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Extending the Definition of the GyrB Quinolone Resistance-Determining Region in *Mycobacterium tuberculosis* DNA Gyrase for Assessing Fluoroquinolone Resistance in *M. tuberculosis*

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Fluoroquinolone (FQ) resistance is emerging in *Mycobacterium tuberculosis*. The main mechanism of FQ resistance is amino acid substitution within the quinolone resistance-determining region (QRDR) of the GyrA subunit of DNA gyrase, the sole FQ target in *M. tuberculosis*. However, substitutions in GyrB whose implication in FQ resistance is unknown are increasingly being reported. The present study clarified the role of four GyrB substitutions identified in *M. tuberculosis* clinical strains, two located in the QRDR (D500A and N538T) and two outside the QRDR (T539P and E540V), in FQ resistance. We measured FQ MICs and also DNA gyrase inhibition by FQs in order to unequivocally 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. All these substitutions are clearly implicated in FQ resistance, underlining the presence of a hot spot region housing most of the GyrB substitutions implicated in FQ resistance (residues NTE, 538 to 540). These findings help us to refine the definition of GyrB QRDR, which is extended to positions 500 to 540.

Fluoroquinolones (FQ) are one of the most important drugs in second-line treatment of multidrug-resistant tuberculosis (13) (MDR-TB, i.e., TB due to a strain resistant to the two most powerful antituberculosis drugs that are currently available, isoniazid and rifampin [31]). Unfortunately, FQ resistance emerged in *Mycobacterium tuberculosis* due to the increase of MDR-TB cases and the wide use of FQ to treat several bacterial infections. Therefore, extensively drug-resistant TB (XDR-TB), defined as MDR-TB with additional resistance to fluoroquinolones and at least one of the injectable drugs amikacin, capreomycin, and kanamycin, had been reported in 69 countries by March 2011 (31).

The exact burden of XDR-TB cases is unknown due to lack of sufficient laboratory capacities in many countries. Drug susceptibility testing (DST), which is constrained by the slow growth of *M. tuberculosis*, is difficult to organize in areas with limited resources. Simple molecular methods, which are a promising path to pursue in order to overcome difficulties in this field, are urgently needed.

The sole target of FQ in *M. tuberculosis* is DNA gyrase, a type II topoisomerase composed of two A and two B subunits encoded by *gyrA* and *gyrB*, respectively. Resistance to FQ typically results from alterations in a small region named the quinolone resistance-determining region (QRDR) of each DNA gyrase subunit. Since 2010, an assay for rapid detection of second-line-drug resistance, including FQ resistance, is commercially available, the MTBDR*sl* test (Hain Lifescience GmbH, Germany). As GyrA houses the most common substitutions, GenoType MTBDR*sl* is designed to detect those substitutions (A90V, S91P, D94A, D94N/Y, D94G, and D94H). The sensitivity of this test for FQ resistance detection ranges from to 75.6% to 90.6% (4, 10, 12, 14, 17), the gap being mainly due to the absence of detection of GyrB substitutions (4). Substitutions in GyrB in *M. tuberculosis* clinical strains are being increasingly reported (2, 4, 6, 8, 9, 16, 19, 21, 25–27, 29, 32). However, the implication of *gyrB* mutations in FQ resistance remains largely unclear, and among the 21 GyrB substitutions described in the literature, only two have been demonstrated to be implicated in FQ resistance so far (N538D and E540V) (2, 15, 27), whereas eight were reported not to be implicated in FQ resistance despite being found in FQ-resistant *M. tuberculosis* clinical strains (D473N, P478A, R485H, S486F, A506G, A547V, G551R, and G559A) (20). Consequently, it is scientifically and medically important to clarify the exact role of new GyrB substitutions in FQ resistance in *M. tuberculosis*.

In this work, we investigated the impact on DNA gyrase sensitivity to quinolones of *M. tuberculosis* GyrB alterations we recently encountered in *M. tuberculosis* clinical strains received at the French national reference center for mycobacteria (NRC) and located in the quinolone-binding pocket (22) at positions D500, N538, T539, and E540 (using the CAB02426.1 numbering system).

MATERIALS AND METHODS

Plasmids and reagents. Expression plasmids pATB and pBTB containing the respective wild-type (WT) *gyrA* and *gyrB* genes of *M. tuberculosis* were

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described previously (1). Plasmids were transformed into *Escherichia coli* BL21-CodonPlus(λDE3)-RP cells (Stratagene) for protein expression.

Enoxacin, ofloxacin, and levofloxacin (Sigma), 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.

Strains and drug susceptibility testing. The four *M. tuberculosis* clinical isolates carrying substitutions in GyrB (D500A, N538T, T539P, and E540V) and the quinolone-susceptible *M. tuberculosis* reference strain H37Rv were obtained from the Centre National de Référence des Mycobactéries et de la Résistance aux Antituberculeux, Groupe Hospitalier Pitié-Salpêtrière, Paris, France. Strains were grown on Lowenstein-Jensen medium. *In vitro* drug susceptibility testing for rifampin (RIF), isoniazid (INH), ethambutol (EMB), streptomycin (STR), ofloxacin (OFX), amikacin (AMK), kanamycin (KAN), capreomycin (CAP), cycloserine (CYC), *p*-aminosalicylic acid (PAS), ethionamide (ETH), thioacetazone (Tb1), and linezolid (LNZ) was performed on Lowenstein-Jensen medium by the proportion method (5), using 2 mg/liter for OFX, Tb1 and EMB, 4 mg/liter for STR, 40 mg/liter for AMK, CAP, ETH, and CYC, 30 mg/liter for KAN, and 1 mg/liter for LNZ and PAS (30) and a critical proportion of 1%. The patients were born in Asia ($n = 1$), North Africa ($n = 2$), and Eastern Europe ($n = 1$).

Determination of MICs. The strains bearing novel GyrB substitutions and the reference strain H37Rv were tested for their susceptibility to the quinolones recommended by the American Thoracic Society (3, 31) for the treatment of tuberculosis, i.e., moxifloxacin (MXF), gatifloxacin (GAT), and levofloxacin (LVX). Ofloxacin (OFX) was added based on the World Health Organization (WHO) definition of FQ resistance, and enoxacin (ENX), a quinolone that is less active against *M. tuberculosis*, was added for its usefulness in detecting low levels of resistance. MICs were determined by the 1% standard proportion method on 7H11 agar supplemented with 10% oleic acid-albumin-dextrose catalase (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 nitrocellulose filters (0.025 μm; Millipore) for 1 h, and 5 μl of this dialyzed product was transformed by electroporation into *E. coli* TOP TEN cells. Primers for mutagenesis (Table 1) were synthesized by Sigma-Genosys, Ltd. After mutagenesis, plasmids were recovered, purified using a Roche High Pure plasmid isolation kit, and entirely sequenced to ensure the absence of unwanted mutations (MilleGen).

DNA sequencing. Genomic DNA was isolated from bacteria grown on Lowenstein-Jensen medium. A loop of culture was suspended in water (500 μl) and heated at 95°C for 15 min. The DNA used for PCR amplification was obtained by heat shock extraction (1 min at 95°C and 1 min on ice, repeated five times). A volume of 5 μl was used in PCR amplifications using the oligonucleotide primers described below. For FQ resistance, the QRDRs of the *gyrA* and *gyrB* genes were amplified and sequenced using primers described previously (7).

Overexpression and purification of wild-type (WT) and mutant gyrase proteins. Gyrase subunits were purified as previously described (2). Protein concentrations were measured with a Nanodrop ND-1000 instrument, and the protein fractions were examined by means of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Enzyme assays. DNA supercoiling assays were carried out as described previously (1). 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 (1). 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 (2) were used for comparison and were referred to as the susceptible control and resistant control, respectively. To facilitate direct comparison, all incubations with WT and mutant enzymes were carried out in parallel on the same day under identical conditions. All enzyme assays were done at least twice, with reproducible results.

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

Mutation in GyrB	Primer[a]	
	Orientation	Sequence
D500A	S	CTG TAT GTC GTA GAA GGT **GCC** TCG GCC GGC GGT TCT GCA
	AS	TGC AGA ACC GCC GGC CGA **GGC** ACC TTC TAC GAC ATA CAG
N538T	S	ATC GAC CGG GTG CTA AAG **ACC** ACC GAA GTT CAG GCG ATC
	AS	GAT CGC CTG AAC TTC GGT **GGT** CTT TAG CAC CCG GTC GAT
T539P	S	GAC CGG GTG CTA AAG AAC **CCC** GAA GTT CAG GCG ATC ATC
	AS	GAT GAT CGC CTG AAC TTC **GGG** GTT CTT TAG CAC CCG GTC
E540V	S	GTG CTA AAG AAC ACC **GTA** GTT CAG GCG ATC ATC
	AS	GAT GAT CGC CTG AAC **TAC** GGT GTT CTT TAG CAC

[a] Based on the sequence gi|1107468|gb|AAA83016.1|. Relevant codons are in bold and underlined. S, sense; AS, antisense.

Molecular modeling. Structural analyses 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 the GyrB C-terminal (Toprim) domains, DNA and MXF, generated by modeling as previously described by Piton et al. (22).

RESULTS

Drug susceptibility profile and typing. Among the four isolates carrying substitutions in GyrB, three were MDR (those with D500A, N538T, and T539P) and one was XDR (that with E540V) (Table 2). The XDR strain was susceptible only to thioacetazone, linezolid, and PAS. All strains (carrying GyrB D500A, N538T, T539P, and E540V) were resistant to OFX by the 1% proportion method, carried out according to WHO recommendations; the proportion of resistant mutants ranged between 1% and 100%.

DNA sequencing of clinical isolates. *gyrA* and *gyrB* QRDR sequencing showed no mutations in *gyrA* and a single mutation in *gyrB*. For one isolate, a heterogeneous peak was found at the mutation site, indicating a mixture of wild-type and mutant cells (Table 3).

Characterization of quinolone susceptibility of *M. tuberculosis* strains bearing GyrB substitutions. FQ MICs are presented in Table 3, except those for the MDR strain carrying T539P, whose culture was too poor to allow reliable MIC measures. For the MDR strain carrying N538T, the MXF MIC measured first was the only MIC available, since later subcultures were too poor to allow reliable MIC measures for other FQ. It was fourfold higher than the *M. tuberculosis* H37Rv MXF MIC (Table 3). According to the

TABLE 2 Demographic characteristics of patients and phenotypic drug susceptibility profiles of *M. tuberculosis* isolates harboring GyrB D500A, N538T, T539P, and E540V

Strain	GyrB mutation	Patient characteristic		Susceptibility to[a]:													
		Country of birth	Sex	OFX	RIF	INH	PZA	EMB	STR	KAN	AMK	CAP	ETH	CYC	TB1	LZD	PAS
CR 08095705	D500A	Georgia	F	R	R	R	R	R	R	S	S	S	S	R	S	S	S
CR 04086216	N538T	Sri Lanka	M	R	R	R	R	S	R	S	S	S	R	S	S	S	R
BK 06021463	T539P	Tunisia	M	R	R	R	R	R	R	S	S	S	R	R	R	S	R
CR 09127069	E540V	Algeria	M	R	R	R	R	R	R	R	S	S	R	R	S	S	S

[a] OFX, ofloxacin; RIF, rifampin; INH, isoniazid; PZA, pyrazinamid; EMB, ethambutol; STR, streptomycin; KAN, kanamycin; AMK, amikacin; CAP, capreomycin; ETH, ethionamide; CYC, cycloserine; Tb1, thiocetazone; LNZ, linezolid; PAS, *para*-aminosalicylic acid; R, resistant; S, sensitive.

WHO definition, MIC measures confirmed that clinical strains carrying D500A and E540V were resistant to FQ, since OFX MICs were ≥2 mg/liter (Table 3). GAT, MXF, LVX, OFX, and ENX MICs against *M. tuberculosis* H37Rv were 0.125, 0.25, 0.25, 0.5, and 8 mg/liter, respectively, identical to those previously reported (1, 2, 18). Compared to MICs found for this strain, MICs against mutants were 4 to 32 times higher depending on the FQ and the strain, except for ENX and MXF, whose MICs for the *M. tuberculosis* clinical strain carrying substitution D500A were equal to those for *M. tuberculosis* H37Rv. The increases in MICs were lowest for the strain with GyrB D500A substitution (1- to 4-fold increases) and highest for the strain with GyrB E540V substitution (8- to 32-fold) and were lowest for ENX (1- to 8-fold) and highest for GAT (4- to 32-fold).

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 mutations on quinolone susceptibility on a biochemical level in order to clarify their role in FQ resistance, since (i) we lack microbiological data for two strains (those carrying GyrB N538T and T539P) whose insufficient subculture did not allow MIC measures and (ii) it has been shown previously that the presence of a mutation in the DNA gyrase of an FQ-resistant *M. tuberculosis* strain does not prove the implication of this mutation in the phenotype (2, 20). The mutant GyrB proteins bearing the substitutions of interest were purified at concentrations ranging from 0.19 to 3.76 mg/ml. The reconstituted mutant proteins exhibited a gyrase activity similar to that of the WT enzyme; specific activities were 4.6×10^3, 5.3×10^3, 2.4×10^3, 3.5×10^3, and 5.7×10^3 U/mg for the wild-type GyrB and proteins carrying D500A, N538T, T539P, and E540V substitutions, respectively.

Inhibition of DNA supercoiling by quinolones and IC_{50} determination. The ability of MXF, GAT, LVX, OFX, and ENX to inhibit DNA supercoiling by mutated and WT *M. tuberculosis*

TABLE 3 Laboratory characterization of clinical strains carrying single GyrB substitutions at residues D500, N538, T539, and E540, described as single substitutions in the literature and encountered in our study

Strain	DNA sequencing		Susceptibility to OFX		MIC[b] (mg/liter) of:					Reference
	gyrB	Heteropeak detected[a]	% resistant[c]	Conclusion	ENX	OFX	LVX	MXF	GAT	
H37Rv	WT	NS[h]	<0.0001	S	8	0.5	0.25	0.25	0.125	This study
CR 08095705	D500A	No	1	R	8	2	1	0.25	0.5	This study
T2-SIMI-0186	D500N[d]	NS	NS	R	NS	NS	NS	NS	NS	21
NS	D500N[d]	NS	NS	R	NS	8	NS	NS	NS	6
NS	D500N	No	NS	R	NS	4	NS	NS	NS	8
H37Rv	D500N	NS	NS	NS	32	4	2	0.5	0.5	23
CR 04086216	N538T	Yes + WT	50	R	NS	NS	NS	1	NS	This study
NS	N538T	Yes + WT	NS	R	NS	NS	NS	NS	NS	8
02-1234	N538T[e]	NS	NS	S	NS	1	NS	0.5	0.5	28
9	N538D	NS	NS	R	NS	8	1	1	NS	29
B	N538D[f]	NS	NS	R	>64	4	2	2	≥1	2
BK 06021463	T539P	No	100	R	NS	NS	NS	NS	NS	This study
NS	T539N[g]	NS	NS	R	NS	NS	≥1	NS	NS	32
CR 09127069	E540V	No	33	R	64	4	2	2	4	This study
NS	E540V	No	NS	R	NS	NS	NS	NS	NS	8
NS	E540D	No	NS	R	NS	12	NS	NS	NS	8

[a] Codon at which heterogeneity was found by sequencing *gyrB*.
[b] ENX, enoxacin; OFX, ofloxacin; LVX, levofloxacin; MXF, moxifloxacin; GAT, gatifloxacin.
[c] Available for studies using the proportion method for OFX drug susceptibility testing.
[d] Described elsewhere as D495N.
[e] Described elsewhere as N510T.
[f] Described elsewhere as N510D.
[g] Described elsewhere as T511N.
[h] NS, not specified.

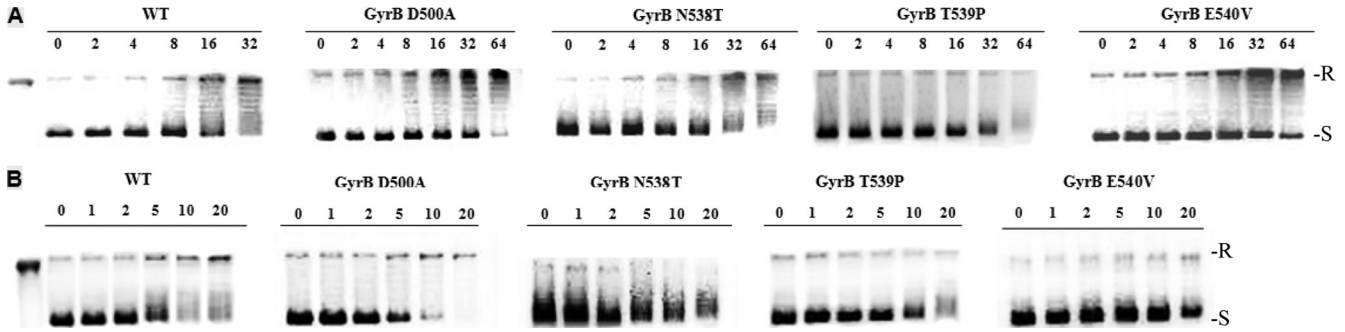

FIG 1 Inhibitory activities of levofloxacin (LVX) (A) and moxifloxacin (MXF) (B) on the supercoiling activity of *M. tuberculosis* WT DNA gyrase and proteins carrying the GyrB substitutions N538T, T539P, E540V, and D500A. R, relaxed pBR322; S, supercoiled pBR322.

gyrase was evaluated. Results of a representative experiment for LVX and MXF are presented in Fig. 1. Concentrations of FQ that inhibited DNA supercoiling (IC_{50}s) are presented in Table 4. Most IC_{50}s of mutated proteins were significantly higher than that observed for the WT enzyme, i.e., increased by a factor of 2 to 5 for proteins carrying D500A, N538T, and T539P and by a factor of 2 to at least 8 for the protein harboring E540V. ENX IC_{50}s for proteins carrying D500A and N538T were similar (less than 2-fold lower) to those of the WT enzyme.

Positioning of the studied mutations within the QBP. We recently characterized the structure of the *M. tuberculosis* DNA gyrase catalytic reaction core (22). It is composed of the GyrA N-terminal (breakage-reunion) and the GyrB C-terminal (Toprim) domains, which include the GyrA and GyrB QRDR, respectively. This 3D model enabled us to define the quinolone-binding pocket (QBP), which is a drug-binding pocket composed of both protein and DNA residues. The four residues studied are all directly located in the QBP. Residues N538, T539, and E540 are located at the beginning of the α2 helix, and D500 is located at the beginning of the α1 helix (Fig. 2B). Thus, they are all in direct contact with the quinolone molecule, as shown in Fig. 2.

DISCUSSION

To improve molecular tests for rapid detection of FQ resistance in *M. tuberculosis*, i.e., one of the two markers defining XDR among MDR strains, it is important to clarify molecular mechanisms of FQ resistance, including the role of *gyrB* mutations. Indeed, single GyrB substitutions in FQ-resistant *M. tuberculosis* clinical strains have been increasingly reported (2, 4, 8, 9, 19, 21, 29), suggesting that substitutions in GyrB should also be considered for screening of MDR strains for XDR. The previous study showing that GyrB substitutions could be not implicated in FQ resistance despite being found in resistant *M. tuberculosis* strains (D473N, P478A, R485H, S486F, A506G, A547V, G551R, and G559A) (20) underlines the need for additional studies of the effect of GyrB substitutions on FQ resistance in *M. tuberculosis*. So far, only two GyrB substitutions have been unequivocally demonstrated to be involved in FQ resistance: N538D (also called N510D in some publications depending on the numbering system used) and, very recently, E540V (2, 15). The present study demonstrates that several GyrB substitutions (D500A, N538T, T539P, and E540V) are responsible for FQ resistance and underlines the presence of a 3-amino-acid hot spot region for substitutions responsible for FQ resistance in *M. tuberculosis*. Since amino acids 539 and 540 are not included in the *M. tuberculosis* GyrB QRDR, these results also help us to refine the QRDR, whose limit can therefore be extended from amino acid 500 to amino acid 540, as suggested by previous studies (15, 22).

Previously, substitutions in GyrB had been described as being mainly associated with GyrA substitutions, leading to high-level resistance (16). In the present study, the strains harbored exclusively GyrB substitutions, without additional GyrA mutations. In the literature, among the 28 strains carrying substitutions at positions D500 ($n = 14$), N538 ($n = 7$), T539 ($n = 5$), and E540 ($n = 2$), 10 were found to have a GyrA substitution and one was found to have another GyrB substitution (6, 8, 16, 19, 21, 23, 28, 32). The heterogeneity of drug susceptibility testing methods among published studies makes it difficult to develop hypotheses regarding differences in level of resistance, but it can be noted that strains with the highest level of resistance to FQ carry both GyrA and GyrB substitutions (8, 16). The FQ susceptibility patterns of the strains carrying substitutions in GyrB can show as much resistance

TABLE 4 Concentrations of quinolones that inhibit *M. tuberculosis* DNA gyrase activity reported in the literature and obtained in our study

	IC_{50} (μg/ml) for GyrB substitution reported in:								
	Our study					Reference 2		Reference 15	
Quinolone	WT	D500A	N538T	T539P	E540V	WT	N538D[a]	WT	E540V
Gatifloxacin	3	8	14	13	>20	2.5	45	9	37
Moxifloxacin	2.5	6	12	12	>20	2	35	16	61
Levofloxacin	8	25	24	17	64	12	500	22	82
Ofloxacin	10	22	28	30	80	10	120	ND[b]	ND
Enoxacin	40	80	30	160	200	ND	ND	84	>320

[a] GyrB N538D was used as a positive control in our study (IC_{50}s obtained were similar to those previously published [2]).
[b] ND, not determined.

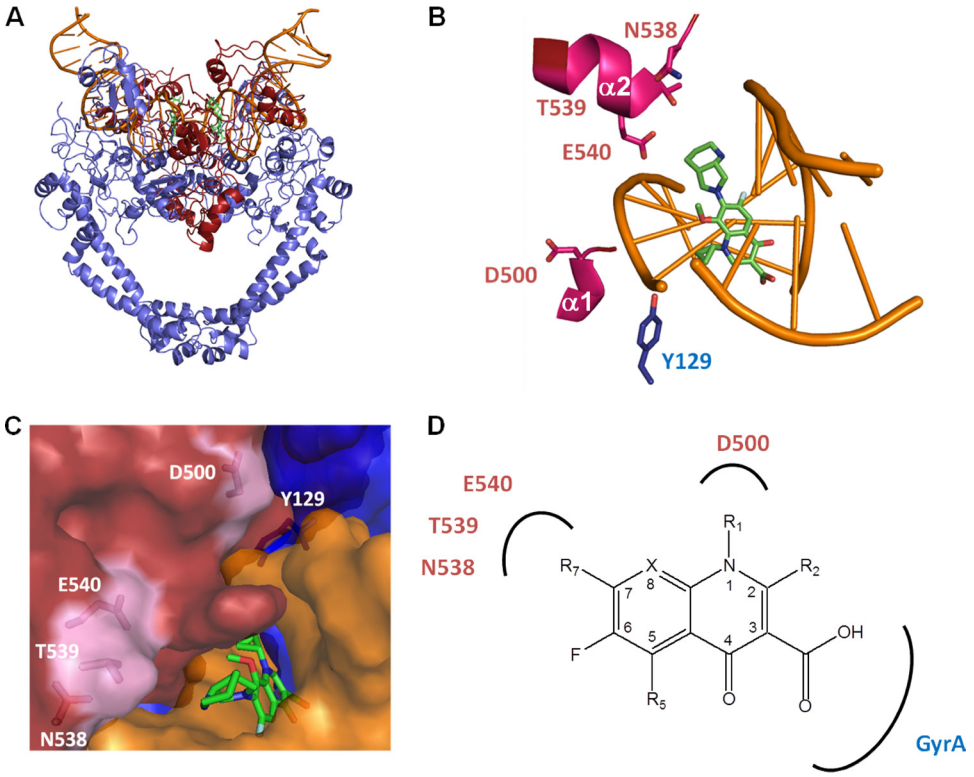

FIG 2 Model of the catalytic reaction core in complex with DNA and moxifloxacin. In each panel, GA57BK is represented in blue, TopBK in red, DNA in orange, and moxifloxacin in green. (A) Overall structure of the complex. (B) Cartoon representation of a close-up view of the catalytic reaction core. The catalytic Tyr129, the residues implicated in resistance, and moxifloxacin are in stick representation. (C) The DNA-protein complex is shown as a molecular surface representation, and moxifloxacin is in stick representation. The residues implicated in resistance are in purple. (D) Schematic representation of the interactions between QBP residues and chemical groups of the quinolone.

as that of strains carrying GyrA substitution. However, our results, as well as those of other studies, suggest that D500A and N538T are responsible for a lower level of resistance, with OFX MICs being close to 2 mg/liter, which is the breakpoint recommended by the WHO for FQ susceptibility testing (Table 3), and E540V is responsible for a higher level of resistance, similar to that occurring in strains carrying GyrA substitutions at position 94 (2).

Since the presence of a mutation in DNA gyrase of an FQ-resistant *M. tuberculosis* strain does not prove the involvement of this mutation in the phenotype (2, 20), we used DNA gyrase inhibition assays to definitively clarify the effect of these substitutions on enzyme susceptibility to FQ. MICs and IC_{50}s measured in our study were consistent for each substitution. Indeed, the ratios of WT to mutant MIC or IC_{50} were similar for each substitution. Therefore, the GyrB substitutions D500A, N538T, T539P, and E540V are doubtless involved in resistance to quinolone in *M. tuberculosis* (Tables 3 and 4). Our work demonstrated for the first time that the GyrB substitutions D500A and T539P can be implicated in FQ resistance, both being involved in low-level resistance (1- to 5-fold increases in MICs and/or IC_{50}s), as suggested by previous data (23). Our results also show that N538T was responsible for a lower level of resistance than N538D (3- to 5-fold increases in MICs and/or IC_{50}s, versus 12- to 42-fold increases) and confirmed the results published very recently regarding substitution E540V (Table 4). The data published by Kim et al. are close to the results we obtained, suggesting that DNA gyrase assay is a reliable tool in demonstrating resistance and allows comparisons between studies (15).

Against the GyrB substitutions studied, MXF and GAT were more active than OFX, showing strong cross-resistance, as classically described for FQ resistance in *M. tuberculosis* as well as other bacteria (2, 11, 24), even for substitution N538T, despite some studies having suggested that N538K could be responsible for a higher level of resistance to MXF than to OFX (28).

According to the recently published structure of the *M. tuberculosis* DNA gyrase catalytic reaction core, the four residues (D500, N538, T539P, and E540V) are located in the heart of the QBP and interact directly with quinolones. Indeed, it has been suggested that quinolone is immobilized by three regions of the Toprim domain in addition to a region of the GyrA subunit. The β1-α1 loop (residues 498 to 501, described as 459 to 462 in reference 22) interacts with the R1 group, the β2-DBL loop (residues 519 to 525; 480 to 486 in reference 22) with the R7-R8 group, and the beginning of α2 (residues 537 to 541; 498 to 502 in reference 22) with the R7 group (Fig. 2). Consequently, both deformation (rise) of the intercalated dinucleotide step forming the DNA saddle and the specific sequence of the QRDRs GyrA and GyrB are required to build up the QBP and determine the geometric characteristics of the binding pocket (volume and shape). The four residues studied are all directly located in the QBP in direct contact with the quinolone, but their localization in the catalytic reaction core is different. D500 is located at the beginning of the α1

helix, whereas N538, T539, and E540 are located at the beginning of the α2 helix (Fig. 2B). The most drastic modification is the substitution of the glutamic acid at position 540 by a valine, which introduces the loss of a positively charged residue and also leads to a conformational change of QPB geometry in GyrB by the replacement of this residue, which has an important steric hindrance, by a smaller one (Fig. 2). This leads to a higher level of resistance regardless of the quinolone. The two other modifications occurring in this region, at positions 538 and 539, are less drastic. Indeed, the mutation of asparagine to aspartic acid at position 538 modifies the global charge in the binding cavity of quinolone, but the conformational change of geometry is minimal, and conversely the replacement of a threonine by a proline at position 539 does not change the polarity but rather introduces a greater congestion in the QBP. This correlates well with the fact that these two substitutions reduce enzyme sensitivity to FQ by a smaller amount than E540V. The replacement of an aspartate by an alanine at position 500 introduces a small hydrophobic residue instead of a negatively charged residue, which leads to the modification of the global charge and the geometry of the QBP, but this residue interacts with the R1 group of quinolones, whereas the three other residues interact with the R7-R8 groups (Fig. 2D). Due to the positioning of this residue in the QBP, the impact of this modification on the sensitivity of the enzyme for quinolones (MICs and IC_{50} increased by a factor less than or equal to 4) is smaller.

This study leads to three important conclusions. First, it is interesting that among these mutations, two are located outside the GyrB QRDR as defined at present. In addition to a recent study demonstrating that substitution E540V is responsible for FQ resistance, our study shows that the QRDR of GyrB could be redefined and expanded to include these changes (T539P and E540V) (15). The QRDR is a conserved region within *gyrA* and *gyrB* which was defined by *in vitro* studies of a selection of *Escherichia coli* mutants resistant to quinolones (33). Later, QRDR was defined by amino acid alignment of GyrA and GyrB sequences of other bacterial species, including *M. tuberculosis* (11, 33). Therefore, the *M. tuberculosis* GyrB QRDR ranges from Asp500 to Asn538, according to the CAB02426.1 numbering system (33). A recent study using three-dimensional structure analysis suggested that the *M. tuberculosis* GyrB QRDR is located from Asn493 to Asn540 (22), which is concordant with the present results. Second, the present results call for adding *gyrB* mutations to molecular detection assays, since it could increase the sensitivity of FQ resistance detection, especially in region 538 to 540, which houses most of the GyrB substitutions involved in FQ resistance, as also suggested in a recent report (4). Indeed, among the 36 FQ-resistant strains harboring GyrB substitutions described in the literature, 14 were described as having mutations at position 500, seven had mutations at position 538, five had mutations at position 539, and two had mutations at position 540; i.e., 78% of substitutions occurring in GyrB are located in a hot spot region formed by amino acids 538 to 540. Finally, the low level of FQ resistance observed for most of these strains (especially those harboring D500A, N538T, or T539P) poses once more the question of the usefulness of moxifloxacin in standard or double dosage among patients infected with such strains. Indeed, a murine model of tuberculosis has demonstrated that moxifloxacin is active against strains with low levels of resistance (MXF MIC for strains carrying D500N, 0.5 μg/ml) and reduces the mortality associated with strains with intermediate resistance (MXF MIC, 2 μg/ml), supporting the current WHO recommendation to use moxifloxacin when there is resistance to early fluoroquinolones, such as ofloxacin (23). Human studies should be conducted to confirm these observations and to assess the long-term tolerability of higher doses of moxifloxacin in TB patients.

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