Correlation between Quinolone Susceptibility Patterns and Sequences in the A and B Subunits of DNA Gyrase in Mycobacteria

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The in vitro activities of seven quinolones and the sequences of the quinolone resistance-determining regions (QRDR) in the A and B subunits of DNA gyrase were determined for 14 mycobacterial species. On the basis of quinolone activity, quinolones were arranged from that with the greatest to that with the least activity as follows: sparfloxacin, levofloxacin, ciprofloxacin, ofloxacin, pefloxacin, flumequine, and nalidixic acid. Based on MICs, the species could be organized into three groups: resistant (Mycobacterium avium, M. intracellulare, M. marinum, M. chelonae, M. abscessus [ofloxacin MICs, ≥8 μg/ml]), moderately susceptible (M. tuberculosis, M. bovis BCG, M. kansasii, M. leprae, M. fortuitum third biovariant, M. smegmatis [ofloxacin MICs, 0.5 to 1 μg/ml]), and susceptible (M. fortuitum, M. peregrinum, M. aurum [ofloxacin MICs, ≤0.25 μg/ml]). Peptide sequences of the QRDR of GyrB were identical in all the species, including the amino acids at the three positions known to be involved in acquired resistance to quinolone, i.e., 426 (Asp), 447 (Arg), and 464 (Asn) (numbering system used for Escherichia coli). The last two residues could be involved in the overall low level of susceptibility of mycobacteria to quinolones since they differ from those found in the very susceptible E. coli (Lys-447 and Ser-464) but are identical to those found in the less susceptible Staphylococcus aureus and Streptococcus pneumoniae. Peptide sequences of the QRDR of GyrA were identical in all the species, except for the amino acid at position 83, which was an alanine in the two less susceptible groups and a serine in the most susceptible one, as in E. coli, suggesting that this amino acid is involved in the observed differences of quinolone susceptibility within the Mycobacterium genus.

Fluoroquinolones, new derivatives of classical quinolones such as nalidixic acid, were demonstrated to be active in vitro against mycobacterial species and effective in the treatment of infections caused by Mycobacterium tuberculosis, M. leprae, and atypical mycobacteria such as M. fortuitum (15, 27, 28). However, mycobacteria were shown to be intrinsically less susceptible to fluoroquinolones than other bacteria such as Escherichia coli (30), and, curiously, the level of susceptibility to these drugs differs markedly according to the mycobacterial species (20, 32).

The intrinsic resistance of mycobacteria to antibiotics is generally attributed to low permeability of the cell wall (14) and production of antibiotic-modifying enzymes such as β-lactamase but may also be due to the low affinity of the target for antibiotics. In other bacterial species, the targets of quinolones are DNA gyrase and topoisomerase IV (8), but DNA gyrase is the only target identified so far in mycobacteria. The interaction between DNA gyrase, a tetrameric protein composed of two A and two B subunits (8), and quinolones seems to involve conserved regions called quinolone resistance-determining regions (QRDR) in the A (34) and B subunits (35). Amino acids at positions 83 and 87 in the A subunit, in the numbering system used for E. coli (90 and 94 in the M. tuberculosis system [25]), and at positions 426, 447, and 464 in the B subunit (495, 516, and 533 in the M. tuberculosis system [25]) are frequently substituted in strains with acquired resistance to quinolones, in mycobacteria as well as in other bacteria (4, 5, 9, 10, 13, 19, 24, 25, 34, 35), and therefore seem to play a key role in the drug-enzyme interaction.

In a previous work, we suggested that the amino acid residue at position 83 in the A subunit of DNA gyrase could play a role in the intrinsic resistance to quinolones in mycobacteria since we found an alanine in M. tuberculosis, M. leprae, and M. avium, for which ofloxacin MICs are ≥1 μg/ml, but, as observed in E. coli, a serine in M. fortuitum, for which the ofloxacin MIC is 10-fold lower (10).

In the present work, we systematically investigated the intrinsic resistance to quinolones in mycobacteria by studying a large set of nontuberculous mycobacterial species, including those that cause major opportunistic infections that can be treated with quinolones. For that purpose, we determined the sequences of the QRDR in GyrA and GyrB and the pattern of susceptibility of each species to seven quinolones, including newer compounds that have promising activity against mycobacteria, in order to investigate the correlation between genetic data and the quinolone susceptibility pattern.

MATERIALS AND METHODS

Strains. A total of 38 strains belonging to 14 slowly and rapidly growing mycobacterial species, including reference strains from the American Type Culture Collection, from the National Collection of Type Cultures, and from the collection of bacterial strains of the Institut Pasteur Tuberculose, and strains isolated from clinical specimens in our laboratory (PS strains) were included in the study (Table 1). E. coli ATCC 25922 was used as the reference strain for MIC determination.

All the clinical strains have been identified by biochemical and phenotypical analyses and 16S rRNA sequencing (18).

Growth conditions and determination of quinolone MICs. Slowly growing species (M. tuberculosis, M. bovis BCG, M. avium, M. intracellulare, M. kansasii, M. marinum) were cultured on Löwenstein-Jensen medium. M. leprae was grown in a mouse footpad (31). Rapidly growing species (M. chelonae, M. abscessus, M. fortuitum, M. fortuitum third biovariant, M. peregrinum, M. smegmatis, M. aurum) were cultured in brain heart infusion agar supplemented with 0.5% Tween 80.

Nalidixic acid and flumequine (Sigma, St. Quentin Fallavier, France), ofloxacin and levofloxacin (Roussel-Uclaf, Paris, France), ciprofloxacin (Bayer Phar-
ma, Puteaux, France), and sparfloxacin (Specia, Paris, France) were tested. Stock solutions of these drugs were prepared in 0.1 N NaOH, except those of flumequine and ciprofloxacin, which were prepared in 5% NH₃ and distilled water, respectively.

For slowly growing species, MICS were determined by the proportion method, as previously described (10), on 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) containing serial twofold dilutions of the quinolone and incubated for 14 to 21 days at 37°C. The MIC was defined as the lowest concentration of quinolone for which the growth was reduced to 1% or less compared with that of the drug-free control culture (12). For rapidly growing species and *M. marinum*, MICS were determined on Mueller-Hinton agar supplemented with 5% OADC and with 5% OADC for *M. marinum* biovariant. MICs of classical quinolones (nalidixic acid and ciprofloxacin) or C. Codons corresponding to amino acids 83 and 87 in the QRDR of GyrA and amino acids 426, 447, and 464 in the QRDR of GyrB are indicated by asterisks. biov., biovariant.

**RESULTS**

**Nucleotide sequences of QRDR of gyrA and gyrB.** Within each given species, the different strains had identical nucleotide sequences of the 120-bp QRDR of gyrA and of the 117-bp QRDR of gyrB in most cases and one- or two-nucleotide differences in the other cases (Fig. 1). In contrast, nucleotide sequences of strains belonging to distinct mycobacterial species were clearly different from each other, the differences ranging between 4 and 29 nucleotides for the QRDR of gyrA (76 to 96% homology) and between 2 and 25 nucleotides for the QRDR of gyrB (79 to 98% homology). Even closely related species had different gyrA and gyrB sequences: *M. avium* differed from *M. intracellulare* (94 to 97% homology), *M. chelonae* differed from *M. abscessus* (88 to 92% homology), and *M. fortuitum* differed from *M. peregrinum* (92 to 96% homology). Within the *M. fortuitum* species, *M. fortuitum* had sequences different from those of *M. fortuitum* third biovariant (92 to 94% homology) (Fig. 1).

**Peptide sequences of the QRDR of GyrA and GyrB.** Peptide sequences of the QRDR of GyrA (amino acids 67 to 106 in the numbering system used for *E. coli*, corresponding to amino acids 74 to 113 in the *M. tuberculosis* system) and of GyrB (amino acids 426 to 464, 495 to 533 in the *M. tuberculosis* system) were deduced from the nucleotide sequences (Fig. 2). Other than the natural polymorphism (Ser versus Thr at position 88) already described for *M. tuberculosis* (25), GyrA QRDR sequences were identical for all the mycobacterial species, except for the amino acid residue at position 83: we found an alanine (Ala-83) in every strain of *M. tuberculosis* and a serine (Ser-83) in *M. avium*. The comparison of the GyrB sequences of strains belonging to distinct mycobacterial species revealed that the differences in the QRDR sequences were more pronounced than those in the GyrA sequences, the highest homology being observed among *M. fortuitum* strains. The QRDR sequences of *M. fortuitum* had sequences different from those of *M. fortuitum* third biovariant (92 to 94% homology) (Fig. 1).

**Quinolone MICs and susceptibility patterns.** The MICs of the seven quinolones tested are presented in Table 1. Within each given species, the different strains had similar susceptibility patterns, i.e., identical MICs or MICs that differed by at most twofold. MICs of classical quinolones (nalidixic acid and...
to a lesser extent flumequine) were much higher than those of fluoroquinolones. For instance, the MICs of nalidixic acid and flumequine were, respectively, 10- to 1,000-fold and 10- to 100-fold higher than those of ofloxacin. Among fluoroquinolones, some important differences were observed. The MICs of pefloxacin were the highest against all the species. The MICs of ofloxacin and ciprofloxacin were mainly similar, but those of ciprofloxacin were two- to fourfold lower against some particular species, e.g., *M. chelonae* and *M. abscessus*. Overall, the MICs of levofloxacin were lower than those of ofloxacin. The MICs of sparfloxacin were the lowest against all the species, except against *M. chelonae* and *M. abscessus*, against which the MICs of ciprofloxacin were the lowest.

With regard to quinolone susceptibility, the mycobacterial species included in this work could be organized into three main groups (Table 1). The first group comprised *M. abscessus*, *M. avium*, *M. chelonae*, *M. intracellulare*, and *M. marinum* and was defined as clearly resistant to quinolones since the MICs (e.g., ofloxacin MICs of ≥8 μg/ml) are above the resistant MIC breakpoints (12). The second group comprised *M. tuberculosis*, *M. bovis* BCG, *M. kansasi*, *M. smegmatis*, and *M. fortuitum* third biovariant and was defined as moderately susceptible since the MICs (e.g., ofloxacin MICs of 0.5 to 1 μg/ml) are equal to or just below the susceptible MIC breakpoints. *M. leprae* was included in this group on the basis of the in vivo activities of quinolones (15). The third group comprised *M. fortuitum*, *M. peregrinum*, and *M. aurum* and was defined as susceptible since the MICs (e.g., ofloxacin MICs of ≤0.25 μg/ml) are clearly below the MIC breakpoints. It should be noted that differences in susceptibility to quinolones between the closely related mycobacterial species mentioned above were observed: *M. intracellulare* was more susceptible than *M. avium* (2- to 4-fold), *M. chelonae* was more susceptible than *M. abscessus* (4- to 16-fold), *M. fortuitum* was more susceptible than *M. peregrinum* (2- to 4-fold), and *M. fortuitum* was more susceptible than *M. fortuitum* third biovariant (2- to 16-fold).

![Alignment of the peptide sequences of the QRDR of GyrA and GyrB from mycobacterial species and from *E. coli*, *N. gonorrhoeae*, *S. aureus*, and *S. pneumoniae* (2, 7, 13, 23, 34, 35). For mycobacteria, dashes represent amino acids identical to those in *M. tuberculosis*. For species other than mycobacteria, dashes represent amino acids identical to those in *E. coli*. The GyrA QRDR extends from amino acid residues 67 to 106, and the GyrB QRDR extends from amino acid residues 426 to 464, in the numbering system used for *E. coli* biovar., biovariant.](http://aac.asm.org/content/50/7/2086)
DISCUSSION

Peptide sequences of the QRDR in the A subunit (GyrA QRDR) and the B subunit (GyrB QRDR) of DNA gyrase are well conserved among procaryotes. Indeed, there is, respectively, 57 to 70 and 69 to 82% identity between the QRDR of GyrA and GyrB of M. tuberculosis on one hand and of E. coli, Neisseria gonorrhoeae, Staphylococcus aureus, and Streptococcus pneumoniae on the other hand. Although each of these regions has 98 to 100% identity within the genus Mycobacterium, the nucleotide sequences are species specific and can even be used to distinguish species phenotypically, biochemically, and genetically close such as M. avium and M. intracellulare; M. chelonae and M. abscessus; and M. fortuitum, M. peregrinum, and M. chelonae third biovariant.

MIC results are in agreement with those reported by several authors (15, 20, 21, 28, 30, 32). It should be stressed that the differences in MICs between the most active (generally sparfloxacin) and the less active (nalidixic acid) quinolones were of the same order (i.e., 2 orders of magnitude) for each mycobacterial species, as observed for other bacteria (20, 30, 32). In other bacteria, the differences in quinolone antibacterial activity are related to the level of anticyrase activity (30). Fluoroquinolone, a classical but fluorinated compound, was more active than the nonfluorinated nalidixic acid, which is consistent with the better activity brought by the fluorine atom at the C-6 position (6). Overall, the newer fluoroquinolones, sparfloxacin and levofloxacin, were more active than ofloxacin and ciprofloxacin, as observed for other gram-positive bacteria (23, 30). The greater activity of ciprofloxacin, compared to that of sparfloxacin, against M. chelonae and M. abscessus should be pointed out. The presence of a porine in the cell wall of M. chelonae, one of the most impermeable of the mycobacterial species, could be particularly crucial for the diffusion of hydrophilic quinolones such as ciprofloxacin (14).

Three groups of mycobacterial species, each comprising slow and rapid growers, can be delineated on the basis of their in vitro susceptibility patterns: susceptible, moderately susceptible, and resistant. Such differentiation is consistent with the in vivo data obtained from humans and from the animal model. Fluoroquinolones are effective and are recommended as first-line agents to treat infections caused by susceptible species such as M. fortuitum (1, 4, 29, 32). Fluoroquinolones are also used in the treatment of infections caused by moderately susceptible species, such as tuberculosis and leprosy, and were shown to be effective when used in combination. They are so far used as second-line agents because the activity levels of the available compounds (ciprofloxacin and ofloxacin) are still lower than those of isoniazid and rifampin (1, 4, 27, 29). Comparative experiments with fluoroquinolones administered alone showed that their in vivo efficacies are largely compound and dose dependent (17, 21). However, newer fluoroquinolones such as sparfloxacin and levofloxacin are more active than ofloxacin and will probably be interesting alternative drugs or the preferred drugs for antituberculosis and antileprosy therapy (15, 21). Finally, fluoroquinolones have also been used in combination with other antimycobacterial agents for infections caused by resistant species, such as M. avium, M. abscessus, and M. chelonae, but as single agents they exhibited only a limited bacteriostatic effect in humans and in the mouse, even at high dosage levels (1, 16).

The GyrA QRDR sequences showed that the mycobacterial species belonging to the resistant and moderately susceptible groups had an alanine residue at position 83 (Ala-83) in the A subunit of DNA gyrase, whereas those belonging to the susceptible group had a serine, as in the most-susceptible bacteria such as E. coli and N. gonorrhoeae (2, 34). The residue at position 87, another key amino acid for quinolone susceptibility, was an aspartate in every mycobacterial species, as in E. coli (34). The importance of the role played by the amino acid at position 83 of the QRDR of GyrA in the quinolone susceptibility patterns of mycobacteria is supported by a recent tridimensional structure analysis of E. coli GyrA, which showed that this amino acid can be exposed to the binding of DNA and of quinolones (22). It has been demonstrated that the substitution of Ala for Ser at position 83 in E. coli led to quinolone resistance at a level comparable to that of moderately susceptible wild-type mycobacterial species which harbor a Ser-83 (11, 33). Moreover, by a systematic screening of in vitro-selected quinolone-resistant mutants of M. aurum and M. peregrinum, species harboring Ser-83, we found that the substitution of Phe for Ser at position 83 led also to resistance levels typical of moderately susceptible species (data not shown).

The observed differences in quinolone MICs between the moderately susceptible and resistant mycobacterial species seem not to be related to the primary structures of the QRDRs in GyrA and GyrB. Differences in cell wall permeability (14) and natural efflux pumps (26) could account for the differences in quinolone susceptibility. A second target of quinolones, such as topoisomerase IV, already identified in some bacteria (8) but still unknown in mycobacteria could also be involved.

Still, the mycobacterial species of the susceptible group are not as susceptible to quinolones as E. coli. In E. coli and other bacteria, it is known that low levels of acquired resistance to quinolones can be associated with substitutions of the amino acid residues at positions 426, 447, and 464 in the GyrB QRDR (9, 13, 35). Interestingly, if all the mycobacterial GyrB QRDR sequences harbor Asp-426, as do those of all other bacteria described so far, they all harbor Arg-447 and Asn-464, in contrast to what was observed for E. coli and N. gonorrhoeae, which harbor Lys-447 and Ser-464 (7, 35). S. aureus and S. pneumoniae, two other gram-negative bacteria naturally less susceptible than E. coli, also harbor Arg-447 and Asn-464 (13, 23). These two residues do not seem to be located near the GyrA QRDR (3), but we could hypothesize that in the mycobacterial GyrB QRDR, Arg-447, which is bulkier than a lysine and which has an additional positive charge, and Asn-464, which is nonhydroxylated and which is bulkier than serine, could decrease the interaction between the gyrase A subunit-DNA complex and the quinolone.

In conclusion, the presence of Arg-447 and Asn-464 in the GyrB QRDR, and of Ala-83 in the GyrA QRDR, are likely involved in the intrinsic resistance of mycobacteria to quinolones. In combination with low cell wall permeability, the presence of the first two amino acids could explain the overall low level of susceptibility of mycobacteria to quinolones and the presence of the last amino acid likely explains, at least in part, the wide range of quinolone susceptibility which characterizes the Mycobacterium genus. A biochemical approach such as the study of purified DNA gyrase from different mycobacterial species would support this hypothesis.

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