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Sequences of Conserved Region in the A Subunit of DNA Gyrase from Nine Species of the Genus *Mycobacterium*: Phylogenetic Analysis and Implication for Intrinsic Susceptibility to Quinolones

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The sequences of a conserved region in the A subunit of DNA gyrase corresponding to the quinolone resistance-determining region were determined for nine mycobacterial species and were compared. Although the nucleotide sequences were highly conserved, they clearly differentiated one species from another. The results of the phylogenetic analysis based on the sequences of the quinolone resistance-determining regions were compared with those provided by the 16S rRNA sequences. Deduced amino acid sequences were identical within the nine species except for amino acid 83, which was frequently involved in acquired resistance to quinolones in many genera, including mycobacteria. The presence at position 83 of an alanine for seven mycobacterial species (*M. tuberculosis*, *M. bovis* BCG, *M. leprae*, *M. avium*, *M. kansasii*, *M. chelonae*, and *M. smegmatis*) and of a serine for the two remaining mycobacterial species (*M. fortuitum* and *M. aurum*) correlated well with the MICs of ofloxacin for both groups of species, suggesting the role of this residue in intrinsic susceptibility to quinolones in mycobacteria.

DNA gyrase is a type II topoisomerase and is the enzyme essential for DNA supercoiling, which is required for DNA replication and gene transcription (7). Two A subunits and two B subunits, encoded by the *gyrA* and the *gyrB* genes, respectively (2,625 and 2,412 bp in *Escherichia coli*, respectively), make up the tetrameric active enzyme. DNA gyrase, especially through its A subunit, is also known to be the target of quinolones (6). The primary structures of the A and B subunits of DNA gyrase are conserved among procaryotes, probably because of the essential function of the gyrase (18). Moreover, a domain of the N-terminal part of the A subunit is highly conserved among procaryotes (10) and between procaryotes and eucaryotes (1). This domain contains the catalytic site of DNA gyrase, i.e., the site of transient double-stranded DNA breakage and covalent linkage between the Tyr residue at position 122 (Tyr-122) and single-stranded DNA (11). In addition, this domain includes, from residues 67 to 106 in the numbering system used in *E. coli*, the quinolone resistance-determining region (QRDR), which is supposed to be the site of interaction between the A subunit of gyrase and quinolones (39). This supposition is based on the association of mutations in the QRDR (especially substitutions of Ser-83 or Asp-87) and acquired quinolone resistance in mutants of *E. coli* and *Staphylococcus aureus* (5, 26, 39; for a review, see reference 2) and on the reduced level of binding of quinolones to the Ser-83→Leu-altered A subunit of *E. coli* gyrase (37).

Species of the genus *Mycobacterium* share long-chain (C-60 to C-90) fatty acids in the cell wall (mycolic acids), a high G+C content (62 to 70%) in chromosomal DNA, and acid-alcohol fastness (Ziehl-Neelsen stain) (8). This genus comprises more than 50 species, including about 20 of medical importance. Besides true mycobacterial pathogens such as *M. tuberculosis* and *M. leprae*, the respective agents of tuberculosis and leprosy, atypical mycobacteria such as *M. avium*, *M. kansasii*, *M. chelonae*, and *M. fortuitum* can cause opportunistic infections in immunocompromised hosts, whereas other mycobacterial species such as *M. aurum* and *M. smegmatis* are not involved in human infections. Differentiation of mycobacterial species is usually based on phenotypic and numerical taxonomy, the composition of mycolic acids in the cell wall, and the sequence of the 16S rRNA (8, 28, 36).

Mycobacteria are naturally less susceptible to quinolones than *E. coli* (16), and their level of susceptibility differs widely according to the species. Quinolones are active in the treatment of tuberculosis and leprosy (14, 31), but relapses because of the selection of resistant mutants can occur during treatment with quinolones (31, 34). In *M. tuberculosis* (3, 30), *M. avium* (4), and *M. smegmatis* (20), mutations affecting the A subunit of DNA gyrase (especially mutations at codons 83 and 87) and associated with acquired resistance to quinolones have been described, allowing us to define the QRDR in the DNA gyrase of mycobacteria (4).

The results about the mechanism of acquired quinolone resistance in mycobacteria that have been obtained let us hypothesize that the QRDR could be also involved in intrinsic quinolone resistance in mycobacteria. In order to verify this hypothesis, we compared nucleotide and amino acid sequences of QRDRs from nine different mycobacterial species with different levels of intrinsic susceptibility to quinolones. We first noticed that although a high degree of conservation of the nucleotide sequences was found, analysis of the differences between the nine species resulted in differentiation of the mycobacterial species and determination of phylogenetic relationships similar to those based on phenotypic, mycolic acid, and rRNA analyses. Interestingly, we also found that the amino acid sequences of the QRDRs from these nine mycobacterial species differed by a single amino acid (at position 83) known

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TABLE 1. Strains used in the study

Group and species	Strain (reference)	Characteristics
Slowly growing species (growth in >7 days)		
M. tuberculosis	H37Rv	Virulent strain
M. bovis	BCG	Live vaccine against tuberculosis; reference strain for genetic studies on mycobacteria
M. leprae	90002 (14)	Isolated from lepromatous patient; cultured in mice
M. kansasii	IP 140110001[a]	Agent of opportunistic infections; photochromogenic
M. avium	101 (17)	Agent of opportunistic infections in patients with AIDS
Rapidly growing species (growth in <7 days)		
M. fortuitum	IP 140410001[a]	Agent of opportunistic infections
M. chelonae	PS4770 (12)	Agent of opportunistic infections
M. aurum	IP 14121305[a]	Nonpathogenic; scotochromogenic
M. smegmatis	mc^2155 (25)	Nonpathogenic; reference species for genetic studies on mycobacteria

[a] Reference strain from the Institut Pasteur collection.

to be involved in acquired resistance to quinolones in *E. coli*, *S. aureus*, *M. tuberculosis*, *M. avium*, and *M. smegmatis*. Comparison of the amino acid sequences with the intrinsic susceptibility to quinolones of the nine mycobacterial species studied showed a good correlation between the level of susceptibility and the amino acid present at position 83 in the QRDR, suggesting the crucial role of the primary structure of the A subunit of DNA gyrase in the natural quinolone resistance of mycobacteria.

The strains included in the study are described in Table 1. All are reference strains or strains previously used in other studies. All have a wild-type susceptibility pattern to quinolones and thus can be considered representative of the corresponding species on this basis. A suspension (10^6 bacteria per ml) of *M. leprae* grown in a mouse footpad was prepared as described by Shepard (24). Other species were grown in Löwenstein-Jensen medium (slowly growing species) or in brain heart infusion (BHI) agar supplemented with 1% Tween 80 (rapidly growing species). The harvested cells were scattered over glass beads and were then suspended in 1 ml of sterile water.

Chromosomal DNA from *M. leprae*, *M. bovis* BCG, *M. avium*, *M. smegmatis*, and *M. fortuitum* was extracted by the freezing-boiling technique of Woods and Cole (38). DNA from *M. tuberculosis*, *M. kansasii*, *M. chelonae*, and *M. aurum* was extracted by a lysis method (32) that was modified as follows. The bacterial suspension that was prepared was inoculated into 5 ml of BHI-Tween broth or Dubos medium. When the culture was at the stationary phase, it was heated at 80°C for 10 min and centrifuged for 10 min (4,000 × *g*). The cells were resuspended in 400 μl of buffer 1 (25% saccharose, 50 mM Tris, 50 mM EDTA [pH 8]), and lysozyme (20 μl, 10 mg/ml) was added. After 30 min at 30°C, 400 μl of buffer 2 (100 mM Tris HCl, proteinase K [0.4 mg/ml], 0.1% sodium dodecyl sulfate) was added. After 30 min of incubation at 55°C, 100 μl of NaCl (5 mM) and 500 μl of phenol-chloroform were added. Total DNA was precipitated from the supernatant with 700 μl of isopropanol and was collected by centrifugation (for 30 min at 4,000 × *g*). The DNA pellet was dissolved in 50 μl of water.

Oligonucleotide primers Pri 9 (5′-CGCCGCGTGCT[G,C]TATGC[A,G]ATG-3′), Pri 8 (5′-[C,T]GGTGG[A,G]TC[A,G]TT[A,G]CC[C,T]GGCGA-3′), and Pri 10 (5′-GACTCGGGCTTCCGTCCGGA-3′) were purchased from Bioprobe Systems (Montreuil-sous-bois, France). PCR was carried out in a final volume of 100 μl containing 10 μl of each oligonucleotide used as a primer (Pri 9 and Pri 8 or Pri 10 and Pri 8; 4 μM), 1 to 5 μl of a DNA suspension used as a template, 10 μl of deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; 2.5 mM each), and 10 μl of *Taq* buffer (15 mM $MgCl_2$) containing 2 U of *Taq* polymerase (Boehringer Mannheim, Meylan, France). After 7 min of denaturation at 94°C, 40 amplification cycles were performed, as follows: denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. A final extension step was performed for 10 min at 72°C. A negative control, in which DNA was replaced by distilled water, was introduced in each run of experiments. The DNA was electrophoresed in 1.5% Tris-borate buffer agarose gels.

For determination of nucleotide sequences, the products of three independent PCRs were concentrated and purified by using the GeneClean kit (Bio 101, Ozyme, France). The nucleotide sequence of each PCR product was determined independently on both strands by using the primers Pri 9 and Pri 8 or the primers Pri 10 and Pri 8, with Pri 9 and Pri 10 annealing to the lagging strand and Pri 8 annealing to the leading strand of the DNA fragment. The sequencing reaction was performed with the T7 sequencing kit (Pharmacia, St Quentin en Yvelines, France) as described previously (3, 20). The products of the sequencing reaction were resolved by electrophoresis on 7% (wt/vol) polyacrylamide gels. Computer analysis of the sequences was performed with the GCG program (Genetics Computer Group, Institut Pasteur, Paris, France), and phylogenetic analysis was performed with PHYLIP (Phylogenetic Inference Package, Joe Felsenstein, Washington, D.C.).

The MICs of ofloxacin (Roussel-Uclaf, Paris, France) were determined for each species (except *M. leprae*) by the agar dilution method. For slowly growing species, MICs were determined by the 1% standard proportion method by using Middlebrook 7H11–10% oleic acid-albumin-dextrose (OADC) agar plates containing serial concentrations of ofloxacin, and the plates were inoculated with 0.05 ml of 10^{-3} mg (estimate, 10^3 CFU in the case of *M. bovis* BCG) and 10^{-5} mg of mycobacterial suspension per ml. The MIC was defined as the lowest concentration at which growth was reduced to ≤1% of that of the control (33). For rapidly growing species, Mueller-Hinton agar plates were inoculated by using a Steers replicator device that delivered 10^3 to 10^4 bacteria per spot.

Analysis of nucleotide sequences. Since the yields of DNA extraction and of the PCR process differed from species to species, we selected the most suitable technique for each species. DNA extraction by the freezing-boiling method was successful for *M. leprae*, *M. bovis* BCG, *M. avium*, *M. smegmatis*, and *M. fortuitum*, whereas the lysis method turned out to be

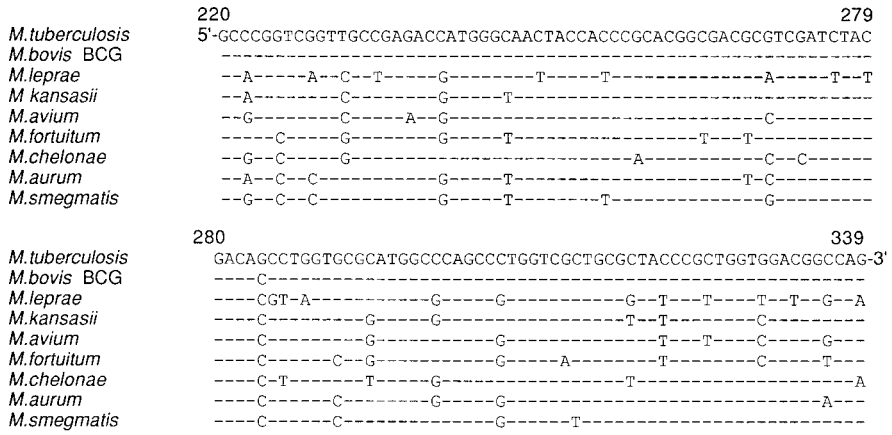

FIG. 1. Nucleotide sequences of the QRDRs (120 bp) in *gyrA* genes from nine mycobacterial species. Identity with the sequence from *M. tuberculosis* is represented by dashes. The numbering system used is that for *M. tuberculosis gyrA* (30).

more efficient for *M. chelonae*, *M. kansasii*, and *M. aurum* and was chosen for *M. tuberculosis* for safety reasons. Pri 9 and Pri 8 were designed on the basis of the alignment of the sequences of the *gyrA* genes of *M. tuberculosis* (30) and *M. smegmatis* (21) for the amplification of a *gyrA* fragment which includes codons 67 to 106 (numbering system in *E. coli* [5]), which delimits the QRDR. Wobbles, indicated by brackets in the primer sequences given above, were introduced at the third positions of some codons to increase the hybridization of primers to sequences possibly distinct from those of *M. tuberculosis* and *M. smegmatis*. For *M. leprae*, we specially designed Pri 10 because no amplification was obtained with Pri 9-Pri 8. Pri 9 was homologous to *gyrA* sequences from *E. coli* (5), *S. aureus* (10, 18), and *Bacillus subtilis* (19), whereas Pri 8 and Pri 10 were homologous only to the *gyrA* genes from mycobacteria. The sizes of the amplified fragments were 192 bp for *M. leprae* and 216 bp for all other species.

Alignment of sequences of the 120-bp fragments defining the QRDR in the *gyrA* genes from the nine mycobacterial species (Fig. 1) showed a high degree of homology ranging from 80% (e.g., *M. tuberculosis* and *M. leprae*) to 98.3% (*M. tuberculosis* and *M. bovis* BCG), as shown in Table 2. Since some strains of *M. tuberculosis* (30) have been reported to have a sequence identical to that of *M. bovis* BCG, the difference found between the two species in the present study (a G for a C at position 284) (Fig. 1) is likely due to the natural polymorphism described previously in *M. tuberculosis* (30) and cannot be used to distinguish *M. tuberculosis* from *M. bovis* BCG.

TABLE 2. Identities between the nucleotide sequences of QRDRs obtained from nine mycobacterial species

Species	% Identity[a]								
	1	2	3	4	5	6	7	8	9
1. *M. tuberculosis*	100	98.3	80	91.7	90.8	88.3	90	90	90.8
2. *M. bovis* BCG		100	80.2	92.5	91.7	89.2	90.8	90.8	91.7
3. *M. leprae*			100	83.3	84.2	77.5	80	80.8	80
4. *M. kansasii*				100	91.7	90.8	89.2	90	88.3
5. *M. avium*					100	90	86.7	89.2	89.2
6. *M. fortuitum*						100	84.2	90.8	89.2
7. *M. chelonae*							100	87.5	87.5
8. *M. aurum*								100	95
9. *M. smegmatis*									100

[a] Numbers in heads indicate the species as numbered in the left column.

Sequences of the 16S rRNA from both species also cannot be distinguished (22, 28). All other species had different sequences of the QRDR in the *gyrA* gene, resulting in agreement with the specificity of each species, as defined by taxonomic studies. For example, there was no higher degree of similarity between *M. chelonae* and *M. fortuitum* than between these two species and any other species, although *M. chelonae* and *M. fortuitum* are usually considered to be very close and are grouped in the *M. fortuitum* complex by phenotypic numerical taxonomy and analysis of mycolic acids (36). In fact, these two species differ on the basis of rRNA analysis (27), pathogenicity (8), and susceptibility to antibiotics (29). Thus, analysis of the QRDR could help in differentiating apparently closely related species of mycobacteria.

Because we found differences in the nucleotide sequences in a highly conserved region of a subunit of an essential protein and despite the small size of the DNA fragments studied, we submitted the results to molecular phylogenetic analysis. That analysis did not consider only the identity values (Table 2) but used a more complex method to compare nucleotide sequences in order to evaluate the phylogenetic distances between species. For that purpose, a distance matrix method (neighbor-joining method) (23) and the parsimony method were applied to the QRDR sequences. Because similar phylogenetic trees were obtained by both methods, only that obtained by the neighbor-joining method is presented (Fig. 2). In the tree in Fig. 2, the slowly growing species are separated from rapidly growing species (except *M. chelonae*), and they have been separated by numerical (8, 36) and rRNA sequence (28) analyses. *M. tuberculosis* and *M. bovis* BCG, both of which belong to the *M. tuberculosis* complex, are grouped together as reported above. In the group of the slowly growing species, *M. leprae* is closely related to *M. avium*, as reported previously (27). *M. fortuitum* is the species that is the most distant from the others, whether they are slowly or fast-growing species, but it still belongs to the group of rapid growers. Such relationships were previously established by 16S rRNA analysis (27). Thus, we conclude that for the mycobacterial species studied, the results of the phylogenetic analysis based on the QRDR in the A subunit of the DNA gyrase compare with those provided by analysis of 16S rRNA, which is the reference method (28). However, the position of *M. chelonae* yielded by our method should be confirmed by additional study, since this position is different from that yielded by studies based on 16S rRNA (27, 28).

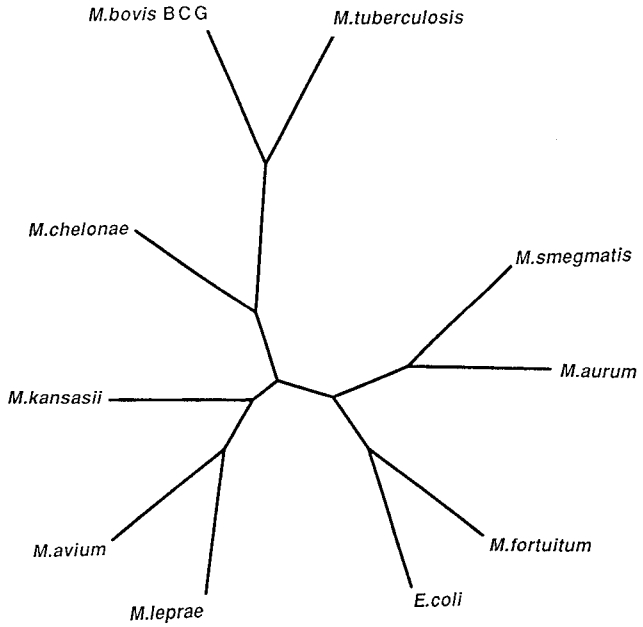

FIG. 2. Phylogenetic tree of sequences of QRDRs in *gyrA* genes from nine mycobacterial species obtained by the neighbor-joining method (23). The sequence from *E. coli* was used as the outgroup element. A similar tree was obtained by the parsimony method (see text).

Analysis of amino acid sequences. Amino acid sequences, which were deduced from nucleotide sequences, shared 95 to 100% identity within members of the genus *Mycobacterium* and 62.5 to 70% identity with sequences from species of other genera (Fig. 3). The overall identity is of the same order of magnitude with gram-negative species (*E. coli*, 70% [5]; *Pseudomonas aeruginosa*, 70% [15]) and gram-positive species (*S. aureus*, 70% [18]; *B. subtilis*, 62.5% [19]), although mycobacteria are gram-positive species. Some residues seem to characterize the genus *Mycobacterium* (Ser-69, Ala-71, Asn-76, and Trp-96).

The amino acids at the residues defining the QRDR (39), i.e., residues 67, 81, 83, 84, 87, and 106 (numbering system in *E. coli*), that are involved in acquired quinolone resistance in *E. coli*, *S. aureus*, and other bacteria (and also in mycobacteria for residues 81, 83, 84, and 87) are globally conserved in all of the species for which results are presented in Fig. 3. The amino acids at residues 67, 81, and 106 were identical in all of the species. The amino acid at residue 87 is either an aspartic acid

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                67                  83                  106
E.coli          ARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLRYMLVDGQ
P.aeruginosa    ----------------T----------------------
C.jejuni        --I--A---R------T----AL-----D--M--P-----
S.aureus        --I---------------SI-EAM-----D--Y--P-----
B.subtilis      --I--E----------ESM-----D-NY--------

M.tuberculosis  --S-AETM-N------ASI--SL------W----P-----
M.bovis BCG     --S-AETM-N------ASI---L------W----P-----
M.leprae        --S-AETM-N------ASI---L------W----P-----
M.kansasii      --S-AETM-N------ASI---L------W----P-----
M.avium         --S-AETM-N------ASI---L------W----P-----
M.fortuitum     --S-AETM-N-------SI---L------W----P-----
M.chelonae      --S-AETM-N------ASI---L------W----P-----
M.aurum         --S-AETM-N-------SI---L------W----P-----
M.smegmatis     --S-AETM-N------ASI---L------W----P-----
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FIG. 3. Alignment of known amino acid sequences of the QRDRs of the gyrase A subunits from *E. coli* (amino acids 67 to 106), *P. aeruginosa* (amino acids 67 to 106), *C. jejuni* (amino acids 70 to 109), *S. aureus* (amino acids 68 to 107), and *B. subtilis* (amino acids 68 to 107) and those obtained from nine mycobacterial species (amino acids 74 to 113 in the numbering system of *M. tuberculosis*).

TABLE 3. Relation between the amino acid present at the position 83 in the gyrase A subunit and susceptibility to ofloxacin

Species	MIC_{50} (μg/ml)[a]	Amino acid at position 83
M. fortuitum	0.125	Ser
M. aurum	0.125	Ser
M. kansasii	0.5	Ala
M. smegmatis	0.5	Ala
M. tuberculosis	1	Ala
M. bovis BCG	1	Ala
M. leprae	1[b]	Ala
M. avium	8	Ala
M. chelonae	8	Ala
E. coli	0.03	Ser
E. coli mutant	0.5[c]	Ala

[a] MIC_{50}, MIC at which 50% of isolates tested are inhibited.
[b] Deduced from experiments in the mouse (14).
[c] From Hallett and Maxwell (9).

or a glutamic acid, but these two amino acids are usually considered equivalent since both have a carboxyl radical and differ only by a $—CH_2$ radical. The amino acids at the two last residues, residues 83 and 84, are different among the species. The amino acid at residue 84 is a serine in all of the mycobacterial species studied as well as in *S. aureus*, whereas it is an alanine in other genera. The amino acid at residue 83 is a serine or a threonine, two amino acids which have a hydroxyl radical, in genera other than mycobacteria, but it is an alanine in most of the mycobacterial species studied. Precisely, the amino acid at residue 83 is an alanine in seven mycobacterial species but a serine in *M. fortuitum* and *M. aurum*. Alanine has never been reported at this position in any bacterial genus other than *Mycobacterium*, whereas a serine is present in *E. coli* (5), *S. aureus* (18), and *B. subtilis* (19). We would like to point out that the amino acid at position 83 is the residue most frequently involved in acquired quinolone resistance in *E. coli* and *S. aureus* (2) as well as in *M. tuberculosis* (3, 30), *M. avium* (4), and *M. smegmatis* (20), suggesting a key role of this residue in quinolone susceptibility.

Interestingly, the intrinsic susceptibility to quinolones, e.g., ofloxacin, of the nine mycobacterial species studied turned out to correlate with the amino acid present at position 83 (Table 3). Indeed, *M. fortuitum* and *M. aurum*, both harboring a Ser-83, were the most susceptible to quinolones and were nearly as susceptible as wild-type *E. coli*, whereas the other mycobacterial species harboring an Ala-83 were 4- to 64-fold less susceptible than the two former species. Strikingly, a Ser-83→Ala mutation obtained by site-directed mutagenesis by Hallett and Maxwell (9) in *E. coli* leads to a decreased susceptibility because of a reduced level of binding of quinolones to the altered gyrase (37). The level of susceptibility to ofloxacin of the Ala-83 mutant of *E. coli* is comparable to that of *M. smegmatis*, *M. tuberculosis*, or *M. kansasii* (Table 3). Thus, these data strongly suggest that the alanine at position 83 in the A subunit of gyrase of mycobacterial species is, at least in part, responsible for the low intrinsic susceptibility of most mycobacterial species to quinolones. The differences in the structures between serine and alanine support that hypothesis. In contrast to serine, alanine cannot provide a hydrogen bond because of its lack of a hydroxyl radical. The loss of a hydroxyl radical at a key amino acid in the gyrase A subunit in quinolone-resistant strains has previously been reported (2, 5, 9, 39). It has been suggested from sequence data for *P. aeruginosa* (15) and *Campylobacter jejuni*, two species harboring a threonine in-

stead of a serine at position 83, that this difference could be involved in the lower intrinsic quinolone susceptibility of these two species (35). In fact, threonine is bulkier than serine, but both amino acids have a hydroxyl radical. More data would be necessary to assess the involvement of Thr-83 in quinolone susceptibility.

The probable relation between susceptibility to quinolones and the differences observed in the QRDRs in the A subunits of DNA gyrases from mycobacteria should be confirmed by further studies such as determination of the affinity of the gyrase for quinolones and genetic studies (e.g., site-directed mutagenesis at residue 83). Differences in the level of intrinsic susceptibility to quinolones between the seven mycobacterial species harboring an Ala-83 is probably due to other mechanisms, particularly differences in the permeability of the cell wall within mycobacteria, as recently published (13).

Nucleotide sequence accession numbers. All of the new nucleotide sequences reported herein will appear in EMBL, GenBank, and DDBJ nucleotide sequences databases under accession numbers X87118, X87119, X87120, X87121, X87122, X87123, and X87124.

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