrook 7H9 broth (Difco) supplemented with bovine serum albumin, glucose, and catalase (ADC; Sigma).

In vitro isolation of ofloxacin-resistant strains. Fluoroquinolone-resistant mutants of *M. tuberculosis* H37Ra were obtained by serial passage in either 7H9-ADC broth or on 7H10-ADC agar containing ofloxacin. Primary mutants were

TABLE 1. Point mutations in *gyrA* and *gyrB* and MICs of fluoroquinolones for *M. tuberculosis* H37Ra and ofloxacin-selected primary and secondary mutants[a]

Strain			Gyrase subunit mutations[b]		MIC (μg/ml)		
Parent	Primary mutant	Secondary mutant	*gyrA*	*gyrB*	Ofloxacin	Ciprofloxacin	Sparfloxacin
H37Ra			None	None	0.62	0.31	0.15
	TKp1		None	None	1.25	1.25	0.31
		TKp11-14	$D_{94} \rightarrow Y$ (GAC→TAC)	None	20	40	2.5
	TK2		$S_{91} \rightarrow P$ (TCG→CCG)	None	2.5	2.5	0.62
	TK3		$A_{90} \rightarrow V$ (GCG→GTG)	None	5	2.5	1.25
		TK31	$A_{90} \rightarrow V$	$D_{495} \rightarrow R$ (GAC→AAC)	160	40	5
		TK32	$A_{90} \rightarrow V + S_{91} \rightarrow P$ (TCG→CCG)	None	>160	80	20
		TK33	$A_{90} \rightarrow V + D_{94} \rightarrow G$ (GAC→GGC)	None	>160	80	10
	TK4		$D_{94} \rightarrow H$ (GAC→CAC)	None	5	5	1.25
	TK5		$A_{90} \rightarrow V$ (GCG→GTG)	None	5	5	1.25
		TK51	$A_{90} \rightarrow V$	$D_{495} \rightarrow H$ (GAC→CAC)	>160	80	5
		TK52	$A_{90} \rightarrow V$	$D_{495} \rightarrow H$ (GAC→CAC)	>160	80	10
		TK53	$A_{90} \rightarrow V + D_{94} \rightarrow G$ (GAC→GGC)	None	>160	40	20
		TK54	$A_{90} \rightarrow V + D_{94} \rightarrow G$ (GAC→GGC)	None	40	40	5

[a] Nucleotide and residue numbers are based upon the H37Rv sequence (GenBank accession number L27512).

[b] Amino acid mutation, with nucleotide substitution given in parentheses.

obtained by passage in broth containing ofloxacin at the MIC, 2× the MIC, or 4× the MIC. After 3 weeks of incubation at 37°C, single colonies were isolated from an appropriately diluted sample by subculturing them onto drug-free agar. To select secondary mutants, broth cultures were prepared from single colonies and were again inoculated onto Middlebrook 7H10-ADC agar plates containing ofloxacin at concentrations at or above the MIC. After 3 weeks, individual colonies growing on the plates containing an ofloxacin concentration greater than the MIC were isolated.

In vitro susceptibility tests. The MICs of ofloxacin, ciprofloxacin, and sparfloxacin, for *M. tuberculosis* H37Ra and its fluoroquinolone-resistant mutants were determined by the agar dilution method (21). In order to determine the mutation rates, a population analysis of parent and mutant cultures exposed to increasing concentrations of ofloxacin, ciprofloxacin, or sparfloxacin was performed. For each organism, 10^8 to 10^9 CFU from a broth culture was inoculated onto antibiotic-containing agar, and the numbers of CFU were counted after 4 to 6 weeks of incubation at 37°C.

Purification of DNA. Genomic DNA was obtained as described previously (19). Briefly, 1 ml (10^8 to 10^9 cells) of *M. tuberculosis* organisms in broth was transferred to a 1.5-ml microcentrifuge tube and washed two times with 10 mM Tris-HCl–1 mM EDTA (pH 8.0) (TE). After the last centrifugation the pellet was resuspended in TE and the mixture was boiled for 20 min. Cellular debris was removed from the boiled sample by centrifugation, and the lysate was used for PCR amplification of the gyrase genes.

Sequence analysis of putative quinolone-binding regions of *gyrA* and *gyrB*. Genomic DNA was amplified by PCR. Oligonucleotide primers were synthesized at the Biomedical Resource Center (University of California, San Francisco). Primers Gyr A1 (5′-CAGCTACATCGACTATGCGA) and Gyr A2 (5′-GGGC TTCGGTGTACCTCAT) were used to amplify a 320-bp region of *gyrA*, and primers Gyr B1 (5′-CCACCGACATCGGTGGATT) and Gyr B2 (5′-CTGCC ACTTGAGTTTGTACA) were used for the PCR amplification of a 375-bp fragment of *gyrB* (42). The PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 mM $MgCl_2$, 200 mM (each) deoxynucleoside triphosphates, 0.5 mM (each) primer, 2 μl of DNA lysate, and 2.5 U of *AmpliTaq* DNA polymerase (Perkin-Elmer). The Mg^{2+} concentration was critical, and amplification was possible only in the range of 1.0 to 1.4 mM. Amplification was performed by denaturation for 3 min at 95°C and then 35 cycles of 95°C, 55°C, and 72°C (1 min each), with a final 5-min extension step at 72°C. The PCR products were purified (QIAquick-spin PCR purification kit; Qiagen) and sequenced (*AmpliTaq* cycle sequencing kit; Perkin-Elmer) according to the manufacturers' instructions. The primers used for sequencing were end-labeled with [γ-³³P]ATP (Amersham), and nucleotide differences were confirmed by sequencing both strands.

Assay of drug accumulation in cells. The assays used to determine the rate of accumulation of [¹⁴C]norfloxacin were an adaptation of the method used by Li et al. (23) to determine the rate of accumulation of tetracycline in *Pseudomonas aeruginosa* isolates. Cells grown to the exponential phase were centrifuged, washed, and resuspended in 50 mM KPO_4 (pH 7.0) to a density of 10 to 20 mg (dry weight) ml⁻¹. The assay was performed at 22°C with aeration. At various time points after the addition of [¹⁴C]norfloxacin (final concentration, 40 μM; specific activity, 14.9 mCi/mmol), a 0.05-ml aliquot was removed and was diluted into 1.5 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 M LiCl. The mixture was immediately filtered through a 0.45-μm-pore-size cellulose acetate membrane filter (Millipore) and was washed with 5 ml of the same buffer. The dried filters were weighed, and the radioactivity retained in the cells was determined by liquid scintillation counting (Beckman L5C scintillation counter).

For experiments examining whether an energy-dependent efflux pump is present in *M. tuberculosis* isolates, the level of accumulation of norfloxacin was also determined in the presence and absence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma), a proton conductor that disrupts the proton motive force across the membrane. After 10 min, samples were divided and either 250 or 500 μM was added to one of the aliquots.

Assay of β-lactam permeation rate across the cell wall. The rate of permeation of the β-lactam across the cell wall was determined by coupling the penetration of cephaloridine across the cell wall with its hydrolysis by periplasmic β-lactamase as described previously (17). *M. tuberculosis* H37Ra contained a sufficient level of constitutive β-lactamase (V_{max}, 0.7 μmol of benzylpenicillin per min/mg of protein hydrolyzed at 22°C), but after a few washings in buffer there was no detectable β-lactamase activity in the medium; thus, it was well-suited for this assay. Because cells spontaneously aggregated, the direct spectrophotometric assay (17) was not possible. Thus, washed, intact cells were incubated with 1 mM cephaloridine at either 22 or 32°C, portions were rapidly centrifuged at 0, 15, 30, and 45 min, and the amount of unhydrolyzed β-lactam antibiotic remaining in the supernatant was determined by scanning absorption spectra in the 240- to 300-nm range after proper dilution.

RESULTS

Selection of fluoroquinolone-resistant mutants of *M. tuberculosis* H37Ra. The MIC of ofloxacin for *M. tuberculosis* H37Ra was 0.62 μg/ml. Five primary mutants that grew after passage of H37Ra in broth containing ofloxacin at its MIC were selected for study. For primary mutants there were 2- to 8-fold increases in the MICs of ofloxacin, 4- to 16-fold increases in the MICs of ciprofloxacin, and 2- to 8-fold increases in the MICs of sparfloxacin (Table 1). When they were inoculated onto agar plates containing increasing concentrations of ofloxacin, 11 secondary mutants for which ofloxacin MICs ranged from 20 to >160 μg/ml were obtained.

Nucleotide sequences of putative quinolone resistance regions of gyrase genes. In order to determine whether the increased levels of resistance to fluoroquinolones detected in the primary mutants were associated with a gyrase mutation, the proposed quinolone-binding region from the *gyrA* gene was amplified and sequenced for the parent strain H37Ra and the five mutants. The nucleotide sequence of the quinolone-binding region of *gyrA* from primary mutant TKp1 was identical to that from strain H37Ra, whereas *gyrA* mutations were present in the remaining four primary fluoroquinolone-resistant mutants (Table 1). TK3 and TK5 had single nucleotide changes that resulted in a substitution of an alanine residue at position 90 for a valine residue ($Ala_{90} \rightarrow Val$). $Ser_{91} \rightarrow Pro$ and $Asp_{94} \rightarrow His$ substitutions were identified in TK2 and TK4, respectively.
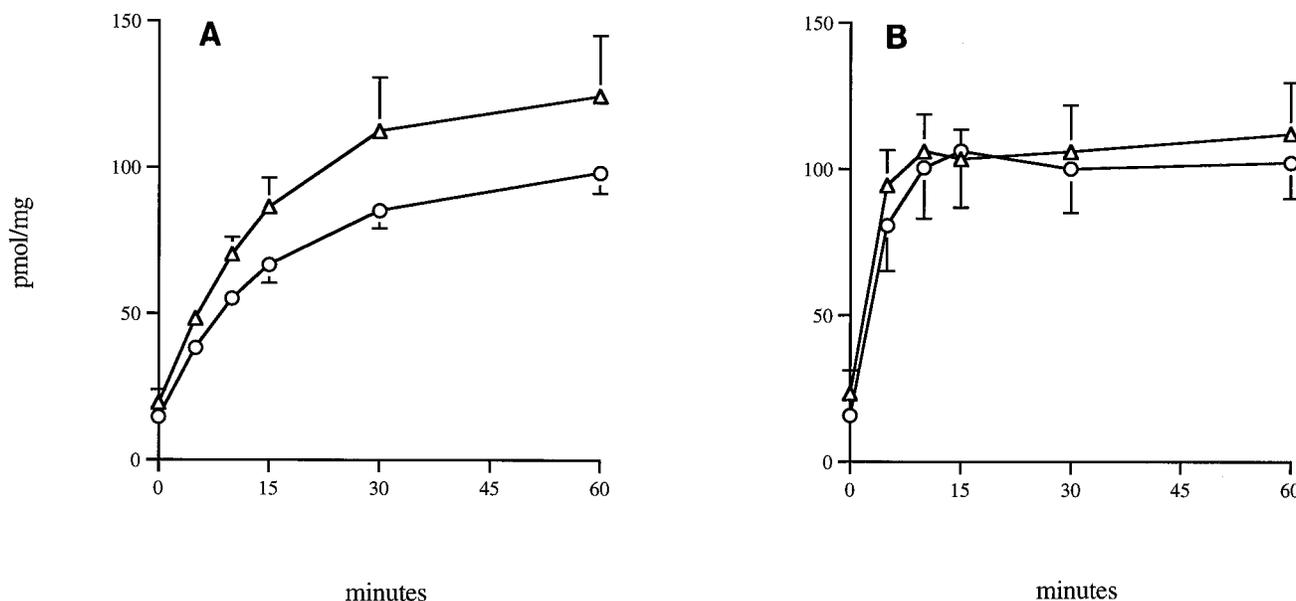
FIG. 1. Accumulation of 10 mM [^{14}C]norfloxacin in intact cells of *M. tuberculosis* H37Ra and TKp1 at 22°C (A) and 32°C (B). After the addition of 10 mM [^{14}C]norfloxacin (specific activity, 14.9 mCi/mmol), aliquots were removed at 1, 5, 10, 15, 30, and 60 min as described in the text. The concentrations of accumulated norfloxacin (picomoles per milligrams of cells) were determined and plotted over time for *M. tuberculosis* H37Ra (triangles) and a primary mutant, TKp1 (circles). Error bars depict standard error of the mean for $n = 4$ (A) and $n = 3$ (B).

Secondary mutants were selected by a single passage of the primary mutants onto ofloxacin-containing agar. Four mutants derived from TKp1 (represented by TKp11) had a single nucleotide change that resulted in a Asp$_{94}$→Tyr amino acid change in GyrA. This mutation was associated with a 16- to 32-fold increase in the ofloxacin and ciprofloxacin MICs compared with the MICs for TKp1 and an 8-fold increase in the MIC of sparfloxacin (Table 1). Four secondary mutants (TK32, TK33, TK53, and TK54) had an additional *gyrA* mutation. This second *gyrA* mutation was associated with 8- to ≥32-fold increases in the ofloxacin MICs, 8- to 32-fold increases in the ciprofloxacin MICs, and 4- to 16-fold increases in the sparfloxacin MICs compared with those for the mutants with single *gyrA* mutations (Table 1).

A region of *gyrB* where mutations that are associated with quinolone resistance have been identified (48) was amplified and directly sequenced from H37Ra as well as from the mutant strains. Additional mutations were not detected in the *gyrB* region from any of the primary mutants or the four secondary mutants that had a double *gyrA* mutation (Table 1). However, three other secondary mutants, TK31, TK51, and TK52, had a substitution of a guanine at position 1444 for an adenine (G$_{1444}$→A) or a G$_{1444}$→C substitution in *gyrB*, resulting in the replacement of Asp$_{495}$ with either arginine or histidine, respectively. For these *gyrA*-*gyrB* double mutants, ofloxacin and ciprofloxacin MICs were ≥160 and 40 to 80 μg/ml, respectively, compared with sparfloxacin MICs of 5 to 10 μg/ml (Table 1).

Accumulation of norfloxacin by *M. tuberculosis* H37Ra and its quinolone-resistant mutants. Since the TKp1 mutant lacked gyrase mutations, the level of uptake of [^{14}C]norfloxacin was assayed to determine whether decreased levels of accumulation (decreased permeability or increased efflux, or both) was a possible mechanism for its low-level resistance. This mutant had decreased levels of accumulation of [^{14}C]norfloxacin in intact cells compared with those of *M. tuberculosis* H37Ra at 22°C (Fig. 1A). The levels of accumulation of [^{14}C]norfloxacin by heat-killed cells of both *M. tuberculosis* H37Ra and TKp1

were similar (data not shown), indicating that the difference observed with intact cells was due to a specific interaction and was not due to different degrees of partition into the cell lipids. The decreased level of accumulation of norfloxacin in this mutant was stable after five passages in medium containing no drug. None of the other primary mutants (all of which had *gyrA* mutations), nor the secondary mutants derived from them, had altered levels of fluoroquinolone accumulation, suggesting that decreased levels of drug accumulation were not involved in the resistance of these strains.

Two mechanisms that could account for decreased levels of intracellular accumulation of fluoroquinolones have been identified in nonmycobacterial species. Decreases in the permeability of the bacterial outer membrane or cell wall are expected to decrease the rate of entry of drugs. Energy-dependent efflux of fluoroquinolones is known to occur through NorA of *S. aureus* and through the MexAB system in *P. aeruginosa* (23, 24, 35). With the former mechanism alone, however, even cells with lower levels of permeability are predicted to accumulate the same final level of the drug (21). In four experiments in which we compared TKp1 directly with parent strain H37Ra, the final steady-state level of accumulation of norfloxacin in TKp1 was lower than that in H37Ra (Fig. 1A), suggesting that active efflux may contribute to the increased level of resistance of TKp1. Seeking additional evidence for an efflux mechanism, we tried to inhibit the putative efflux pump by collapsing the proton motive force through the addition of CCCP, a protonophore. CCCP did increase the level of accumulation of norfloxacin by about 20% in both TKp1 and H37Ra. Although this result is consistent with the presence of an active efflux pump (23), it is not decisive evidence, because norfloxacin tends to be excluded somewhat from the cytoplasm because of the presence of a proton gradient, and a modest increase in the level of norfloxacin accumulation is predicted to occur upon the addition of CCCP, even in the complete absence of a specific active efflux proteins (see Figure 4 of Nikaido and Thanassi [31]).

In gram-negative bacilli, decreased levels of accumulation

can be due to impaired diffusion of drug across the outer membrane, often in association with decreased levels of porins that form the channels that allow small polar molecules to enter the cell. To assess the penetration mechanisms of fluoroquinolones across the outer cell wall, the levels of accumulation of [^{14}C]norfloxacin were assayed at both 22 and 32°C. If drug primarily enters the cell via a protein channel, then increasing the temperature from 22 to 32°C would be expected to have only a slight effect (e.g., a 20 to 30% increase) on the drug accumulation rate. On the other hand, if a drug enters via the lipid domains of the cell wall, such a temperature shift would cause a larger ($\geq$100%) increase in the penetration rate (28). A 100 to 150% increase in the initial accumulation rate (i.e., within the first 10 min of the assay) was observed at 32°C compared with the rate at 22°C (Fig. 1B) for both TKp1 and H37Ra, and the differences between the strains in [^{14}C]norfloxacin uptake that were observed at 22°C were abolished. This result is consistent with the penetration of fluoroquinolone at least partially though the lipid domains of the cell wall.

Penetration rates of cephaloridine through the cell wall. β-Lactams have at least one negatively charged group with a low pK$_a$ value, and thus, large amounts are unlikely to traverse the lipid domains of the cell wall. Indeed, the small effects of lipophilicity and temperature on their penetration rates (17) suggest that the penetration of β-lactams occurs mainly through the porin channels known to be present in the mycobacterial cell wall (44). Since there was a modest increase in the MIC of amoxicillin-clavulanate for TKp1 compared with that for the parent, (from 0.25 to 1.0 μg/ml; determined by the BACTEC broth dilution method [15]), we thought that it would be important to measure β-lactam antibiotic permeation across the cell wall of TKp1. The TKp1 cell wall was less permeable to cephaloridine in comparison with the permeability of the cell wall of parent strain, strain H37Ra (6). The permeability coefficient of cephaloridine at 22°C was 9.4 nm/s for H37Ra and 0.7 nm/s for TKp1. We confirmed earlier results obtained with *Mycobacterium chelonae* and strain H37Ra (6) that temperature has only a small effect on β-lactam permeation for TKp1, with an average increase of 10% at 32°C compared with that at 22°C (data not shown).

Frequency of emergence of resistant mutants. The frequency at which resistant mutants emerge is an important element of treatment failure during chemotherapy of tuberculosis. To determine whether the phenotype of a reduced level of drug accumulation was associated with an increase in the frequency of high-level resistant mutants, population analyses were performed for strains H37Ra and TKp1 with increasing concentrations of ciprofloxacin, ofloxacin, or sparfloxacin. The mutation frequencies of strain H37Ra for the three fluoroquinolones were similar when the results are analyzed from the perspective of multiples of the MIC (Fig. 2). Spontaneously resistant colonies arose at a frequency of 1 in 10^6 to 1 in 10^7 when exposed to drug at 4× the MIC, and this rate decreased substantially ($\leq$1 in 10^8) when the organisms were subjected to antibiotic concentrations of 16× the MIC or higher. However, when activity was compared on the basis of weight, sparfloxacin was the most active drug. No resistant mutants of H37Ra were obtained with a single passage with sparfloxacin concentrations above 1.25 μg/ml. The frequency of resistant mutants of TKp1 at concentrations above 1.25 μg/ml was increased by 10- to 100-fold compared with that of the parent. Still, no resistant mutants of TKp1 were obtained with sparfloxacin concentrations above 2.5 μg/ml.
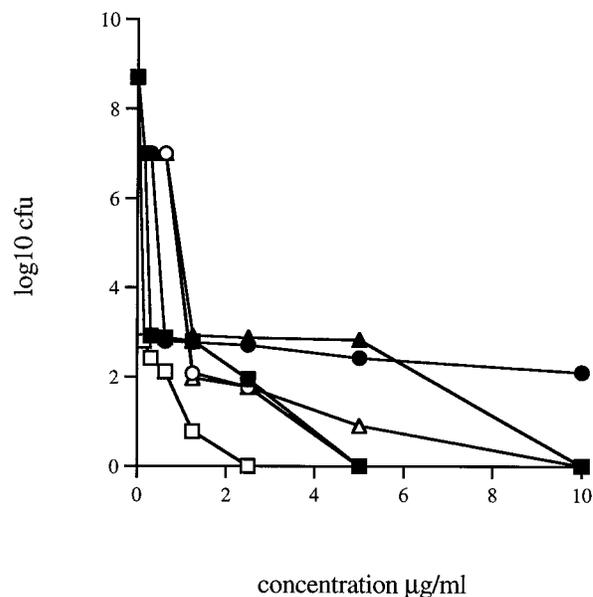
FIG. 2. Population analysis of *M. tuberculosis* H37Ra and its single-step mutant TKp1. A total of 10^8 to 10^9 CFU of each strain was plated onto agar containing 0.16, 0.32, 0.64, 1.25, 2.5, 5.0, and 10.0 μg of ciprofloxacin (circles), ofloxacin (triangles), and sparfloxacin (squares) per ml. After 4 weeks, the numbers of CFU that grew on each plate were counted, and the log CFU was plotted versus the concentration of antibiotic. Open symbols are H37Ra, and closed symbols are TKp1.

DISCUSSION

Alteration of DNA gyrase and decreased levels of drug accumulation have been identified as two principal mechanisms of resistance to fluoroquinolones in several species of bacteria (4, 11, 18, 34, 38). Not surprisingly, these experiments indicate that similar mechanisms are likely present in fluoroquinolone-resistant strains of *M. tuberculosis*. Selection for fluoroquinolone-resistant mutants of *M. tuberculosis* H37Ra by passage in the presence of ofloxacin produced two types of primary mutants: mutants with a point mutation in *gyrA* and a mutant that lacked a gyrase mutation but that exhibited reduced levels of drug accumulation. The level of resistance of these primary mutants, particularly the mutant exhibiting reduced levels of drug accumulation and for which MICs were in the susceptible range, was relatively low, e.g., two- to eightfold increases in the MIC of ofloxacin. Interestingly, these primary mutants remained susceptible to sparfloxacin (MICs, $\leq$1.25 μg/ml). The types of gyrase mutations were similar to those identified in clinical isolates and strain H37Rv, which is a virulent variant of H37Ra (1, 42).

The frequency at which resistant mutants (i.e., approximately 1 in 10^6) occur and identification of a point mutation in *gyrA* in primary mutants indicate that clinically significant resistance (MIC, >2 μg/ml) to ciprofloxacin or ofloxacin can be achieved with a single gyrase mutation. At least two mutations (two mutations in *gyrA*, mutations in *gyrA* plus *gyrB*, or a mutation affecting drug accumulation plus a *gyrA* mutation) appeared to be required for the highest level of resistance. The *gyrB* mutations, which have not been previously associated with resistance in mycobacteria, were at the same amino acid residue as has been reported in *E. coli gyrB* mutants (48). Presumably, the gyrase B subunit, although it does not contain the fluoroquinolone-binding site, participates in drug binding.

As in other species, gyrase mutations conferring resistance in *M. tuberculosis* isolates are confined to a very small region

(5, 14, 37, 40, 41, 45, 47). Laboratory-selected fluoroquinolone-resistant mutants of *M. tuberculosis* H37Ra showed exactly the same changes at GyrA residues 90, 91, and 94 with the replacement of the same amino acids previously described in clinical isolates (42). It appears that to inhibit binding of the fluoroquinolone while maintaining a functional gyrase, there is a strict preference for altering certain amino acids at specific positions. $Ala_{90}\rightarrow Val$ and $Ser_{91}\rightarrow Pro$ were the only types of amino acid changes at their respective positions. Five different amino acid substitutions at the 94th position of GyrA all resulted in the exchange of a residue with a negatively charged side chain with a residue that contained a neutral or a positively charged group. When double gyrase mutants were selected from the strains with an $Ala_{90}\rightarrow Val$ mutation, the secondary mutations occurred again either at the 91st or the 94th residue, emphasizing the importance of a preference for these sites in the development of resistance.

Laboratory selection of fluoroquinolone-resistant mutants of strain H37Ra confirms the results reported by others and accurately models what is occurring or what is likely to occur clinically (1), indicating that the H37Ra strain is suitable for investigation of fluoroquinolone resistance. The ability to use an attenuated strain such as H37Ra offers an advantage over the use of virulent strains, which require biosafety level 3 containment for experimental manipulation.

These results suggest that *gyrA* mutations are probably the first step for clinically important levels of resistance and that DNA gyrase is the primary target of fluoroquinolones. Gyrase mutations were identified in four of the five primary mutants that were examined, and higher-level resistance was associated with the acquisition of a new gyrase mutation in every case. However, as with other bacterial species, other mechanisms might also contribute to resistance. For example, Ferrero and coworkers (12) have shown that topoisomerase IV is probably the primary target for fluoroquinolones in *S. aureus* on the basis of the findings that only mutations in *grlA* are found in first-step mutants and that only after selection for higher levels of resistance is enhanced (NorA-mediated) efflux or a *gyrA* mutation found. Since the homologous genes in *M. tuberculosis* isolates were not sequenced, a contribution of a mutation at this locus to resistance cannot be excluded. Unidentified mutations might account for differences in the MICs for mutants TK33, TK53, and TK54, which have the same gyrase genotypes. However, TK53 and TK54, which were the two mutants for which fluoroquinolone MICs were different, were selected from the same parent not in broth but on agar, implying that two mutations were selected in a single step.

The contribution of altered levels of drug accumulation to resistance in TKp1, assuming that this is mechanistically related and not just a marker, was modest. An alteration in the levels of accumulation is not a required step for resistance, because no other primary or secondary mutant exhibited a similar phenotype. Moreover, selection of secondary mutants of TKp1 on agar produced mutants with a single *gyrA* mutation, further evidence that gyrase is the primary target. However, a single gyrase mutation in this background resulted in a higher level of resistance than other single mutations in the primary mutants (e.g., ofloxacin MICs, 20 versus 2.5 to 5 μg/ml), suggesting that nongyrase mutations, perhaps affecting uptake of drug, contribute to resistance. Because the primary mutants were selected in broth, it is possible that other unrecognized steps led to resistance. This does not seem likely, however, given that primary mutants were selected at the MIC, requiring a relatively small incremental increase in resistance that is readily accomplished in a single step in all other bacterial species that have been examined.

The basis of the decreased level of accumulation of fluoroquinolone by TKp1 is obscure. Decreased levels of accumulation of antimicrobial agents in bacterial cells and resistance are both often associated with low levels of permeation through the outer membrane in gram-negative bacteria (3, 4, 10, 25, 26, 46). Yet even with low levels of permeation, the penetration of many antimicrobial agents is such that equilibrium half-lives are on the order of a few minutes (50). An additional event such as enzymatic inactivation, modification of drug, or removal of drug through active efflux pumps is required to create a significant level of resistance (31). Accordingly, active efflux is a well-defined mechanism of fluoroquinolone resistance in several species of bacteria (13, 22, 23, 31, 49). Although the uptake experiments were inconclusive, they do not exclude that possibility of a coexisting active efflux mechanism, in view of the lowering of the steady-state accumulation level observed at 22°C.

There may also be a role for a low level of permeability of the mycobacterial cell envelop in producing resistance. The presence of an effective external barrier is a necessary condition for an active efflux mechanism to produce significant levels of resistance, as was demonstrated with *P. aeruginosa* isolates with their outer membranes with characteristically low levels of permeability (23, 24) and demonstrated by mathematical modeling of the tetracycline efflux process in *E. coli* isolates (43). In *E. coli* isolates it has been shown that *marR* mutations and the interaction of antibiotics with the *mar* system not only increase the level of drug efflux activity but also decrease the cell envelope permeability through decreased levels of expression of the wider porin, OmpF (2). Similar changes might be occurring in the TKp1 mutant, because the decrease in the level of penetration of cephaloridine suggests that the level of expression of porins could also be reduced. Hydrophilic antimicrobial agents, such as β-lactams, use aqueous channels (porins in the case of gram-negative bacteria) as a major route of entry into the cells. When the presence of a porin was discovered in *M. chelonae* isolates, it became clear that the low level of hydrophilic permeability of mycobacteria was due to the fact that, unlike the porins of gram-negative bacteria, this porin was a minor protein of the cell wall and produced permeability far lower than that produced by an equal weight of *E. coli* porin (44). Since aqueous channels available for penetration are scarce, some drug may penetrate through the lipids in mycobacteria. Efflux could play an important role in keeping drug concentrations inside the cell low. The precise genetic and biochemical mechanisms of permeability and efflux and whether they in fact account for the resistance of TKp1 require further study.

Apart from the altered permeability possibly due to porins, the intrinsically low levels of permeability of the lipid domains of the cell envelope may also contribute to reduced levels of drug accumulation. Mycobacteria do not have an outer membrane comparable to that of gram-negative bacteria, but the mycobacterial cell wall contains a very complex lipid bilayer on the surface, the inner leaflet of which is presumably composed of mycolic acid residues with very long chains and very low fluidity (30), which probably acts like an outer membrane and limits the penetration of many antimicrobial agents. Fluoroquinolones are relatively lipophilic drugs, and their route of penetration appears to be mainly through the lipid domains of mycobacterial cell walls. The evidence for this route of entry was that an increase in the rate of norfloxacin accumulation in *M. tuberculosis* H37Ra and TKp1 paralleled an increase in temperature. A rise in temperature increases the fluidity of the lipids and thus renders the membrane more permeable to the chemicals which use lipids as a way of entering the cells.

Sparfloxacin was significantly more active than either ciprofloxacin and ofloxacin against primary and secondary mutants. Alangaden et al (1) reported similar results. On the basis of sequence analysis and corresponding susceptibility data, two gyrase mutations were required to achieve high-level resistance to sparfloxacin, with double *gyrA* mutants expressing the highest level of resistance (MICs, 20 µg/ml). Single gyrase mutants and the slow-accumulation mutant remained susceptible to sparfloxacin (MICs, ≤1.25 µg/ml). This is within the range of the achievable concentrations of sparfloxacin in serum, which peaks at 1 to 2 µg/ml after the administration of a 400-mg oral dose (9). The apparent requirement of at least two gyrase mutations for resistance to sparfloxacin (i.e., for MICs of 4 µg/ml or higher, the clinical interpretative breakpoint for resistance to fluoroquinolones) suggests that it may be more difficult to select for resistant mutants with sparfloxacin than with other fluoroquinolones. Population analysis of strain H37Ra and the TKp1 mutant supports this hypothesis. This also may explain why Alangaden et al. (1) could not select for sparfloxacin-resistant mutants at sparfloxacin concentrations of 8 µg/ml and higher.

Since stepwise mutation is the mechanism by which *M. tuberculosis* strains develop resistance clinically, our data strongly suggest that sparfloxacin has a significant advantage compared with ofloxacin and ciprofloxacin, the two fluoroquinolones that are used to treat tuberculosis. Limited data from studies with animals indicate that sparfloxacin may have unique antimycobacterial activity compared with the activities of other fluoroquinolones (20). Unfortunately, clinical trials of sparfloxacin have not gone forward because the holder of the patent apparently has chosen to focus on developing the drug for indications other than for the treatment of tuberculosis. Given the critical need for new antituberculosis agents and the possibility that the fluoroquinolones that are now in use may be suboptimal with respect to the emergence of resistance, the decision not to develop sparfloxacin as a drug for treating tuberculosis needs to be reconsidered.

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