

Purification and inhibition by quinolones of DNA gyrases from *Mycobacterium avium*, *Mycobacterium smegmatis* and *Mycobacterium fortuitum* bv. *peregrinum*

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The DNA gyrases from *Mycobacterium avium*, *Mycobacterium smegmatis* and *Mycobacterium fortuitum* bv. *peregrinum*, which are species naturally resistant, moderately susceptible and susceptible to fluoroquinolones, respectively, were purified by affinity chromatography on novobiocin-Sepharose columns. The DNA gyrase inhibiting activities (IC₅₀ values) of classical quinolones and fluoroquinolones were determined from the purified enzymes and were compared to the corresponding antibacterial activities (MICs). Regarding *M. fortuitum* bv. *peregrinum*, which is nearly as susceptible as *Escherichia coli*, the corresponding MIC and IC₅₀ values of quinolones were significantly lower than those found for *M. avium* and *M. smegmatis* (e.g. for ofloxacin, MICs of 0.25 versus 32 and 1 µg ml⁻¹, and IC₅₀ values of 1 versus 8 and 6 µg ml⁻¹, respectively). Such a result could be related to the presence of Ser-83 in the quinolone-resistance-determining region of the gyrase A subunit of *M. fortuitum* bv. *peregrinum*, as found in wild-type *E. coli*, instead of Ala-83 in *M. avium* and *M. smegmatis*, as found in fluoroquinolone-resistant *E. coli* mutants. The IC₅₀ values of quinolones against the *M. avium* and *M. smegmatis* DNA gyrases were similar, while the corresponding MICs were 32-fold higher for *M. avium* when compared to *M. smegmatis*, suggesting that an additional mechanism, such as a low cell wall permeability or a drug efflux, could contribute to the low antibacterial potency of quinolones against *M. avium*.

Keywords: *Mycobacterium*, DNA gyrase, quinolone inhibition assays, fluoroquinolone resistance

INTRODUCTION

DNA gyrase is a type II topoisomerase which catalyses ATP-dependent negative supercoiling of covalently closed circular DNA. The enzyme is a tetramer composed of two A and two B subunits, which are encoded by the *gyrA* and *gyrB* genes, respectively (Gellert *et al.*, 1976, 1977; Sugino *et al.*, 1977). DNA gyrase is inhibited by quinolones through the formation of a ternary complex between the DNA, the quinolone and the DNA gyrase, which results in the inhibition of DNA synthesis (Gellert *et al.*, 1977). As reported previously, conserved regions in the DNA gyrase subunits A and B, referred to as the quinolone-resistance-determining regions (QRDR

GyrA and GyrB, respectively), are involved in quinolone resistance (Drlica & Zhao, 1997). Indeed, the DNA gyrases from most of the quinolone-resistant mutants of Gram-negative and Gram-positive species harbour substitutions of amino acid residues within the QRDRs of GyrA and/or GyrB, mostly at positions 83 and 87 in the QRDR of GyrA (Yamagishi *et al.*, 1986; Cullen *et al.*, 1989; Ito *et al.*, 1994). Purification and biochemical studies of DNA gyrases from such quinolone-resistant mutants have shown that the corresponding enzymes are at least 10-fold less sensitive to the inhibitory effects of quinolones, when compared to the corresponding wild-type DNA gyrases (Sato *et al.*, 1986; Okuda *et al.*, 1991).

Mycobacteria are naturally less susceptible to quinolones than other bacteria (Wolfson & Hooper, 1989). Moreover, they are characterized by a wide range of

Abbreviation: QRDR, quinolone-resistance-determining regions.

quinolone susceptibility patterns (Leysen *et al.*, 1989; Yew *et al.*, 1994). Indeed, we have shown previously that several mycobacterial species, such as *Mycobacterium fortuitum* and *M. peregrinum*, are less susceptible to quinolones than *Escherichia coli*, but are more susceptible to these drugs than other mycobacterial species such as *M. smegmatis* and *M. tuberculosis* (Guillemin *et al.*, 1995, 1998). By contrast, other species, such as *M. avium*, display a very high level of resistance to quinolones (Guillemin *et al.*, 1998). It is likely that the natural differences in the level of resistance to quinolones within the genus *Mycobacterium* is, at least in part, related to the primary structure of the DNA gyrase subunits A and B, and more specifically to the residue at position 83 in the QRDR GyrA (Cambau *et al.*, 1994; Guillemin *et al.*, 1995). However, such a hypothesis, based on genetic studies, has not been confirmed yet by biochemical experiments. In this report, we describe the purification and the biochemical properties of the DNA gyrases from *M. avium*, *M. smegmatis* and *M. fortuitum* bv. *peregrinum*, which are representative of the various quinolone susceptibility patterns observed in mycobacteria. The inhibition by quinolones of the supercoiling activity of the purified enzymes has been investigated and compared to that observed for the DNA gyrase purified from *E. coli*.

METHODS

Strains, growth conditions and determination of MICs. *M. avium* 101 was from our laboratory collection (Lounis *et al.*, 1995). *M. fortuitum* bv. *peregrinum* NGU was a clinical strain isolated from a patient at the Pitié-Salpêtrière hospital. The wild-type strain of *M. smegmatis* used was *M. smegmatis* mc²155 (Snapper *et al.*, 1990). The strains were grown at 30 °C in brain heart infusion medium (BHI) supplemented with 0.5% Tween 80, for 10 d for *M. avium* and 3 d for the two other species. *E. coli* ATCC 25922 was grown for 18 h at 37 °C in BHI. For mycobacteria, MICs were determined by the 1% standard proportion method, on 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase for *M. avium*, and on Mueller-Hinton agar for *M. smegmatis* and *M. fortuitum* bv. *peregrinum* (Guillemin *et al.*, 1995). The MIC values were defined as the lowest concentrations of quinolone inhibiting more than 99% of the bacterial growth (Inderlied & Nash, 1996). For *E. coli*, the MICs were determined by the agar dilution method on Mueller-Hinton plates and were defined as the lowest concentration of quinolones for which no visible growth was observed (Inderlied & Nash, 1996).

Antibiotics. The following antimicrobial agents were used: nalidixic acid, flumequine (Sigma), pefloxacin, sparfloxacin (Rhône-Poulenc Rorer), ofloxacin, levofloxacin (Roussel-Uclaf) and ciprofloxacin (Bayer Pharma).

DNA gyrase purification. DNA gyrases were purified by affinity chromatography on novobiocin-Sepharose columns by the method of Bazile *et al.* (1992), with the following modifications. All purification steps were carried out at 4 °C. The bacterial pellets obtained from 6-litre cultures (approx. 20 g cells) of *M. avium*, *M. smegmatis*, *M. fortuitum* bv. *peregrinum* or *E. coli*, were suspended in 50 mM Tris/HCl (pH 7.5) containing 2 mM DTT and 1 mM PMSF. Bacteria were lysed in a cell homogenizer (B. Braun ScienceTec) for 3 × 30 s with glass beads (Sigma) of 212–300 µm diameter for

E. coli, and for 4 × 1 min with beads of 106 µm for *M. avium*, *M. smegmatis* and *M. fortuitum* bv. *peregrinum*.

The supernatant was recovered after 20 min centrifugation at 12000 g. To precipitate nucleic acids and ribosomes, KCl and magnesium acetate were added at final concentrations of 0.66 M and 5 mM, respectively. After 90 min ultracentrifugation at 53000 g, ammonium sulfate (Sigma) was added to the supernatant at a final saturation of 37%. After stirring for 1 h, the suspension was centrifuged for 20 min at 12000 g. The ammonium sulfate concentration in the supernatant was then adjusted to 55% for mycobacteria and 42% for *E. coli*. The precipitate was recovered after 20 min of centrifugation at 12000 g. The pellet was resuspended in 1–2 ml 25 mM HEPES, 1 mM EDTA, 6 mM β-mercaptoethanol, 200 mM KCl, 10% (v/v) ethylene glycol (buffer A, pH 8), and was dialysed overnight against 1 litre of the same buffer.

After dialysis, proteins were loaded onto a 1 × 2 cm novobiocin-Sepharose column previously equilibrated in buffer A. The column was washed with buffer A until the A₂₈₀ returned to the base line. The adsorbed proteins were eluted, first with buffer A containing 20 mM ATP, and then with buffer A containing 5 M urea. The protein content of each ATP and urea fraction was examined by SDS-PAGE. The eluates were finally dialysed overnight against buffer B (50 mM KH₂PO₄, 1 mM DTT, 0.2 mM EDTA, pH 7.6), and were concentrated by dialysis against the same buffer containing 50% (v/v) glycerol.

DNA supercoiling assay. The DNA gyrase supercoiling activity was assessed by measuring the conversion of relaxed plasmid pBR322 DNA to the supercoiled form, as described previously (Bazile *et al.*, 1992; Revel-Viravau *et al.*, 1996). The relaxed pBR322 DNA was prepared from the supercoiled form (Boehringer Mannheim) by treatment with prokaryotic topoisomerase I (Eurogentec), as described previously (Moreau *et al.*, 1990).

Supercoiling assays were carried out in 15 µl reaction mixtures containing the DNA gyrase assay buffer (20 mM HEPES, 25 mM KCl, 6 mM magnesium acetate, 2 mM spermine, 4 mM DTT, 3% ethylene glycol, v/v, 30 µg *E. coli* tRNA ml⁻¹, 1 mM ATP) (pH 8.0), 150 ng relaxed pBR322, and 2–3 µl purified DNA gyrase. The mixture was incubated for 3 h at 30 °C for *M. avium*, for 30 min at 30 °C for *M. smegmatis* and *M. fortuitum* bv. *peregrinum*, and for 30 min at 37 °C for *E. coli*. The reaction was stopped by the addition of 50% glycerol containing 0.25% bromophenol blue, and the total reaction mixture was subjected to electrophoresis on 1% agarose gel in 1 × TBE buffer (Tris/borate/EDTA, pH 8.3). After a run of 3 h at 90 V, the gel was stained with ethidium bromide (0.7 µg ml⁻¹). Supercoiling activity was assessed by tracing the brightness of the bands corresponding to the supercoiled pBR322 DNA, using a Densylab densitometer (Bio-Rad). One unit (U) of enzyme activity was defined as the amount of DNA gyrase that converted 150 ng relaxed pBR322 to the supercoiled form in 30 min at 30 °C for *M. smegmatis*, *M. fortuitum* and *M. avium*, and at 37 °C for *E. coli*.

Inhibition by quinolones of DNA gyrase supercoiling activity. Inhibition of the supercoiling activity of the purified DNA gyrases was performed using the method of Staudenbauer & Orr (1981), modified as described previously (Revel-Viravau *et al.*, 1996). In brief, a reaction mixture in the gyrase assay buffer (15 µl), containing 150 ng relaxed pBR322 DNA, 1 U purified DNA gyrase and a serial twofold dilution of the quinolone, was incubated as described above. The inhibitory effect of quinolones on DNA gyrase was assessed by deter-

mining the concentration of drug required to inhibit 50% of the supercoiling activity of the enzyme (IC₅₀).

RESULTS

Quinolone susceptibility patterns

The MICs of various quinolones and fluoroquinolones were determined against *M. avium*, *M. smegmatis*, *M. fortuitum* bv. *peregrinum* and *E. coli* (see Table 1). The MICs of flumequine, a fluorinated classical quinolone, were 2- to 16-fold lower than those of nalidixic acid. The fluoroquinolones displayed MICs 8- to 4000-fold lower than those of nalidixic acid and flumequine and ranked, from the lowest to the highest values, as follows: sparflloxacin, levofloxacin, ciprofloxacin, ofloxacin, pefloxacin.

The quinolone susceptibility patterns of the mycobacteria differed according to the species. The MIC values of quinolones against *M. fortuitum* bv.

peregrinum were approximately twofold lower than those against *M. smegmatis*, and 8- to 128-fold lower than those against *M. avium*. The MICs of quinolones against *M. fortuitum* bv. *peregrinum* were 4- to 128-fold higher than those found against *E. coli*.

DNA gyrase purification and supercoiling assays

The experimental conditions used to purify the mycobacterial DNA gyrases by chromatography on novobiocin-Sepharose columns were somewhat different from those used for *E. coli*. The DNA gyrase activity was recovered in the 37–42% ammonium sulfate fraction for *E. coli*, whereas it was found in the 37–55% fraction for mycobacteria. For every species, the supercoiling activity was detected both in the fractions eluted with ATP and urea (Fig. 1). However, the highest supercoiling activity was found in the fractions eluted with ATP for *E. coli* (data not shown) and in those

Table 1. MICs ($\mu\text{g ml}^{-1}$) and IC₅₀ values ($\mu\text{g ml}^{-1}$) of quinolones against purified mycobacterial and *E. coli* DNA gyrases

Quinolone	<i>M. avium</i>		<i>M. smegmatis</i>		<i>M. fortuitum</i> bv. <i>peregrinum</i>		<i>E. coli</i>	
	MIC	IC ₅₀	MIC	IC ₅₀	MIC	IC ₅₀	MIC	IC ₅₀
Nalidixic acid	> 2048	1400	256	1050	256	310	2	50
Flumequine	1024	130	32	180	16	25	0.25	4
Pefloxacin	64	70	2	42	2	8	0.125	1.2
Ofloxacin	32	6.2	1	7.9	0.25	1	0.03	0.35
Ciprofloxacin	16	2	0.5	3.2	0.25	0.5	0.007	0.3
Levofloxacin	8	2	0.25	3	0.125	0.5	0.015	0.29
Sparflloxacin	4	1	0.125	1.4	0.06	0.5	0.015	0.2

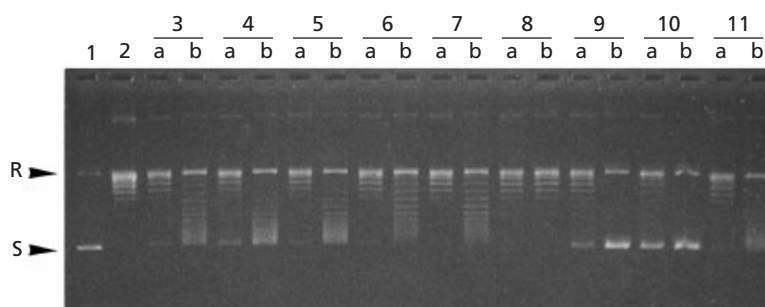


Fig. 1. Supercoiling activity of the DNA gyrase purified from *M. avium*. Lanes: 1, supercoiled pBR322 DNA (S); 2, relaxed pBR322 DNA (R); 3–11, reaction mixtures containing 2 μl (a) and 6 μl (b), respectively, of the fractions eluted with 20 mM ATP (lanes 3–8), or 5 M urea (lanes 9–11).

Table 2. Characteristics of the purified mycobacterial and *E. coli* DNA gyrases

Bacterium	Molecular mass (kDa)		$10^{-4} \times$ Specific activity [U (mg protein) ⁻¹]
	A subunit	B subunit	
<i>M. avium</i>	92	77	0.3
<i>M. smegmatis</i>	91	78	2.5
<i>M. fortuitum</i> bv. <i>peregrinum</i>	93	77	2.0
<i>E. coli</i>	105	92	3.4

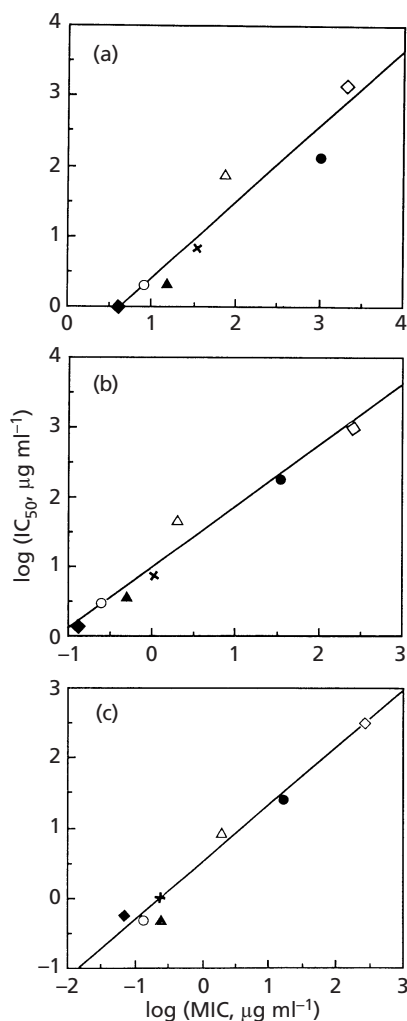


Fig. 2. Correlation between the antibacterial activity (MICs) of quinolones and the corresponding concentration of quinolones inhibiting the supercoiling activity (IC_{50} values) of the DNA gyrases from *M. avium* (a), *M. smegmatis* (b) and *M. fortuitum* bv. *peregrinum* (c). \diamond , Nalidixic acid; \bullet , flumequine; \triangle , pefloxacin; \times , ofloxacin; \blacktriangle , ciprofloxacin; \circ , levofloxacin; \blacklozenge , sparfloxacin.

eluted with urea for mycobacteria (Fig. 1). The highest supercoiling activity for the purified DNA gyrases was reached after 3 h of incubation at 30 °C for *M. avium*, 30 min at 30 °C for *M. smegmatis* and *M. fortuitum* bv. *peregrinum*, and 30 min at 37 °C for *E. coli*. SDS-PAGE of the active fractions revealed, for each enzyme, two major bands corresponding to the A and B subunits (data not shown). The apparent molecular masses of the subunits and the specific activities of these DNA gyrases are presented in Table 2.

Inhibition of DNA gyrase supercoiling activity by quinolones

The IC_{50} values of quinolones determined from the purified DNA gyrases are presented in Table 1. The IC_{50} values of the fluoroquinolones were at least twofold

lower than those of the classic quinolones. Among the latter, IC_{50} values were 10-fold lower for flumequine than for nalidixic acid. The fluoroquinolones ranked, from the lowest to the highest IC_{50} values, as follows: sparfloxacin, levofloxacin and ciprofloxacin, ofloxacin, pefloxacin.

The IC_{50} values were far higher for the DNA gyrases from mycobacteria than for that from *E. coli* (see Table 1). The DNA gyrase from *M. fortuitum* bv. *peregrinum* displayed IC_{50} values two- to eightfold lower than those from *M. smegmatis* and *M. avium*. The enzymes from the latter two species displayed very similar IC_{50} values. A good correlation was found for the three purified DNA gyrases between the IC_{50} values and the corresponding MICs, as shown in Fig. 2 (correlation coefficient values, r : *M. avium*, 0.91; *M. fortuitum*, 0.98; *M. smegmatis*, 0.97).

DISCUSSION

Analysis of the DNA gyrases purified from three different mycobacterial species indicated that they consisted of A and B subunits with very similar apparent molecular masses (see Table 2), and also similar to those reported previously (Madhusudan & Nagaraja, 1995; Revel-Viravau *et al.*, 1996; Staudenbauer & Orr, 1981; Wu & Shahied, 1995).

For *M. smegmatis* and *M. fortuitum* bv. *peregrinum*, the maximum supercoiling activity was obtained after an incubation time of 30 min, as found for *E. coli*. By contrast, 3 h was required to reach this maximum with the DNA gyrase from *M. avium*, which had a low specific activity (0.3×10^4 U mg^{-1}) compared with the values found for the gyrases purified from the three other bacteria ($2\text{--}3.4 \times 10^4$ U mg^{-1}) (see Table 2). It must be noted here that the specific activity measured for the *M. avium* DNA gyrase is similar to the one reported previously for *M. bovis* BCG (Wu & Shahied, 1995).

Site-directed mutagenesis experiments in *E. coli* have demonstrated that the substitution of Ser-83 with Ala can lead to a 10-fold increase in both MIC and IC_{50} values of quinolones (Hallet & Maxwell, 1991). The fact that the IC_{50} values found for the DNA gyrases from *M. avium* and *M. smegmatis*, which both naturally have an alanine at position 83, were significantly higher than those inhibiting the enzyme from *M. fortuitum* bv. *peregrinum*, characterized by a serine residue at this position, confirms the important role played by residue 83 of GyrA in the intrinsic quinolone resistance of mycobacteria (Cambau *et al.*, 1994; Revel *et al.*, 1994; Guillemin *et al.*, 1995). The recently determined three-dimensional structure of the *E. coli* DNA gyrase indicates that the serine residue at position 83 in the A subunit is located in a region where protein-DNA contacts occur (Morais Cabral *et al.*, 1997). Therefore, it is tempting to speculate that the natural presence of an alanine at position 83 in GyrA from *M. avium* and *M. smegmatis* could impair the binding of quinolones, probably by modifying the quinolone binding site in the DNA-DNA gyrase complex.

It is evident that the IC₅₀ values found for the DNA gyrase from *M. avium* were similar to those found for *M. smegmatis*, while the MICs against the former species were markedly (30-fold) higher than those against the latter (see Table 1). This may indicate that the natural resistance of *M. avium* to quinolones is not solely related to the low susceptibility of its DNA gyrase to these drugs, but also to other factors such as the low permeability of the mycobacterial cell wall to hydrophilic compounds (Jarlier & Nikaido, 1994). *M. avium* is assumed to have a particularly low permeability, although this has not been accurately measured so far (Rastogi *et al.*, 1981), which is likely to be an additional factor in its natural resistance. Finally, an efflux pump for quinolones, LfrA, has recently been described in ciprofloxacin-resistant mutants of *M. smegmatis*. The presence of such a pump has also been suggested in wild-type *M. avium* (Takiff *et al.*, 1996) and could be involved in the low intrinsic susceptibility of this species to quinolones, if it really is present and functional.

In conclusion, the data reported here highlight the important role played by the DNA gyrase in the natural low susceptibility of mycobacteria to quinolones. However, further investigations on the mode of entrance of the quinolones through the mycobacterial cell wall and the efflux pumps recently identified in mycobacteria will be necessary to fully understand why some mycobacterial species, such as *M. avium*, exhibit such high levels of resistance to these drugs.

ACKNOWLEDGEMENTS

This study was supported by grants from the Association Française Raoul Follereau, Institut National de la Santé et de la Recherche Médicale (grant CRI 950601) and Hoechst Roussel Uclaf.

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Received 26 October 1998; revised 8 April 1999; accepted 18 May 1999.