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Cloning and Nucleotide Sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* Genes and Detection of Quinolone Resistance Mutations

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The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* has resulted in increased interest in the fluoroquinolones (FQs) as antituberculosis agents. To investigate the frequency and mechanisms of FQ resistance in *M. tuberculosis*, we cloned and sequenced the wild-type *gyrA* and *gyrB* genes, which encode the A and B subunits of the DNA gyrase, respectively; DNA gyrase is the main target of the FQs. On the basis of the sequence information, we performed DNA amplification for sequencing and single-strand conformation polymorphism analysis to examine the presumed quinolone resistance regions of *gyrA* and *gyrB* from reference strains ($n = 4$) and clinical isolates ($n = 55$). Mutations in codons of *gyrA* analogous to those described in other FQ-resistant bacteria were identified in all isolates ($n = 14$) for which the ciprofloxacin MIC was >2 μg/ml. In addition, we selected ciprofloxacin-resistant mutants of *Mycobacterium bovis* BCG and *M. tuberculosis* Erdman and H37ra. Spontaneously resistant mutants developed at a frequency of 1 in 10^7 to 10^8 at ciprofloxacin concentrations of 2 μg/ml, but no primary resistant colonies were selected at higher ciprofloxacin concentrations. Replating of those first-step mutants selected for mutants with high levels of resistance which harbored *gyrA* mutations similar to those found among clinical FQ-resistant isolates. The *gyrA* and *gyrB* sequence information will facilitate analysis of the mechanisms of resistance to drugs which target the gyrase and the implementation of rapid strategies for the estimation of FQ susceptibility in clinical *M. tuberculosis* isolates.

The resurgence of tuberculosis and its incidence in human immunodeficiency virus-positive populations in both developing countries and the industrialized world have been accompanied by the alarming emergence of virulent multidrug-resistant tuberculosis (MDR-TB) strains in North American cities (7). Many of these strains have acquired resistance to almost all first- and second-line antituberculosis agents. For this reason, there is an increasing interest in the antimycobacterial actions of the fluoroquinolones (FQs). Against *Mycobacterium tuberculosis*, the FQs show moderate in vitro activity (4), with sparfloxacin (MIC, 0.25 to 0.5 μg/ml) perhaps being the most effective compound (17). The principal target of the quinolones is the DNA gyrase, a type II DNA topoisomerase that is composed of two A and two B subunits (30) encoded by *gyrA* and *gyrB*, respectively. Mutations in the putative FQ-binding region of the A subunit have been found to confer high-level FQ resistance in several bacterial species (8, 19, 22, 31, 33). Other mutations that confer resistance to quinolones have been found in *gyrB*, in genes that lower the intracellular concentration of the drug (although these tend to confer lower-level resistance than do the *gyrA* mutations [32, 34]), or mapped to loci involved in uncharacterized novel mechanisms of resistance to these agents (27).

The effectiveness of the FQs in the treatment of tuberculosis may depend on the frequency and mechanisms by which *M. tuberculosis* develops resistance and the ability to determine rapidly whether the strain from a particular patient remains susceptible to FQs. Rapid susceptibility determination is especially important for the slow-growing *M. tuberculosis*, because by conventional methods it can take up to 6 to 12 weeks to determine whether the infecting strain is susceptible, a period equal to the median survival time for human immunodeficiency virus-positive patients diagnosed as having MDR-TB infections (7).

In this report we describe the cloning and sequencing of the *M. tuberculosis* genes *gyrA* and *gyrB*. On the basis of those sequences, we studied clinical *M. tuberculosis* isolates to find mutations in these genes associated with FQ resistance and then used rapid techniques to identify those mutants. In addition, we selected spontaneous FQ-resistant mutants of *Mycobacterium bovis* BCG and several *M. tuberculosis* strains and describe here the frequency and nature of these mutations.

MATERIALS AND METHODS

Cloning of the *M. tuberculosis* DNA gyrase genes. A genomic library of *M. tuberculosis* H37Rv, constructed in the shuttle cosmid vector pYUB18, was the gift of Lisa Pascopella (15).

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The library was screened by colony hybridization to a 0.9-kb *Bgl*II fragment of the novobiocin-resistant *Streptomyces sphaeroides gyrB* gene on plasmid pLST182, which was provided by Eric Cundliffe and Amrik Thiara (26). To confirm that the cosmids that hybridized to *gyrB* contained the correct region of the chromosome, they were digested with appropriate restriction enzymes and were then subjected to Southern blotting and hybridization with a series of gene-specific probes: a 1.8-kb *Eco*RI-*Bam*HI fragment of the *Streptomyces coelicolor dnaA* gene, from plasmid pTB9018, given by Michael Calcutt (3), a 0.7-kb PCR fragment carrying the *oriC* locus of *Streptomyces lividans* supplied by Hilde Schrempf (35), and a fragment of *Mycobacterium smegmatis gyrA* amplified by using primers representing conserved regions of gyrase A (13). Two very similar cosmids, T776 and pIV305, hybridized to all four probes and were then used as sources of DNA to generate a series of subclones in vector pUC118 or pUC119; these were then sequenced by using the Sequenase 2.0 kit with 7-deaza-dGTP (United States Biochemicals, Cleveland, Ohio).

In vitro isolation of ciprofloxacin-resistant strains. *M. bovis* BCG Pasteur and *M. tuberculosis* H37Rv, H37Ra, and Erdman were grown in Middlebrook 7H9–OADC–Tween (15) (the OADC supplement was from Carr Scarborough Microbiologicals Inc., Decatur, Ga.) to a density of 10^8 to 10^9 CFU/ml, and 100 μl of culture was plated onto 7H10-OADC or 7H11-OADC plates containing concentrations of ciprofloxacin ranging from 0.25 to 3.0 μg/ml. The frequencies of selection of resistant strains were determined by simultaneously plating culture dilutions on plates without antibiotics. Individual resistant colonies formed on plates containing 0.5 to 2.0 μg of ciprofloxacin per ml were streaked onto plates containing the same concentration of ciprofloxacin for clonal purification and to new plates containing increasing concentrations of ciprofloxacin.

Direct identification of *gyrA* mutations in clinical isolates. On the basis of the sequence of *gyrA*, primers were designed to amplify the putative FQ-binding region of 4 reference strains, 6 ciprofloxacin-resistant laboratory mutants of the *M. tuberculosis* complex, a collection of 54 clinical isolates of *M. tuberculosis*, and 1 clinical isolate of *Mycobacterium africanum*. Strain preparation for PCR was performed by mechanical disintegration as reported previously (25). Studies of the susceptibilities of the strains to ciprofloxacin were done by radiometric methods (20).

PCR for direct sequencing and SSCP. DNA lysate preparations (2.5 μl) were used for amplification of the *gyrA* region with biotinylated primer GyrA1[b] (5′-CAGCTACATCGACTATG CGA) and GyrA2 (5′-GGGCTTCGGTGTACCTCAT). The PCR mixture (50 μl) contained 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 10% glycerol; 200 μM (each) dATP, dTTP, dCTP, and dGTP; 0.5 μM (each) primer; and 1.25 U of *Taq* DNA polymerase (Boehringer Mannheim). For single-strand conformation polymorphism (SSCP) analysis, the concentration of dCTP was reduced to 100 μM and 0.5 μl (5 μCi) of [α-^{32}P]dCTP was added to the reaction mixture. Amplification was performed for 40 cycles (1 min at 94°C, 1 min at 55°C, 1 min at 72°C); this was followed by a 10-min extension at 72°C to generate a 320-bp PCR product. For sequencing, PCR products were converted to single strands by using alkaline denaturation in conjunction with streptavidin-coated magnetic beads (Dynabeads M-280; Dynal, Oslo, Norway) as outlined previously (14). GyrA2 was used as the sequencing primer with T7 DNA polymerase (Sequenase). PCR-SSCP was performed as described previously (24). In addition, primers were designed for the *gyrB* region (5′-CCACCGACATCGGTGGATT and 5′-CTGCCACTTGAGTTTGTACA), where mutations conferring resistance to quinolones (34) have been reported to occur, and were used in PCR-SSCP analysis of 18 selected strains of *M. tuberculosis* for which ciprofloxacin MICs ranged from 0.25 to 8 μg/ml.

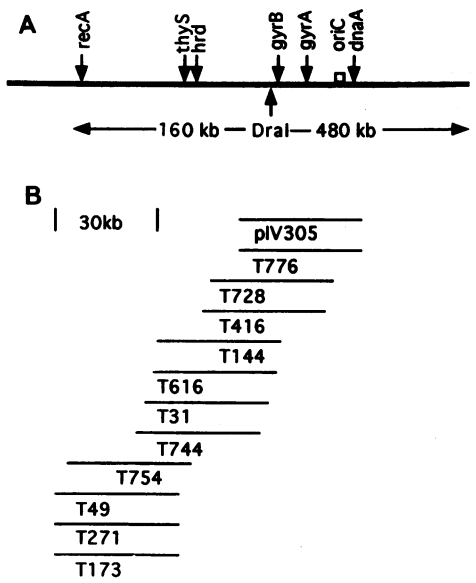

FIG. 1. Organization of a 100-kb segment of the chromosome of *M. tuberculosis* encompassing the *gyrB* and *gyrA* genes (A) and the individual cosmids comprising this part of the contig (B). The position of a rare restriction site, *Dra*I, separating two fragments of 160 and 480 kb is shown. The order and approximate positions of the genes were established by hybridization to specific probes. In the case of *oriC* and *dnaA*, the exact order is not known because these markers are on the same *Bam*HI restriction fragment.

Nucleotide sequence accession number. The complete nucleotide sequences of *gyrA* and *gyrB* have been deposited in GenBank under accession number L27512.

RESULTS

***M. tuberculosis* DNA gyrase genes.** Several screenings of mycobacterial genomic libraries with probes representing *gyrA* or *gyrB* from *Escherichia coli* and *Bacillus subtilis* were unsuccessful. However, strongly hybridizing cosmids were obtained by using the *gyrB* probe that was recently isolated from *S. sphaeroides* (26), which is phylogenetically much closer to the mycobacteria. These clones were then subjected to hybridization analysis with a set of probes for genetic loci known to be linked to *gyrB* in other gram-positive bacteria. Two cosmids (T776 and pIV305) with similar restriction profiles were found to hybridize with the *gyrA*, *gyrB*, *oriC*, and *dnaA* probes, whereas several others hybridized with one to three of these probes. A 5-kb *Bam*HI fragment carried the *oriC* and *dnaA* loci, while the DNA gyrase genes appeared to be located on several *Bam*HI fragments: 2.3 kb (*gyrA*), 0.9 kb (*gyrBA*), and 1.5 kb and two fragments of <0.5 kb (*gyrB*).

As part of the *M. tuberculosis* genome project, an ordered collection of cosmid clones is being established by a combination of fingerprinting and hybridization analyses and the resultant contig map is being correlated with the *Dra*I macrorestriction map of the chromosome. The DNA gyrase genes are located on a 480-kb *Dra*I fragment in *M. tuberculosis* H37Rv. A 100-kb segment of the contig carrying the *gyrB-dnaA* region is depicted in Fig. 1, where it can be seen that three

TABLE 1. Frequency of primary spontaneously ciprofloxacin-resistant colonies from *M. bovis* BCG and three *M. tuberculosis* strains

M. tuberculosis complex strain	Frequency of resistant colonies with the following ciprofloxacin concn (μg/ml) in the plates:			
	0.5	1.0	2.0	3.0
BCG	1×10^{-6}–1×10^{-7}	1×10^{-6}–1×10^{-7}	1×10^{-8}	0
H37Ra	—[a]	1×10^{-6}	0	0
H37Rv	—	5×10^{-6}	1×10^{-7}	0
Erdman	—	2×10^{-6}	1×10^{-8}	0

[a] —, scant growth in patches, with occasional isolated colonies.

other known genes are located nearby (6, 19a). These encode the RecA recombinase (*recA*) and the putative thymidylate synthase (*thyS*) and sigma factor (*hrd*). Details of the construction of the map will be published elsewhere.

Nucleotide sequence analysis of DNA gyrase genes. Suitable restriction fragments from cosmids pIV305 and T776 were subcloned, and their nucleotide sequences were determined. On examination of the resultant composite DNA sequence, the *gyrA* gene (2,517 bp) was found to be located 36 bp downstream of *gyrB* (2,060 bp). The deduced *M. tuberculosis* GyrB protein showed 79 and 63% amino acid similarities with the *S. sphaeroides* and *E. coli* proteins, respectively (Fig. 2). The deduced GyrA protein showed 69 and 68% similarities with the *E. coli* and *Staphylococcus aureus* GyrA proteins, respectively (Fig. 3). The gyrase of *M. tuberculosis* also exhibited considerable homology with the type IV topoisomerase of *E. coli* ParC and ParE (16): 62% amino acid similarity between ParC and GyrB and 59% amino acid similarity between ParE and GyrA. The nucleotide sequence corresponding to the GyrA region associated with FQ resistance in *M. tuberculosis* is shown in Fig. 4.

Generation of ciprofloxacin-resistant mutants. *M. bovis* BCG was plated onto 7H11 plates with 0.5 μg to 3.0 mg of ciprofloxacin per ml, and spontaneously resistant colonies appeared at the frequencies given in Table 1. Colonies were selected from plates with 0.5 or 1.0 μg of ciprofloxacin per ml, and colonies from the first two restreakings were resistant to only 0.5 to 1.0 μg of ciprofloxacin per ml. However, by the third restreaking the colonies grew well on 7H10 plates with 3 μg of ciprofloxacin per ml. Four mutants were isolated in this way, and a fifth mutant was isolated when growth was found in a flask containing *M. bovis* BCG inoculated into Middlebrook 7H9–OADC-Tween with 1 μg of ciprofloxacin per ml.

Spontaneous ciprofloxacin-resistant mutants were similarly selected from the avirulent strain *M. tuberculosis* H37Ra and from the virulent strains H37Rv and Erdman, and the frequencies with which they appeared are given in Table 1. The appearances of resistant colonies were roughly the same for all strains. Colonies resistant to 1.0 μg of ciprofloxacin per ml were found 10 to 50 times more frequently than colonies resistant to 2.0 μg of ciprofloxacin per ml. No primary resistant colonies were isolated on plates containing more than 2 μg of ciprofloxacin per ml, even when 10^8 CFU per plate was used.

SSCP and sequence evaluation of reference strains, laboratory mutants, and clinical isolates. Screening of the region for FQ resistance of *gyrA* for polymorphisms by PCR-SSCP revealed the existence of several SSCP patterns (Fig. 5 and Table 2). Two SSCP patterns were found to be associated with susceptibility to FQs. Sequencing of this 320-bp region showed that codon 95 could present a natural polymorphism encoding either serine (in *M. tuberculosis* H37Rv and H37Ra and 11 FQ-susceptible clinical isolates) or threonine (in *M. tuberculosis* Erdman, *M. bovis* BCG, *M. africanum*, and 28 susceptible clinical isolates). Eight different SSCP patterns were found to be associated with resistance to ciprofloxacin (MIC, ≥2 μg/ml) (Fig. 5). The corresponding mutations (Table 2) involved four codons within the well-described region of FQ resistance (Fig. 4) (31). One strain for which the ciprofloxacin MIC was 2 μg/ml presented with a genotype of ciprofloxacin susceptibility. Without exception, all laboratory strains, laboratory mutants, and ciprofloxacin-resistant clinical isolates were evaluated by PCR-SSCP and sequenced. Ciprofloxacin-susceptible clinical isolates were characterized by PCR-SSCP, sequencing, or both. Screening of the putative *gyrB* quinolone resistance region previously described in *E. coli* and other bacteria resulted in a single SSCP pattern for all evaluated strains.

DISCUSSION

Although the amino acid sequences of the DNA gyrases are well conserved in various bacteria (13), the *M. tuberculosis* DNA gyrase genes could not be identified by hybridization to probes from either *E. coli* or *B. subtilis* but were easily selected with a probe for the *S. sphaeroides gyrB* gene. These findings can be explained by the much closer dG+dC content of *Mycobacterium* and *Streptomyces* species, and this interpretation is supported by the cross-hybridization observed between their *dnaA* and *oriC* loci. Nevertheless, the putative FQ resistance-determining region of *M. tuberculosis gyrA* was found to be quite similar to those from other organisms; this region is located at the highly conserved (interspecies) N-terminal protein region (31), and the mutations encountered in ciprofloxacin-resistant *M. tuberculosis* clinical isolates and spontaneously arising ciprofloxacin-resistant BCG, Erdman, and H37Ra strains were located in codons equivalent to those

TABLE 2. Summary of the evaluation of *gyrA* mutations associated with FQ resistance[a]

Strain (no. of isolates)	Phenotype (MIC [μg/mg])	Genotype	
		SSCP	Mutation
Reference strains			
M. tuberculosis H37Rv (1)	S	A	S (I)
M. tuberculosis H37Ra (1)	S	A	S (I)
M. tuberculosis Erdman (1)	S	B	S (II)
M. bovis BCG (1)	S	B	S (II)
Laboratory mutants			
BCG mutant (3)	R (≥3)	C	Ala-90→Val
BCG mutant (1)	R (≥3)	D	Asp-94→Asn
Erdman mutant (1)	R (≥3)	C	Ala-90→Val
H37Ra mutant (1)	R (≥3)	E	Gly-88→Cys
Clinical isolates			
FQ resistant (3)	R (4–8)	C	Ala-90→Val
FQ resistant (1)	R (4)	F	Ser-91→Pro
FQ resistant (1)	R (≥4)	G	Asp-94→His
FQ resistant (1)	R (>8)	D	Asp-94→Asn
FQ resistant (5)	R (>8)	H	Asp-94→Gly
FQ resistant (2)	R (>8)	I	Asp-94→Tyr
FQ resistant (1)	R (4)	J	Asp-94→Ala
FQ resistant (1)	M (2)	B	S (II)
Susceptible strains (11)	S	A	S (I)
Susceptible strains (28)	S	B	S (II)
M. africanum (1)	S	B	S (II)

[a] S, susceptible (MIC, ≤1 μg/ml); S(I) and S(II), the presence of a serine or threonine polymorphism in codon 95, respectively; R, resistant; M, moderate susceptibility.

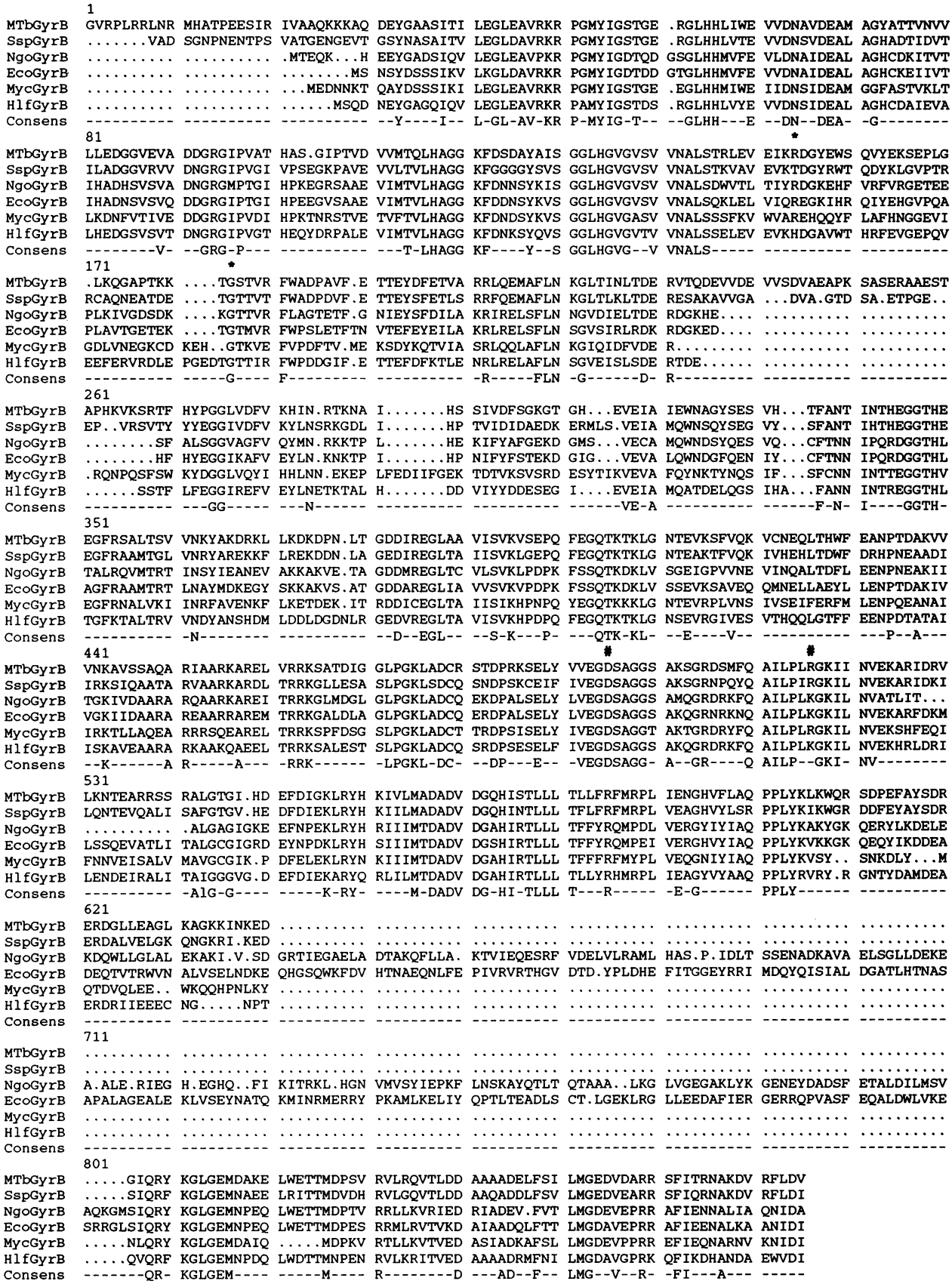

FIG. 2. Alignment of the deduced amino acid sequence of *M. tuberculosis gyrB* (MTbGyrB) with the sequences from *S. sphaeroides* (SspGyrB), *Neisseria gonorrhoeae* (NgoGyrB), *E. coli* (EcoGyrB), *Mycoplasma pneumoniae* (MycGyrB), and *Haloferax* sp. (HlfGyrB) (GenBank accession numbers Z17305, M59981, X00870, X53555, and M38373, respectively). Identical amino acids are listed on the consensus line (Consens). Marked are those positions in *gyrB* where mutations have been associated with resistance to coumarin (*) or quinolone drugs (#) in *E. coli* and other bacteria (5, 34).

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                                                                                           *               *
          1
MTbGyrA   MTDTTLAPDD ..SLDRIEPV DIEQEMQRSY IDYAMSVIVG RALPEVRDGL KPVHRRVLYA MFDSGFRPDR SHAKSARSVA ETMGNYHPHG
BsuGyrA   ......MSEQ ..NTPQVREI NISQEMRTSF LDYAMSVIVS RALPDVRDGL KPVHRRILYA MNDLGMTSDK PYKKSARIVG EVIGKYHPHG
SauGyrA   ......MAEL ..PQSRINER NITSEMRESF LDYAMSVIVA RALPDVRDGL KPVHRRILYG LNEQGMTPDK SYKKSARIVG DVMGKYHPHG
EcoGyrA   ......MSDL ..A.REITPV NIEEELKSSY LDYAMSVIVG RALPDVRDGL KPVHRRVLYA MNVLGNDWNK AYKKSARVVG DVIGKYHPHG
CjeGyrA   ......MENI FSKDSDIELV DIENSIKSSY LDYSMSVIIG RALPDARDGL KPVHRRILYA MQNDEAKSRT DFVKSARIVG AVIGRYHPHG
Consens   ---------- ---------- -I------S- -DY-MSVI-- RALP--RDGL KPVHRR-LY- ---------- ---KSAR-V- ---G-YHPHG
           ** *                     *                 #
MTbGyrA   DASIYDSLVR MAQPWSLRYP LVDGQGNFGS PDNDPPAAMR YTEARLTPLA MEMLREIDEE TVDFIPNYDG RVQEPTVLPS RFPNLLANGS
BsuGyrA   DSAVYESMVR MAQDFNYRYM LVDGHGNFGS VDGDSAAAMR YTEARMSKIS MEILRDITKD TIDYQDNYDG SEREPVVMPS RFPNLLVNGA
SauGyrA   DSSIYEAMVR MAQDFSYRYP LVDGQGNFGS MDGDGAAAMR YTEARMTKIT LELLRDINKD TIDFIDNYDG NEREPSVLPA RFPNLLANGA
EcoGyrA   DSAVYDTIVR MAQPFSLRYM LVDGQGNFGS IDGDSAAAMR YTEIRLAKIA HELMADLEKE TVDFVDNYDG TEKIPDVMPT KIPNLLVNGS
CjeGyrA   DTAVYDALVR MAQDFSMRYP SITGQGNFGS IDGDSAAAMR YTEAKMSKLS HELLKDIDKD TVDFVPNYDG SESEPDVLPS RVPNLLLNGS
Consens   D---Y---VR MAQ----RY- ---G-GNFGS -D-D--AAMR YTE------- -E-------- T-D---NYDG ----P-V-P- --PNLL-NG-
          171
MTbGyrA   GGIAVGMATN IPPHNLRELA DAVFWALENH DADEEETLAA VMGRVKGPDF PTAGLIVGSQ GTADAYKTGR GSIRMRGVVE VE.EDSRGRT
BsuGyrA   AGIAVGMATN IPPHQLGEII DGVLAVSENP DI....TIPE LMEVIPGPDF PTAGQILGRS GIRKAYESGR GSITIRAKAE IE.QTSSGKE
SauGyrA   SGIAVGMATN IPPHNLTELI NGVLSLSKNP DI....SIAE LMEDIEGPDF PTAGLILGKS GIRRAYETGR GSIQMRSRAV IE.ERGGGRQ
EcoGyrA   SGIAVGMATN IPPHNLTEVI NGCLAYIDDE DI....SIEG LMEHIPGPDF PTAAIINGRR GIEEAYRTGR GKVYIRARAE VEVDAKTGRE
CjeGyrA   SGIAVGMATN IPPHSLNELI DGLLYLLDNK DA....SLEE IMQFIKGPDF PTGGIIYGKK GIIEAYRTGR GRVKVRAKTH IE..KKTNKD
Consens   -GIAVGMATN IPPH-L-E-- ---------- D--------- -M----GPDF PT---I-G-- G---AY--GR G----R---- -E--------
          261
MTbGyrA   SLVITELPYQ VNHDNFITSI AEQVRDGKLA GISNIEDQSS DRVGLRIVIE IKRDAVAKVV INNLYKHTQL QTSFGANMLA IVDGVPRTLR
BsuGyrA   RIIVTELPYQ VNKAKLIEKI ADLVRDKKIE GITDLRDE.S DRTGMRIVIE IRRDANANVI LNNLYKQTAL QTSFGINLLA LVDGQPKVLT
SauGyrA   RIVVTEIPFQ VNKARMIEKI AELVRDKKID GITDLRDETS LRTGVRVVID VRKDANASVI LNNLYKQTPL QTSFGVNMIA LVNGRPKLIN
EcoGyrA   TIIVHEIPYQ VNKARLIEKI AELVKEKRVE GISALRDE.S DKDGMRIVIE VKRDAVGEVV LNNLYSQTQL QVSFGINMVA LHHGQPKIMN
CjeGyrA   VIVIDELPYQ TNKARLIEQI AELVKERQIE GISEVRDE.S NKEGIRVVIE LKREAMSEIV LNNLFKSTTM ESTFGVIMLA IHNKEPKIFS
Consens   -----E-P-Q -N----I--I A--V------ GI----D--S ---G-R-VI- ----A----- -NNL---T-- ---FG----A -----P----
          351
MTbGyrA   LDQLIRYYVD HQLDVIVRRT TYRLRKANER AHILRGLVKA LDALDEVIAL IRASETVDIA RAGLIE.... .......... ..........
BsuGyrA   LKQCLEHYLD HQKVVIRRRT AYELRKAEAR AHILEGLRVA LDHLDAVISL IRNSQTAEIA RTGLIEQ... .......... ..........
SauGyrA   LKEALVHYLE HQKTVVRRRT QYNLRKAKDR AHILEGLRIA LDHIDEIIST IRESDTDKVA MESLQQR... .......... ..........
EcoGyrA   LKDIIAAFVR HRREVVTRRT IFELRKARDR AHIPEALAAA LANIDPIIEL IRHAPTPAEA KTALVANPWQ LGNVAAMLER AGDDAARPEW
CjeGyrA   LLELLNLFLT HRKTVIIRRT IFELQKARAR AHILEGLKIA LDNIDEVIAL IKNSSDNNTA RDSLVAK... .......... ..........
Consens   L--------- H---V--RRT ---L-KA--R AHI---L--A L---D--I-- I--------A ---L------ ---------- ----------
          441
MTbGyrA   .......... LLDIDEIQAQ AILDMQLRRL AALERQRIID DLAKIEAEIA DLEDILAKPE RQRGIVRDEL AEIVDRHGDD RRTRIIAAD.
BsuGyrA   .......... .FSLTEKQAQ AILDMRLQRL TGLEREKIEE EYQSLVKLIA ELKDILANEY KVLEIIREEL TEIKERFNDE RRTEIVTSGL
SauGyrA   .......... .FKLSEKQAQ AILDMRLRRL TGLERNKIEA EYNELLNYIS ELEAILADEE VLLQLVRDEL TEIRDRFGDE RRTEIQLGGF
EcoGyrA   LEPEFGVRDG LYYLTEQQAQ AILDLRLQKL TGLEHEKLLD EYKELLDQIA ELLRILGSAD RLMEVIREEL ELVREQFGDK RRTEI.TANS
CjeGyrA   .......... .FGLSELQAN AILDMKLGRL TGLEREKIEN ELAELMKEIA RLEEILKSET LLENLIRDEL KEIRSKFDVP RITQI.EDDY
Consens   ---------- -----E-QA- AILD--L--L --LE------ --------I- -L--IL---- ------R-EL ---------- R-T-I-----
          531
MTbGyrA   GDVSDEDLIA REDVVVTITE TGYAKRTKTD LYRSQKRGGK GVQGAGLKQD DIVAHFFVCS THDLILFFTT QGRVYRAKAY DLPEASRTAR
BsuGyrA   ETIEDEDLIE RENIVVTLTH NGYVKRLPAS TYRSQKRGGK GVQGMGTNED DFVEHLISTS THDTILFFSN KGKVYRAKGY EIPEYGRTAK
SauGyrA   EDLEDEDLIP EEQIVITLSH NNYIKRLPVS TYRAQNRGGR GVQGMNTLEE DFVSQLVTLS THDHVLFFTN KGRVYKLKGY EVPELSRQSK
EcoGyrA   ADINLEDLIT QEDVVVTLSH QGYVKYQPLS EYEAQRRGGK GKSAARIKEE DFIDRLLVAN THDHILCFSS RGRVYSMKVY QLPEATRGAR
CjeGyrA   DDIDIEDLIP NENMVVTITH RGYIKRVPSK QYEKQKRGGK GKLAVTTYDD DFIESFFTAN THDTLMFVTD RGQLYWLKVY KIPEGSRTAK
Consens   -----EDLI- -E--V-T--- --Y-K----- -Y--Q-RGG- G--------- D--------- THD------- -G--Y--K-Y --PE--R---
          621
MTbGyrA   GQHVANLLAF QPEERIAQVI QIRGY.TDAP YLVLATRNGL VKKSKLTDFD SNRSGGIVAV NLRDNDELVG AVLCSAGDD. ..........
BsuGyrA   GIPIINLLEV EKGEWINAII PVTEFNAE.L YLFFTTKHGV SKRTSLSQFA NIRNNGLIAL SLREDDELMG VRLTDGTKQ. ..........
SauGyrA   GIPVVNAIEL GNDEVISTMI AVKDLESEDN FLVFATKRGV VKRSALSNFS RINRNGKIAI SFREDDELIA VRLTSGQED. ..........
EcoGyrA   GRPIVNLLPL EQDERITAIL PVTEFE.EGV KVFMATANGT VKKTVLTEFN RLRTAGKVAI KLVDGDELIG VDLTSGEDE. ..........
CjeGyrA   GKAVVNLINL QAEEKIMAII PTTDFD.ESK SLCFFTKNGI VKRTNLSEYQ NIRSVGVRAI NLDENDELVT AIIVQRDEDE IFATGGEENL
Consens   G----N---- ---E-I---- ---------- -----T--G- -K---L---- -----G--A- -----DEL-- ---------- ----------
          711
MTbGyrA   .......... .......... .....LLLVS ANGQSIRFSA TDEALRPMGR ATSGVQGMRF NIDDRLVSLN VVREGTY..L LVATSGGYAK
BsuGyrA   .......... .......... .....IIIGT KNGLLIRFPE TD..VREMGR TAAGVKGITL TDDDVVVGME ILEEES..HV LIVTEKGYGK
SauGyrA   .......... .......... .....ILIGT SHASLIRFPE ST..LRPLGR TATGVKGITL REGDEVVGLD VAHANSVDEV LVVTENGYGK
EcoGyrA   .......... .......... .....VMLFS AEGKVVRFRE SS..VRAMGC NTTGVRGIRL GEGDKVVSLI VPRGDG..AI LTATQNGYGK
CjeGyrA   ENQEIENLDD ENLENEESVS TQGKMLFAVT KKGMCIKFPL AK..VREIGR VSRGVTAIKF KEKNDELVGA VVIENDEQEI LSISAKGIGK
Consens   ---------- ---------- ---------- -------F-- -----R--G- ---GV----- ---------- ---------- L-----G--K
          801
MTbGyrA   RTAIEEYPVQ GRGGKGVLTV MYDRRRGRLV GALIVDDDSE LYAVTSGGGV IRTAARQVRK AGRQTKGVRL MNLGEGDTLL AIARNAEESG
BsuGyrA   RTPAEEYRTQ SRGGKGLKTA KITENNGQLV AVKATKGEED LMIITASGVL IRMDINDISI TGRVTQGVRL IRMAEEEHVA TVALVEKNEE
SauGyrA   RTPVNDYRLS NRGGKGIKTA TITERNGNVV CITTVTGEED LMIVTNAGVI IRLDVADISQ NGRAAQGVRL IRLGDDQFVS TVAKVKEDAD
EcoGyrA   RTAVAEYPTK SRATKGVISI KVTERNGLVV GAVQVDDCDQ IMMITDAGTL VRTRVSEISI VGRNTQGVIL IRTAEDENVV GLQRVAEPVD
CjeGyrA   RTNAGEYRLQ SRGGKGVICM KLTEKTKDLI SVVIVDETMD LMALTSSGKM IRVDMQSIRK AGRNTSGVIV VNVENDE.VV SIAKCPKEEN
Consens   RT----Y--- -R--KG---- ---------- ---------- ----T--G-- -R-------- -GR---GV-- ---------- ----------
          891
MTbGyrA   DDNAVDANGA DQTGN..... .......... .......... .......... .......... .......... .
BsuGyrA   DENEEEQEEV .......... .......... .......... .......... .......... .......... .
SauGyrA   EVNEDEQSTV SEDGTEQQRE AVVNDETPGN AIHTEVIDSE ENDEDGRIEV RQDFMDRVEE DIQQSSDEDE E
EcoGyrA   EEDLDTIDGS AAEGDDEIAP EVDVDDEPEE E......... .......... .......... .......... .
CjeGyrA   DEDELSDENF GLDLQ..... .......... .......... .......... .......... .......... .
Consens   ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
```

FIG. 3. Alignment of the deduced amino acid sequence of *M. tuberculosis gyrA* (MTbGyrA) with the sequences from *B. subtilis* (BsuGyrA), *S. aureus* (SauGyrA), *E. coli* (EcoGyrA), and *Campylobacter jejuni* (CjeGyrA) (modified from reference 31). Identical amino acids are listed on the consensus line (Consens). Codons where mutations in *M. tuberculosis* or other bacteria have been associated with resistance are marked with an asterisk. Tyrosine-122 (#), the active site which links to DNA, was found to be fully conserved among the tested species.

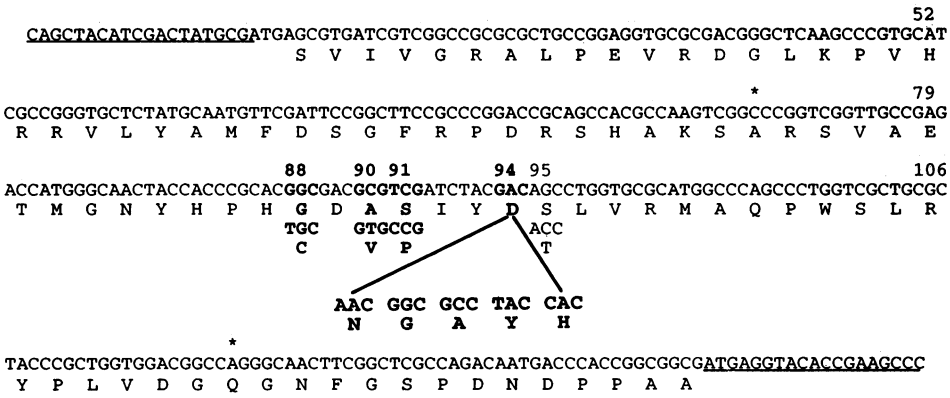

FIG. 4. Nucleotide sequence of the *gyrA* FQ resistance region amplified with primers GyrA1 and GyrA2 (underlined) corresponding to nucleotides 78 to 397 in *M. tuberculosis gyrA*. The deduced amino acid sequence is shown together with mutations in codons 88, 90, 91, and 94 (boldface type), which were found to be associated with ciprofloxacin resistance. Codon 95 may display a serine or a threonine in ciprofloxacin-susceptible strains. An asterisk indicates two additional positions were mutations associated with FQ resistance have been reported in other bacteria. The complete nucleotide sequences of *gyrA* and *gyrB* are deposited in GenBank under accession number L27512.

in the *gyrA* genes of other ciprofloxacin-resistant bacteria (8, 19, 22, 31, 33). Similarly, the regions of *gyrB* where mutations have been described in *E. coli* (5, 34) and which lead to resistance to coumarin compounds (coumermycin A1, novobiocin, and clorobiocin) and to the quinolone compound nalidixic acid were found to be highly conserved among *M. tuberculosis* and other bacteria.

We found *gyrA* mutations in all strains for which the ciprofloxacin MIC was >2 μg/ml, a level which appears to be useful in the evaluation of clinical isolates and which has been proposed as a cutoff for clinical resistance (9). The development of acquired resistance has been described after single-drug therapy of the rapidly growing mycobacterium *M. fortuitum*, and resistant strains have been isolated in vitro from *M. fortuitum* and *M. smegmatis* at frequencies of 10^{-5} to 10^{-7} (22a, 29). Spontaneously resistant mutants of *M. bovis* of BCG and *M. tuberculosis* H37Ra, H37Rv, and Erdman have been isolated at frequencies ranging from 10^{-6} to 10^{-8}. These frequencies appear to be slightly higher than those seen in the enteric bacteria (11).

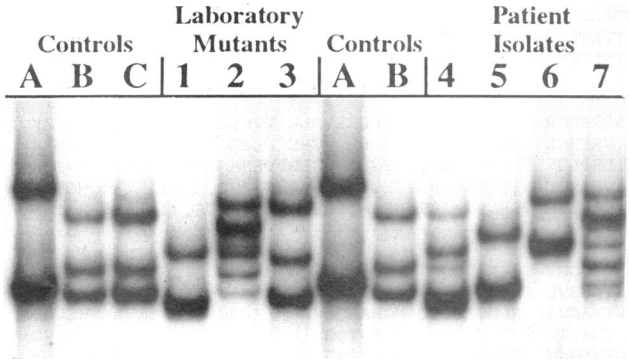

FIG. 5. Evaluation by PCR-SSCP of the FQ resistance region. The control strains *M. tuberculosis* H37Rv (lanes A), *M. bovis* BCG (lanes B), and *M. tuberculosis* Erdman (lane C) represent the two SSCP polymorphisms for FQ-susceptible strains. FQ-resistant laboratory mutants are strains of BCG with a Val-90 mutation (lane 1) and an Asn-94 mutation (lane 2) and a strain of *M. tuberculosis* H37Ra displaying a Cys-88 mutation (lane 3). An isolate from a patient (lane 4) had a mixed pattern, reflecting the presence of a mixed population: a Val-90 mutant and a susceptible organism. The other clinical isolates (lanes 5 to 7) contained the mutations Pro-91, His-94, and Asn-94, respectively.

Are these the only mutations in these strains involved in their resistance to ciprofloxacin? The initial level of ciprofloxacin at which the resistant mutants were selected, 0.5 to 2.0 μg/ml, is only two to eight times the reported MIC range (0.25 to 1.0 μg/ml) for *M. tuberculosis* (9). Primary mutants resistant to more than 2 μg of ciprofloxacin per ml were not obtained in the initial selection. Difficulties in obtaining high-level resistance in a single step and the need for repeated subculturing in low concentrations of FQs for the selection of high-level FQ-resistant strains have been described for other bacteria (1, 12, 18). In *S. aureus*, resistance to quinolones is achieved in two sequential steps: a first event resulting in a moderate increase in MICs and a second step in which a mutation in *gyrA* takes place (12). Accumulation of additional mutations in the FQ resistance region of *gyrA* may lead to even higher levels of resistance (10). Thus, in vitro data suggest that more than one step is required for the development of high-level resistance, and such multistep development of resistance may occur during therapy (21). We did not determine the MICs for our preliminary mutants and we did not look for *gyrA* mutations in our preliminary mutants prior to replating of the mutants in higher concentrations of ciprofloxacin. Those mutants are no longer available for testing, and thus, we cannot rule out the possibility that, as in *S. aureus*, our method would first select for mutations involving drug uptake or other resistance mechanisms which result in a lower level of resistance (27, 32) and that the *gyrA* mutations were a subsequent event (12). It is also possible that the *M. bovis* BCG and *M. tuberculosis* mutants or clinical isolates contained other mutations in their DNA gyrase genes. However, we screened by SSCP the region where mutations that confer quinolone resistance most commonly arise in *E. coli gyrB* (34) in 18 *M. tuberculosis* isolates (ciprofloxacin MIC range, 0.25 to 8 μg/ml) and found no polymorphisms suggestive of the presence of mutations.

The technique used in the present study to amplify and analyze the presumed FQ-binding region, PCR-SSCP, is easy and rapid and should be useful in estimating whether clinical *M. tuberculosis* isolates are resistant to FQs. One of the advantages of this method is that material can be used from minimally grown cultures and that SSCP can be performed in

an automated, nonradioactive fashion (24). In addition, it is possible to amplify and establish the *gyrA* genotypes of mycobacteria directly from sputum specimens, in which significant numbers of acid-fast bacilli are present (24). At least 10 SSCP patterns were identified (2 among susceptible strains and 8 among FQ-resistant isolates), and the identities of the mutations were established by DNA sequencing. No FQ-resistant isolates with a wild-type SSCP pattern were encountered.

Will the FQs be useful in treating MDR-TB? Although we found that the tested organisms developed a relatively high frequency of resistance developed in vitro, the frequency of emergence of resistant strains should be lower in the clinical setting, where the FQs will be part of a multidrug regimen. However, the use of FQs in a regimen that is failing or in noncompliant patients will facilitate the emergence of resistant strains. This is illustrated by two of the clinical isolates investigated here: MDR-TB strains from New York City, which shared the same rifampin resistance *rpoB* mutation (23) and appeared to be clonal on the basis of fingerprint analysis (28). However, they were found to have different *gyrA* mutations, which suggests that both of the patients from whom the two isolates were obtained were infected with the same MDR-TB organism and, later on, developed FQ resistance independently.

May specific mutations predict the level of resistance? In *E. coli*, different *gyrA* substitutions are associated with different ciprofloxacin MICs (33). This could be the case in *M. tuberculosis*, because our preliminary assessment of clinical isolates identified some mutations which were associated with ciprofloxacin MICs of 4 to 8 μg/ml, and others were associated with MICs of >8 μg/ml. It is also conceivable that not all mutations will result in resistance to different compounds from the same family; *M. tuberculosis* displays discrepant susceptibility patterns to rifabutin and rifampin that are dependent on the type and location of the mutation within the rifampin resistance region of *rpoB* (2). However, because resistance to FQs results from more than one mutational event, it may not be possible to establish a firm relationship between specific *gyrA* mutations, ciprofloxacin MICs, and variable susceptibilities to other FQs.

The knowledge of the nucleotide and amino acid sequences of the gyrases and the delineation of the antibiotic resistance regions (to quinolones and coumarin drugs) provided by the results of the present study will serve as useful bases for the assessment of the mechanisms of action and the molecular basis of resistance to new compounds of those families or other drugs which putatively target the gyrase.

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