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DNA Gyrase Inhibition Assays Are Necessary To Demonstrate Fluoroquinolone Resistance Secondary to *gyrB* Mutations in *Mycobacterium tuberculosis*[▽]

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The main mechanism of fluoroquinolone (FQ) resistance in *Mycobacterium tuberculosis* is mutation in DNA gyrase ($GyrA_2GyrB_2$), especially in *gyrA*. However, the discovery of unknown mutations in *gyrB* whose implication in FQ resistance is unclear has become more frequent. We investigated the impact on FQ susceptibility of eight *gyrB* mutations in *M. tuberculosis* clinical strains, three of which were previously identified in an FQ-resistant strain. We measured FQ MICs and also DNA gyrase inhibition by FQs in order to clarify the role of these mutations in FQ resistance. Wild-type GyrA, wild-type GyrB, and mutant GyrB subunits produced from engineered *gyrB* alleles by mutagenesis were overexpressed in *Escherichia coli*, purified to homogeneity, and used to reconstitute highly active gyrase complexes. MICs and DNA gyrase inhibition were determined for moxifloxacin, gatifloxacin, ofloxacin, levofloxacin, and enoxacin. We demonstrated that the eight substitutions in GyrB (D473N, P478A, R485H, S486F, A506G, A547V, G551R, and G559A), recently identified in FQ-resistant clinical strains or encountered in *M. tuberculosis* strains isolated in France, are not implicated in FQ resistance. These results underline that, as opposed to phenotypic FQ susceptibility testing, the DNA gyrase inhibition assay is the only way to prove the role of a DNA gyrase mutation in FQ resistance. Therefore, the use of FQ in the treatment of tuberculosis (TB) patients should not be ruled out only on the basis of the presence of mutations in *gyrB*.

Drug-resistant tuberculosis (TB), especially multidrug-resistant tuberculosis (MDR-TB), is becoming an alarming public health problem. In 2009, the WHO estimated the number of MDR-TB cases in the world to be 440,000 (38). Curing MDR-TB requires the use of second-line antituberculosis drugs, among which fluoroquinolones (FQs) are the main drugs described as a factor in a successful outcome (9). The WHO warned recently against the emergence of a form of tuberculosis called extensively drug-resistant tuberculosis (XDR-TB), defined as multidrug-resistant TB that is also resistant to fluoroquinolones and to one of the three injectable second-line anti-TB drugs (amikacin, kanamycin, or capreomycin). XDR-TB, which represents 4 to 19% of MDR-TB cases (6, 7, 8, 25, 38), is difficult to treat, and the death rate can be as high as 50%, i.e., close to the historical rate of death observed in untreated TB (7, 17, 18, 22). Alarmingly, XDR-TB is likely to increase in the future due to the poor management of MDR-TB patients and the increasing use of fluoroquinolones for empirical treatment of a large range of nonmycobacterial infections, such as urinary and respiratory tract infections, diarrhea, and typhoid fever, which are common infections in high-tuberculosis-incidence areas (16). Analysis of drug susceptibility of *Mycobacterium tuberculosis* strains is essential for the following reasons: (i) to guide the clinical management of cases and ensure rapid and adequate chemotherapy of tuberculosis and (ii) to determine the need for institutional isolation of patients and so prevent the spread of XDR-TB strains.

Since *M. tuberculosis* grows slowly (21-day culture), molecular tests can facilitate rapid diagnosis of resistance in *M. tuberculosis*, including MDR and XDR, with a net time gain of up to 2 to 3 months over conventional mycobacterial culture plus anti-TB susceptibility testing (10). The sole commercially available assay for rapid detection of second-line-drug resistance, including FQ resistance, the MTBDRsl test (Hain Lifescience GmbH, Germany), detects only the most common mutations found in the quinolone resistance determining region (QRDR) of *gyrA* (5, 19, 21, 33). The sensitivity of this assay for detection of FQ resistance is far from perfect and ranges from 75% (21) to 90% (5, 19, 33). The first step to improve the reliability of this assay is to decipher the molecular mechanisms of FQ resistance that are still not fully elucidated. FQ resistance is defined by the ability of the bacilli to grow on medium containing 2 mg/liter ofloxacin (37). Quinolone resistance is mediated mainly by substitutions in the QRDR located in the GyrA N-terminal domain and in the GyrB C-terminal domain of the sole target of quinolone in *M. tuberculosis*, i.e.,

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TABLE 1. Primers used in site-directed mutagenesis of *M. tuberculosis gyrB* genes

Substitution in GyrB	Use[a]	Nucleotide sequence of the primer[b]
D473N	S	5′-CGG CGT AAG AGC GCC ACC **AAC** ATC GGT GGA TTG CCC GGC-3′
	AS	5′-GCC GGG CAA TCC ACC GAT **GTT** GGT GGC GCT CTT ACG CCG-3′
P478A	S	5′-ACC GAC ATC GGT GGA TTG **GCC** GGC AAG CTG GCC GAT TGC-3′
	AS	5′-GCA ATC GGC CAG CTT GCC **GGC** CAA TCC ACC GAT GTC GGT-3′
R485H	S	5′-CTG GCC GAT TGC **CAT** TCC ACG GAT CCG-3′
	AS	5′-CGG ATC CGT GGA **ATG** GCA ATC GGC CAG-3′
S486F	S	5′-AAG CTG GCC GAT TGC CGT **TTC** ACG GAT CCG CGC AAG TCC-3′
	AS	5′-GGA CTT GCG CGG ATC CGT **GAA** ACG GCA ATC GGC CAG CTT-3′
A506G	S	5′-TCG GCC GGC GGT TCT **GGA** AAA AGC GGT CGC GAT-3′
	AS	5′-ATC GCG ACC GCT TTT **TCC** AGA ACC GCC GGC CGA-3′
A547V	S	5′-CAG GCG ATC ATC ACG **GTG** CTG GGC ACC GGG ATC-3′
	AS	5′-GAT CCC GGT GCC CAG **CAC** CGT GAT GAT CGC CTG-3′
G551R	S	5′-ACG GCG CTG GGC ACC **AGG** ATC CAC GAC GAG TTC-3′
	AS	5′-GAA CTC GTC GTG GAT **CCT** GGT GCC CAG CGC CGT-3′
G559A	S	5′-CAC GAC GAG TTC GAT ATC **GCC** AAG CTG CGC TAC CAC AAG-3′
	AS	5′-CTT GTG GTA GCG CAG CTT **GGC** GAT ATC GAA CTC GTC GTG-3′

[a] S, sense; AS, antisense.
[b] Based on the sequence NCBI AAA83016.1. Relevant codons are in bold and underlined.

DNA gyrase ($GyrA_2GyrB_2$) (3, 15, 27, 28, 31). It has been shown previously that the presence of a mutation in DNA gyrase of an FQ-resistant *M. tuberculosis* strain does not prove the implication of this mutation in the phenotype, underlining the usefulness of using biochemical studies to avoid false interpretations of molecular diagnosis of FQ resistance (3). Although the most common mutations involved in FQ resistance in *M. tuberculosis* clinical strains are located in *gyrA*, one mutation in *gyrB* was demonstrated to be implicated in FQ resistance (3, 34). However, the discovery of unknown *gyrB* mutations whose implication in FQ resistance is unclear is becoming more frequent (3, 14, 15, 28, 29, 31, 32, 34, 36). Therefore, clarification of the role of every new *gyrB* mutation in FQ resistance in *M. tuberculosis* is urgently needed.

In the present study, we aimed to investigate whether GyrB alteration recently observed in clinical *M. tuberculosis* FQ-resistant strains at positions R485 (12, 15), S486 (14), and G551 (12) alter *M. tuberculosis* DNA gyrase quinolone sensitivity. Moreover, we also examined the quinolone complexes bearing GyrB D473N, P478A, A506G, A547V, and G559A substitutions we had recently encountered in *M. tuberculosis* clinical strains received at the French national reference center for mycobacteria (NRC).

MATERIALS AND METHODS

Strains, plasmids, and reagents. The 10 *M. tuberculosis* clinical isolates and the quinolone-susceptible *M. tuberculosis* strain H37Rv used in this study were obtained from the Laboratory of Bacteriology, Centre National de Réference de la Résistance des Mycobactéries aux Antituberculeux, Groupe Hospitalier Pitié-Salpêtrière, Paris, France. Among the 10 isolates carrying novel substitutions in GyrB (D473N, P478A, R485H, S486F, A506G, A547V, G551R, and G559A [3 strains]), four were MDR (R485H, S486F, G551R, and G559A). Strains were grown on Löwenstein-Jensen medium.

The expression plasmids pATB, pBTB, and GyrBN538D, containing the respective *M. tuberculosis* wild-type (WT) *gyrA* gene, WT *gyrB* gene, and *gyrB* gene containing the N538D substitution, have been described previously (2, 3). Plasmids were transformed into *E. coli* BL21-CodonPlus(λDE3)-RP cells (Stratagene) for protein expression.

Enoxacin (Sigma), ofloxacin and levofloxacin (Aventis), moxifloxacin (Bayer Pharma), and gatifloxacin (Grünenthal) were provided by the manufacturers. Supercoiled plasmid pBR322 DNA was purchased from New England BioLabs, and relaxed plasmid pBR322 DNA was from John Innes Enterprises, Ltd.

Determination of MICs. The 10 clinical strains bearing novel *gyrB* mutations were tested for their susceptibilities to the quinolones recommended for the treatment of tuberculosis by the American Thoracic Society (4), i.e., moxifloxacin, gatifloxacin, and levofloxacin. Ofloxacin was added due to WHO-defined FQ resistance regarding ofloxacin MICs. Enoxacin, which is a second-generation quinolone less active against *M. tuberculosis*, was also included in order to detect low levels of resistance. Quinolone-susceptible *M. tuberculosis* strain H37Rv was tested against the quinolones for comparison. MICs were determined by the 1% standard proportion method on 7H11 agar supplemented with 10% oleic acid-albumin-dextrosecatalase (OSI) (1). MICs were defined as the lowest concentration of quinolone that inhibited more than 99% of the bacterial growth.

***In vitro* mutagenesis.** Plasmids expressing mutant *M. tuberculosis gyrB* genes were generated from pBTB using the QuikChange site-directed mutagenesis kit (Stratagene) with minor modifications of the manufacturer's instructions. Mutagenesis products were dialyzed on a nitrocellulose filter (0.025 μm; Millipore) for 1 h, and 5 μl of this dialyzed product was transformed by electroporation into *E. coli* TOP10 cells. Primers for mutagenesis (Table 1) were synthesized by Sigma-Genosys, Ltd. After mutagenesis, plasmids were recovered, purified using the Roche High Pure plasmid isolation kit, and entirely sequenced to ensure the absence of unwanted mutation (MilleGen).

Overexpression and purification of wild-type (WT) and mutant gyrase proteins. DNA gyrase subunits were purified as described previously (3). Protein concentrations were measured with a Nanodrop ND-1000 instrument, and the protein fractions were examined by means of SDS-PAGE.

Enzyme assays. DNA supercoiling assays were carried out as described previously (2). One unit of enzyme activity was defined as the amount of DNA gyrase that converted 400 ng of relaxed pBR322 to the supercoiled form in 1 h at 37°C (2). DNA products were analyzed by electrophoresis in 1% agarose, stained with ethidium bromide, photographed, and quantified with an Alpha Innotech digital camera and associated software. Wild-type *M. tuberculosis* DNA gyrase and DNA gyrase harboring the GyrB N538D substitution previously characterized (3) were used for comparison as the "susceptible control" and "resistant control," respectively. To facilitate direct comparison, all incubations with wild-type and mutant enzymes were carried out and processed in parallel on the same day under identical conditions. All enzyme assays were done at least twice, with reproducible results.

Molecular modeling. Structural analysis of the effect of the GyrB mutations was done using the three-dimensional (3D) model of the *M. tuberculosis* DNA gyrase catalytic core, composed of the crystal structures of the GyrA N-terminal (breakage-reunion domain) and GyrB C-terminal (Toprim) domains, DNA, and moxifloxacin (30).

RESULTS

Characterization of *M. tuberculosis* strains bearing GyrB alterations. Drug MICs of the studied strains are presented in Table 2, except for strains carrying substitutions D473N,

TABLE 2. Concentrations of quinolones that inhibit GyrB mutant DNA gyrase activity and GyrB mutant *M. tuberculosis* growth

Strain	Substitution in GyrB	Inhibitory concn (mg/liter)[e]									
		ENX		OFX		LVX		MXF		GAT	
		IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
H37Rv	None	30	8	10	0.5	8	0.25	3	0.25	3	0.125
CR07066000	D473N	18	ND[f]	9	ND	5	ND	4	ND	3	ND
CR08095735	P478A	10	8	10	0.5	4	0.5	2	0.25	2	<0.125
CR07076077	R485H	50	ND	6.5	ND	12	ND	6	ND	4	ND
CR08105905	S486F[a]	25	8	12	1	13	0.5	2.5	0.25	6	<0.125
CR09036185	A506G	57	16	16	1	14	0.5	4.5	0.25	4	0.125
CR04066124	N538D[b]	>320	>64	120	4	500	2	35	2	45	≥1
CR09026138	A547V	25	ND	16	ND	4	ND	4.5	ND	4	ND
CR09127079	G551R[c]	30	8	5	1	6	0.5	3	0.25	3	<0.125
CR05096580	G559A[d]	25	16	8	1	7	0.5	2.5	0.5	3	0.25
CR07086184	G559A[d]	25	16	8	1	7	0.5	2.5	0.5	3	0.25
CR07106313	G559A[d]	25	16	8	1	7	0.5	2.5	0.5	3	0.25

[a] Described in reference 14.
[b] Described and studied previously (3).
[c] Described in reference 12.
[d] The 3 strains carrying GyrB G559A harbored the same MICs.
[e] ENX, enoxacin; OFX, ofloxacin; LVX, levofloxacin; MXF, moxifloxacin; GAT, gatifloxacin.
[f] ND, not determined.

R485H, and A547V, whose subculture was too poor to allow reliable MIC measures (mainly MDR strains). The moxifloxacin, gatifloxacin, levofloxacin, ofloxacin, and enoxacin MICs for the wild-type *M. tuberculosis* strain H37Rv were, respectively, 0.25, 0.125, 0.25, 0.5, and 8 mg/liter. These values were identical to those reported previously (2, 3, 26). MIC values of strains bearing substitutions of interest in the GyrB subunit were similar to those of the H37Rv strain (a less than 2-fold difference). Moreover, according to the WHO definition, all these strains carrying GyrB substitutions (GyrB D473N, P478A, R485H, S486F, A506G, A547V, G551R, and G559A) are susceptible to FQ, since ofloxacin MICs were <2 mg/liter.

Construction, purification, and DNA supercoiling activity of DNA gyrase proteins with substitutions in the GyrB subunit. We aimed to also examine the effects of these substitutions on quinolone susceptibility at a biochemical level in order to clarify their role in FQ resistance, since some strains harboring GyrB substitutions studied in this work were found in FQ-resistant strains in other studies (12, 14, 15, 28) and we lack microbiological data for strains whose insufficient subculture did not allow MIC measurement. The eight mutant GyrB proteins bearing substitutions of interest were generated by site-specific mutagenesis of GyrB alleles. *M. tuberculosis* WT GyrA, *M. tuberculosis* WT, and mutant GyrB subunits were overexpressed in *E. coli*, purified, and used to reconstitute active gyrase complexes. Protein concentrations ranged from 0.13 to 0.50 mg/ml. Their specific activities were determined in a DNA supercoiling assay in the presence of the complementary wild-type GyrA subunit. The reconstituted activity of all the mutant proteins was similar to that of the WT enzyme. Indeed, the wild-type protein specific activity was 4.6×10^3 U/mg, and specific activities were 6.7×10^3, 7.7×10^3, 1.1×10^3, 1.3×10^3, 3.1×10^3, 3×10^3, 4.8×10^3, and 2×10^4 U/mg, respectively, for the GyrB proteins carrying the D473N, P478A, R485H, S486F, A506G, A547V, G551R, and G559A substitutions.

Inhibition of DNA supercoiling by quinolones and IC_{50} determination. The abilities of moxifloxacin, gatifloxacin, levofloxacin, ofloxacin, and enoxacin to inhibit DNA supercoiling by mutated and wild-type *M. tuberculosis* gyrase were evaluated. A representative experiment for gatifloxacin is presented in Fig. 1. Concentrations of quinolone that inhibited 50% of DNA supercoiling, i.e., IC_{50}s, are represented in Table 2. Biochemical studies correlated with MIC measurements. Indeed, IC_{50}s of mutated proteins carrying D473N, P478A, R485H, S486F, A506G, A547V, G551R, and G559A substitutions were similar to those measured for the wild-type enzyme (identical or a less than 2-fold difference), whereas IC_{50}s of an enzyme carrying GyrB N538D were at least 11-fold higher than WT IC_{50}s.

Positioning of the studied substitutions within the QBP. We recently characterized the structure of the *M. tuberculosis* DNA gyrase catalytic reaction core (30). It is composed of the GyrA N-terminal (breakage-reunion) and the GyrB C-terminal (Toprim) domains, which include the GyrA and GyrB QRDRs, respectively. This 3D model permitted us to define the quinolone-binding pocket (QBP), which is a drug-binding pocket composed of both protein and DNA residues. The studied residues can be separated into two groups. The first group contains the amino acids D473, P478, R485 and S486,

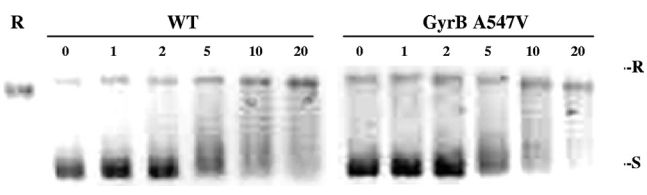

FIG. 1. DNA supercoiling activity of *M. tuberculosis* WT and A547V GyrB DNA gyrase is sensitive to inhibition by gatifloxacin (GAT) (R, relaxed pBR322; S, supercoiled pBR322). GAT concentrations are expressed in mg/liter. GAT IC_{50}s are estimated at 3 mg/liter for the WT enzyme and at 4 mg/liter for the GyrB A547V mutant.

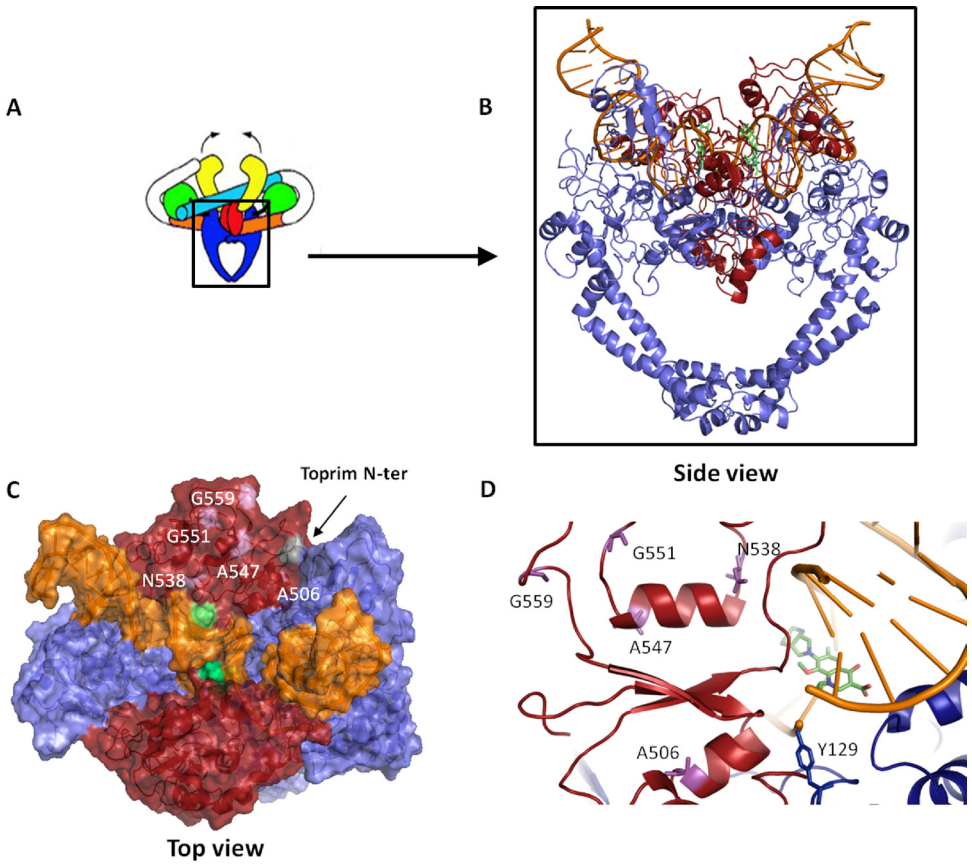

FIG. 2. (A) Schematic representation of the *Mycobacterium tuberculosis* DNA gyrase in complex with DNA. Protein domains are colored as follows: for GyrB, the ATPase domain is in yellow and the Toprim domain is in red; the GyrA breakage-reunion domain is in blue, and the C-terminal domain domain is in green. DNA within the catalytic core is in orange (11). The same color code is followed for the other figures. (B) Side view of the catalytic core in ribbon representation in complex with DNA in orange and moxifloxacin in green (30). (C) Top view of the catalytic core represented in molecular surface. The pink surface indicates the substituted amino acids, and the surface in gray and the black arrow indicate the beginning of the Toprim N-terminal GyrB structure. (D) Cartoon representation of a closeup view of the catalytic core in complex with DNA and moxifloxacin. The catalytic Tyr129 and the substituted amino acids are in stick representation.

which are localized in the hinge region between the ATPase (GyrB N-terminal) domain and the Toprim domain (Fig. 2A). They do not belong to the catalytic core and therefore cannot be positioned in the model. The second group contains the amino acids A506, A547, G551, and G559. These four residues have been positioned in the catalytic core 3D model (Fig. 2C and D).

DISCUSSION

The first step to improve the reliability of the molecular assay for detecting FQ resistance in *M. tuberculosis* is to decipher the molecular mechanisms of FQ resistance that have still not been fully elucidated, especially the implications of *gyrB* mutations in FQ resistance. In the 1990s, mutations in *gyrB* were thought to occur together with a *gyrA* mutation, leading to high level of resistance (20, 23). Over the last 10 years, however, single *gyrB* mutations have been observed in FQ-resistant *M. tuberculosis* strains (3, 5, 14, 15, 28, 29, 36). By comparison with GyrA substitutions, GyrB substitutions are less frequent, occur in a greater variety of amino acids, are located along a sequence of nearly 200 amino acids situated inside and outside the QRDR, and are poorly studied (15, 35). Indeed, among all the GyrB substitutions found in *M. tuberculosis* clinical strains which have been published, only one has been demonstrated to be implicated in FQ resistance, i.e., N538D (also called N510D in some publications) (3, 28). Therefore, our work demonstrating that 8 GyrB substitutions (D473N, P478A, R485H, S486F, A506G, A547V, G551R, and G559A) are not responsible for quinolone resistance, although they have been observed previously in FQ-resistant strains, underlines the considerable significance of biochemical studies of the impact of DNA gyrase mutations in FQ resistance (12, 14, 15).

We first studied GyrB alterations in residues previously identified in *M. tuberculosis* FQ-resistant strains in the relevant literature, i.e., GyrB R485, S486, and G551 (12, 14, 15). Surprisingly, none of the clinical strains harboring the substitutions R485 and S486, observed exclusively in FQ-resistant strains in the literature, were resistant to FQ in our study (Table 2). The strain harboring substitution G551R was also susceptible to FQ, but this mutation had been identified previously in both FQ-susceptible and -resistant strains (12). In order to clarify the role of these substitutions in FQ resistance,

we studied the sensitivity to FQ of DNA gyrase complexes in which they were present. IC_{50}s were similar to the WT values, definitively demonstrating that R485, S486, and G551 are not implicated in FQ resistance. Therefore, the isolates harboring one of the substitutions described as conferring resistance to FQ either could have been misclassified as resistant or are truly resistant to FQ but due to another mechanism of resistance, such as overexpression of efflux pumps (13).

In the second part of our study, we investigated the impact of 5 GyrB substitutions, D473N, P478A, A506G, A547V, and G559A, found in *M. tuberculosis* clinical strains studied in the French NRC and never described previously. The IC_{50}s demonstrated that these 5 new GyrB substitutions do not affect the enzyme sensitivity to quinolones (Table 2) and so are not implicated in FQ resistance. Biochemical studies were indispensable in demonstrating that the GyrB substitutions D473N and A547V were not implicated in FQ resistance, since the poor subculture of the strains carrying these substitutions did not make it possible to measure the MIC.

The fact that the concentration of FQ required to inhibit DNA supercoiling by gyrase is substantially higher than that required to inhibit growth (Table 2) has been attributed to the poisoning effect of quinolone interacting with topoisomerases (2, 24). However, since MICs and IC_{50}s are parallel, the use of IC_{50}s of different proteins to study DNA gyrase sensitivity to quinolone and therefore implication of amino acid substitutions in FQ resistance is valuable using well-known references values of the wild-type enzyme (2, 3).

From a structural point of view, the 8 residues studied in this report are localized in distinct areas. On one side, amino acids at position 473, 478, 485, and 486 are localized in the hinge region between the ATPase and the Toprim domain (Fig. 2A), meaning that they are on the surface of the catalytic core, close to the N terminus of the Toprim domain (indicated by an arrow in Fig. 2C), and consequently far from the *M. tuberculosis* QBP, supporting our observation. On the other side, none of the other residues (A506, A547, G551, and G559) are located in the QBP (Fig. 2B, C, and D). However, the GyrB A506G mutation draws our attention since it is localized inside the QRDR. As shown in Fig. 2, residue A506 is localized at the end of helix α1, in the QBP, but its side chain is far away from this pocket. Furthermore, its modification into a glycine cannot change the secondary structure of helix α1 and the environment of this residue. To summarize, the substitution of amino acids localized outside the QBP has no effect on the gyrase sensitivity to quinolones, whereas the substitution of amino acids localized inside the QBP, such as the amino acid at position 538 (Fig. 2C and D), induces a high level of resistance to quinolones (Table 2) (3).

Our results lead us to conclude that mutation in the QRDR of *M. tuberculosis* DNA gyrase is not necessarily synonymous with resistance to quinolones, underlining once more that biochemical analysis is essential to improve our molecular comprehension of the mechanisms of resistance to FQ, since this, as far as we know, is the only way to prove the implication of DNA gyrase mutations in FQ resistance in *M. tuberculosis.*

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