The Molecular Genetics of Fluoroquinolone Resistance in Mycobacterium tuberculosis

CLAUDINE MAYER¹²³ and HOWARD TAKIFF⁴
¹Unité de Microbiologie Structurale, Institut Pasteur; ²UMR 3528 du CNRS; ³Université Paris Diderot, Sorbonne Paris Cité, Cellule Pasteur, 75015, Paris, France; ⁴Laboratorio de Genética Molecular, CMBC, IVIC, Caracas, Venezuela

ABSTRACT The fluoroquinolones (FQs) are synthetic antibiotics effectively used for curing patients with multidrug-resistant tuberculosis (TB). When a multidrug-resistant strain develops resistance to the FQs, as in extensively drug-resistant strains, obtaining a cure is much more difficult, and molecular methods can help by rapidly identifying resistance-causing mutations. The only mutations proven to confer FQ resistance in M. tuberculosis occur in the FQ target, the DNA gyrase, at critical amino acids from both the gyrase A and B subunits that form the FQ binding pocket. GyrA substitutions are much more common and generally confer higher levels of resistance than those in GyrB. Molecular techniques to detect resistance mutations have suboptimal sensitivity because gyrase mutations are not detected in a variable percentage of phenotypically resistant strains. The inability to find gyrase mutations may be explained by heteroresistance: bacilli with a resistance-conferring mutation are present only in a minority of the bacterial population (>1%) and are therefore detected by the proportion method, but not in a sufficient percentage to be reliably detected by molecular techniques. Alternative FQ resistance mechanisms in other bacteria—efflux pumps, pentapeptide proteins, or enzymes that inactivate the FQs—have not yet been demonstrated in FQ-resistant M. tuberculosis but may contribute to intrinsic levels of resistance to the FQs or induced tolerance leading to more frequent gyrase mutations. Moxifloxacin is currently the best anti-TB FQ and is being tested for use with other new drugs in shorter first-line regimens to cure drug-susceptible TB.

Mycobacterium tuberculosis strains resistant to other antibiotics, especially multidrug-resistant TB (MDR-TB) —strains resistant to at least isoniazid and rifampin (1, 2). They are also being proposed as first-line agents to reduce the duration of treatment for pan-susceptible strains, particularly when given in new combinations with other recently developed drugs (3–5). A major problem with the FQs is the development of resistant strains. MDR strains that have developed resistance to the FQs and also to any of the injectable drugs are termed extensively drug-resistant (XDR-TB)—strains that are extremely difficult to treat (6), with studies showing from 65% (7) to less than 50% having favorable outcomes (8). For the best chance of curing XDR-TB, resistance to FQs should be identified promptly so that alternative or additional antibiotics can be prescribed. So while there is intellectual interest in investigating the mechanisms and mutations through which M. tuberculosis develops FQ resistance, and the hope

Received: 16 April 2013, Accepted: 6 August 2013, Published: 11 July 2014
Editors: Graham F. Hatfull, University of Pittsburgh, Pittsburgh, PA, and William R. Jacobs, Jr., Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, NY
Correspondence: H. Takiff, htakiff@gmail.com © 2014 American Society for Microbiology. All rights reserved.
that this information will eventually lead to the design of more effective FQs or quinolone derivatives that are less susceptible to these resistance mechanisms, there is also an urgent need to define the mutations conferring resistance so they can be incorporated into rapid molecular tests to identify FQ-resistant strains.

This review will describe the current use of FQs to treat TB, their interaction with the drug target, their mechanism of action, and the mutations known to confer resistance. It will also describe limitations to the rapid detection of resistance mutations and additional potential mechanisms of resistance.

**FQs AND THEIR INTRODUCTION INTO ANTI-TB THERAPY**

The first quinolone, nalidixic acid, was discovered in 1962 (9) and introduced into clinical use in 1967 for the treatment of Gram-negative urinary tract infections (10). The quinolones are synthetic molecules that contain a 4-oxo-1,4-dihydroquinoline ring system with a carboxylic acid attached at position 3 (Fig. 1), and are amenable to multiple modifications. After it was found that the addition of a fluorine atom at the 6-position of the quinoline ring greatly improved their antibacterial potency and broadened their activity, the other side groups were modified, and thousands of different FQs were synthesized. They are classically divided into two subfamilies: the “older” agents, such as ciprofloxacin, ofloxacin, norfloxacin, and pefloxacin, and the “newer” FQs that were developed after 1990, such as levofloxacin, sparfloxacin, gatifloxacin, moxifloxacin, and gemifloxacin. Garenoxacin, one of the last quinolone generation, is a des-F(6)-quinolone. Among the side groups the three positions R1, R7, and R8 are the most variable and have been exploited to design FQs with increased efficacy.

When the first “blockbuster” FQ, ciprofloxacin, was introduced, its heralded broad-spectrum activity raised hopes that it might even be capable of replacing injected drugs for the treatment of serious infections such as *Staphylococcus aureus* osteomyelitis. Unfortunately, it soon became apparent that the usefulness of ciprofloxacin would be limited by the development of resistant strains. Within just a few years after it was introduced, a high percentage of nosocomial isolates (11), especially Gram-positive bacteria, were found...
THE FQ DRUG TARGET

The targets of the FQs are type II topoisomerases. Topoisomerases are ubiquitous nucleic acid–dependent nanomachines essential to cell life that solve the topological problems of DNA during replication, transcription, or recombination (28). They have been divided into two classes, type I and type II, according to their basic mechanism of action (28, 29). Type I DNA topoisomerases introduce transient single-stranded breaks to force the passage of one DNA strand through the other, whereas type II DNA topoisomerases introduce transient double-stranded breaks to force the passage of one segment of double-stranded DNA through another. All organisms contain at least one type I and one type II topoisomerase. Most bacterial genomes encode two type II topoisomerases, DNA gyrase and topoisomerase IV, which together help manage chromosome integrity and topology (28). DNA gyrase tends to be the primary FQ target in Gram-negative bacteria, whereas topoisomerase IV is preferentially targeted by most quinolones in Gram-positive bacteria. DNA gyrase is unique in introducing negative supercoils into DNA, an activity mediated by the carboxy-terminal domain of its DNA-binding GyrA subunit, and is therefore responsible for the DNA unwinding at replication forks. Although very similar to DNA gyrase, topoisomerase IV has a specialized function in mediating the decatenation of interlocked daughter chromosomes that result from replication of the closed circular DNA (30) and also relaxes positive supercoils. A few bacteria, such as Treponema pallidum, Helicobacter pylori, and mycobacteria, possess only one type II topoisomerase, the DNA gyrase, which is therefore the unique target of FQs in these organisms, as has been demonstrated for M. tuberculosis (31).

Based on evolutionary considerations, type II topoisomerases have been subclassified into two families, type IIA and type IIB (28, 29). Bacterial DNA gyrase and topoisomerase IV, together with eukaryal and viral topoisomerases, belong to type IIA, whereas type IIB includes archaeal topoisomerase VI and homologues in plants, a few protists, and a few bacteria (29, 32). Bacterial type IIA topoisomerases, DNA gyrase, and topoisomerase IV consist of two subunits, GyrA and GyrB, and ParC and ParE, respectively, which form the catalytic active heterotetrameric (A2B2 or C2E2) complex (Fig. 2A) of nearly 350 kDa. Subunit A consists of two domains, the N-terminal breakage-reunion domain (BRD) and the carboxy-terminal domain (represented in blue and green, respectively, in Fig. 2). Subunit B consists of the ATPase domain followed by the Topprim
domain, a conserved catalytic domain in type IA and II topoisomerases, DnAG-type primases, OLD family nuclease, and RecR proteins (33) (represented in yellow and red, respectively, in Fig. 2).

Biochemical in vitro work combined with structural studies of isolated domains over the past 20 years has allowed several authors to propose a global quaternary structural model and a catalytic mechanism of the holoenzyme (34) (Fig. 2). The BRD binds a DNA segment termed the “gate” or G-segment at the DNA-gate created by the dimeric interface formed by the so-called catalytic core, constituted by the Toprim domain of GyrB and the BRD of the GyrA subunit. The GyrB N-terminal ATPase domains dimerize upon ATP binding, capturing the DNA duplex to be transported, termed the T-segment. A double-stranded break is then introduced into the G-segment by the BRDs, and each end of the cleaved DNA is covalently bound to a critical tyrosine residue of the GyrA subunits. The T-segment is then passed through the break in the G-segment that is opened, the DNA is resealed, and the T-segment is released through a protein gate at the other end of the complex, the C-gate, prior to resetting of the enzyme to the open clamp form to begin another cycle (Fig. 2C).

**FIGURE 2** (A) Schematic representation of the sequence and domain organization of type IIA topoisomerases formed by the association of two subunits, A and B. Bacterial type IIA topoisomerases are A2B2 heterotetramers. The names of the four conserved domains are indicated. (B, C) Proposed atomic and schematic model of the type IIA topoisomerase architecture. The three gates are indicated. doi:10.1128/microbiolspec.MGM2-0009-2013.f2
Two FQ molecules, one for each protomer, bind at the ternary interface formed by the DNA, the BRD, and the Toprim domain (Fig. 3). FQs inhibit the religation of the broken DNA by intercalating in the bound DNA between the dinucleotides that form the covalent bond between the DNA and the enzyme—a phosphodiester bond between the oxygen of the tyrosine and the phosphor atom of a phosphate group of the DNA (Fig. 3D). Because the catalytic core is dimeric, two FQ molecules are bound and separated by 4 base pairs (Fig. 3E). The consequence of this intercalation is that the two ends of the DNA are moved farther away from each other—more than 9 Å—making it structurally impossible for the O3′-P religation to occur (Fig. 3).

INTERACTIONS WITH DIFFERENT FQs AND RELATION OF STRUCTURE TO RESISTANT DEVELOPMENT

The mutations causing FQ resistance have been investigated in many different bacteria. Most bacteria have gyrase and Topo IV, and resistance-conferring mutations can occur in both, with the stepwise accumulation of several mutations leading to progressively higher-level resistance. In Gram-negative bacteria, the mutations tend to occur first in the A subunit of the gyrase, within the quinolone-resistance-determining region (QRDR) of gyrA (QRDR-A), followed by mutations in the equivalent region of the gene for the A subunit of topoisomerase IV, parC. In Gram-positives, such as *Streptococcus pneumoniae*, the first mutations tend
to be in the QRDR region of ParC, but this depends upon the specific FQ (37). Subsequent mutations can occur in the other A subunit (GyrA or ParC) and also in the QRDR-B of the B subunits of both enzymes (GyrB and ParE). Because mycobacteria do not have topoisomerase IV, the FQ resistance mutations occur in the genes encoding the gyrase, principally in the QRDR of gyrA (20), but also less frequently in the QRDR of gyrB. Moxifloxacin appears to preferentially target the gyrase, which may contribute to its effectiveness against \textit{M. tuberculosis} (38).

The substitutions conferring FQ resistance in the QRDR-A of \textit{M. tuberculosis} occur most commonly in amino acid 94, but also in amino acids 89, 90, and 91 (39) and, less frequently, in amino acids 88 (40) and 74 (41) (Fig. 4). The only commercial test for resistance to second-line agents, the line probe assay MTBDRs\textsubscript{l} (Hain Lifescience GmbH, Germany) (42), is designed to detect the substitutions A90V, S91P, D94A, D94N/Y, D94G, and D94H. GyrB substitutions were initially described for an \textit{in vitro} selected FQ-resistant strain (43), but for many years they were not reported in clinical isolates of \textit{M. tuberculosis}, and their relation to FQ resistance was questioned, although most studies did not look for gyrB mutations in FQ-resistant strains. With the increased use of FQs for TB, and more studies sequencing the QRDR-B, GyrB mutations have been reported more frequently (44), most consistently in amino acids 500, 538, 539, and 540 (45–47) (amino acids 461, 499, 500, and 501 in the new proposed numbering system (48)).

Several studies have sequenced the entire gyrA and gyrB genes, some 4,500 nucleotides, from FQ-resistant strains and found a variety of mutations, mostly outside of the QRDR regions, which were possibly related to FQ resistance. Mutations in gyrB outside of the QRDR-B appear to be more common than gyrA mutations outside of the QRDR-A. Part of the difficulty in clearly showing the effect of the these mutations on FQ susceptibility, especially those in gyrB, was that they are often found in association with gyrA mutations known to cause resistance, and most of the confirmed gyrB mutations cause lower-level resistance than the validated QRDR-A mutations. The causative role of the mutations at the sites mentioned above, and the lack of association of several other mutations, were proven in four ways: (i) determining whether the mutations are also found in FQ-sensitive strains (48); (ii) careful studies of the FQ susceptibility of strains that contain only the mutations in question and no other mutations (45, 46); (iii) \textit{in vitro} studies with overexpressed gyrase proteins containing the mutations in question to determine if their enzyme activity is inhibited by the FQs (46, 49); and (iv) allelic replacement to insert the mutations individually into two FQ-sensitive strain backgrounds (H37R\textsubscript{v} and Erdmann) and then test the FQ sensitivity of the genetically engineered strains (43).

These studies have confirmed that FQ resistance is caused by the GyrA mutations in the QRDR-A and also by GyrB substitutions at amino acids 500, 538, 539, and 540 (45, 46) (Fig. 4). Although there were some differences in the levels of resistance to different FQs conferred by particular amino acid substitutions in the genetically engineered strains compared to the \textit{in vitro} enzyme assay, there was good agreement on the amino acids involved in resistance. Both studies found that the N538D and E540V substitutions conferred high-level resistance to all FQs. The \textit{in vitro} enzyme study also found resistance with the D500H, D500N, E540D, and T539P amino acid substitutions and low-level resistance with the D500A change (46), while the sensitivity of the engineered strains varied with the FQ tested and the strain background, and often the level of resistance did not meet the clinical definition of resistance with some or all of the FQs tested (2 μg/ml) (45). Reports that have tested many clinical isolates with different QRDR mutations against different FQs have shown that while the A90V substitution routinely confers resistance to ciprofloxacin and ofloxacin, with MICs >3 μg/ml, it only modestly increases resistance to moxifloxacin, with most strains showing moxifloxacin MICs of <0.5 to 1.0 μg/ml (50–52). A similar tendency has been observed in strains with the D94A substitution (50).

The model of the \textit{M. tuberculosis} DNA gyrase catalytic core also establishes that the QRDR residues of both subunits are spatially close and form the quinolone-binding pocket (QBP) (Fig. 3). In this pocket, the FQ is maintained on one side by three residues of the QRDR-A (G88, D89, and A90) that are in close contact with the two conserved groups of the quinolone, the carboxylic and carbonyl functions (R3 and R4 group [Fig. 3]). On the other side, five residues of the QRDR-B (D500, R521, N538, T539, and E540) [D461, R482, N499, T500, and E501] are in close contact with the most variable groups, R1, R7, and R8 (Fig. 3). Modification of any of these residues from either QRDR has been shown to affect the level of resistance to FQs (47, 51, 53–57). The three residues from the QRDR-A belong to the DNA-recognition helix from the CAP-like DNA binding domain (a4) and are localized in the bottom of the QBP, whereas the residues of the QRDR-B lie at the top entry of the QBP (Fig. 3). The 3D model shows that the geometry of the QBP is crucial for the recognition and the stability of the
FQ molecule in the pocket. This could explain, for example, why nalidixic acid, a very small quinolone, has a very high MIC for *M. tuberculosis* and other bacteria, whereas several of the bulkiest quinolones (sparfloxacin, sitafloxacin, gatifloxacin, and moxifloxacin) are highly efficient gyrase inhibitors because they are perfectly adapted to fit snugly into the QBP. This also accounts for the observed difference in MIC between moxifloxacin and ciprofloxacin for most bacteria, because the R7 and R8 groups are very different for these two molecules (Fig. 1).

**FIGURE 4** The schematic diagram in the center shows the arrangement of the gyrA and gyrB genes, encoding the GyrA and GyrB subunits of the *M. tuberculosis* DNA gyrase. Also shown are the locations of the TOPRIM region of GyrB, the BRD of GyrA, and the sites of the QRDRs of both subunits, QRDR-B or QRDR-A. Above the diagram is an alignment of the region of GyrB containing QRDR-B, illustrating that this region is highly conserved in the B subunits of the gyrase and the B subunits of the topoisomerase IV enzymes (ParE), as illustrated by the Gram-positive *S. pneumoniae* and the Gram-negative *E. coli*. Below the diagram is the alignment of segments including the QRDR-A for the A subunits of the gyrase and topoisomerase IV from the same bacteria. The underlined letters in bold indicate amino acids where mutations confer FQ resistance. The blue Y in the GyrA alignment indicates the tyrosine that is covalently bound to the cleaved G segment DNA (see text). On the top and bottom of the figure are the nucleotide and amino acid sequences of the QRDR-A and QRDR-B regions, with the amino acid substitutions shown to confer FQ resistance. Amino acid 95 of the QRDR-A is polymorphic and can be either serine or threonine depending upon the phylogeny of the strain, but has not been implicated in FQ resistance (20, 155). Below the sequence of the QRDR-B are the amino acid numbers in both the old and new numbering systems.

doi:10.1128/microbiolspec.MGM2-0009-2013.f4
For a given FQ, moxifloxacin for example, any modification of the amino acids belonging to the QBP leads to either a direct or indirect change of the geometry of the pocket that could alter its affinity for the gyrase (Fig. 5). When the side chain of the modified amino acid is smaller, the pocket becomes too large to stabilize the FQ. In contrast, when the side chain of the modified amino acid is bulkier, the pocket becomes too small to fix the FQ. The most common substitutions change either D94 or A90 in GyrA. The substitution of a serine for the alanine at amino acid 90 generates a more adapted pocket for moxifloxacin, increasing the susceptibility to this inhibitor. In contrast, substitution to a valine, frequently observed in resistant strains (48), creates a smaller pocket with steric hindrance between the side chain and moxifloxacin (Fig. 5). However, there is no structural explanation yet of why strains carrying the A90V substitution are more resistant to ciprofloxacin than to moxifloxacin (48) as this residue interacts with the conserved part of the FQ (R3 and R4).

An indirect and more complex effect on the geometry of the pocket is observed when amino acid substitutions are localized in the DNA recognition α-helix (α4) that interacts with the major groove of DNA. As DNA is complexed with the gyrase to form the QBP (Fig. 5), modification of the structure of the DNA also modifies the pocket geometry and can lead to destabilization of the FQs in the pocket. This mechanism could explain why substitutions of the amino acid D94 (the position most commonly mutated in FQ-resistant strains of M. tuberculosis) have paradoxical effects; e.g., the substitution of the aspartate by an amino acid with a smaller side chain, such as alanine or glycine, increases the resistance to the same level as substitutions by amino acids with bulky side chains, such as histidine (45).

There are a couple of interesting interactions for which there are as yet no good structural explanations. While the A74S substitution produces only a very modest increase in resistance to all FQs, when it occurs together with the D94G mutation there appears to be a synergistic effect, making the doubly mutant strain more resistant than strains carrying either mutation alone. Other reports have also suggested that strains with more than one mutation in the QRDR-A, or mutations in both QRDR-A and QRDR-B, have higher levels of resistance than strains with the corresponding single mutations (43, 50). Another unexplained observation is that while the T80A mutation by itself has no effect on FQ susceptibility, when it occurs together with the A90G mutation the strain becomes slightly more susceptible to ofloxacin than strains without any GyrA substitutions (43, 49).

The resistance of the M. tuberculosis DNA gyrase to FQs results from two mechanisms. The effect of mutations in the QRDR that confer “acquired” FQ

**FIGURE 5** (A) Close view of the quinolone-binding pocket. The DNA-protein complex is represented in transparent molecular surface and moxifloxacin, in sticks (color code is the same as in Fig. 3). The residues of the QRDR-B (Toprim) belonging to the QBP are indicated in pink, purple, and yellow. Residue A90 of the QRDR-B is represented in light green in the background of the pocket. (B) Effect of the substitution of A90 (QRDR-A) on the geometry of the quinolone-binding pocket. (Left) Quinolone-binding pocket of the wild-type M. tuberculosis DNA gyrase. The A90 is colored in yellow. (Middle) Substitution of A90 to serine (S90 is represented in green). (Right) Substitution of A90 to valine (V90 is represented in magenta). doi:10.1128/microbiolspec.MGM2-0009-2013.f5
resistance is superimposed upon the baseline “intrinsic” resistance due to the low natural affinity of the M. tuberculosis DNA gyrase catalytic core for the FQs. This is principally attributable to the amino acids at positions 81 and 90 in the QRDR-A and 500 (482 in the new numbering) in the QRDR-B (55). The amino acids at two of these positions in the QBP play a crucial role in both intrinsic and acquired resistance: position 90 in the QRDR-A, which is an alanine in the M. tuberculosis sequence, and position 500 (482), an arginine in the M. tuberculosis QRDR-B. Both amino acids are essential for the shape of the QBP and, as a result, for the binding of the quinolone. In the QRDR-B, the side chain of residue R521 (R482), which is located in a loop of the DNA binding Toprim domain, points toward the DNA minor groove and forms a “flap” that blocks the FQ in the pocket through contact with the R7 group (Fig. 5). The increase in the susceptibility to FQs by the substitution of a lysine for the arginine at position 521 (482) can be explained by the lower energy cost of moving an arginine rather than a lysine in the DNA minor groove (58). This means that the flap created by this amino acid would find it easier to open up and destabilize the FQ when the residue is an arginine rather than a lysine.

**FQ RESISTANCE WITHOUT GYRASE MUTATIONS**

While mutations in the QRDRs are found in most FQ-resistant isolates, almost all studies report that at least a few FQ-resistant clinical isolates of M. tuberculosis lacked mutations in both of these regions, and the percentage of these strains without QRDR mutations has varied from 0 to 50% (3). Some studies that sequenced the entire gyrA and gyrB genes found additional mutations outside of the QRDR regions, but most of these were shown not to have a role in FQ resistance (see above).

How can these strains be resistant if no mutations are found in either QRDR-A or QRDR-B? There are several possible explanations. A recent study found that strains with high-level resistance (ofloxacin 10 μg/ml) are more likely to have QRDR mutations than those with low-level resistance (ofloxacin 2 μg/ml), although a majority even of those with this lower level of resistance also had QRDR mutations (59). Strains with even lower levels of resistance, below the 2 μg/ml ofloxacin cutoff for the clinical definition of resistance, are more likely to lack QRDR-A mutations, but some QRDR-B mutations, such as T533P and E540D, conferred resistance to only 1 μg/ml ofloxacin (45). The MICs for FQs in “susceptible” strains of M. tuberculosis never exposed to FQs vary over an 8-fold range from 0.125 to 1 μg/ml ofloxacin (21), so perhaps some of the low-level “resistance” below 2 μg/ml merely represents strains at the upper limits of this range. The reasons for this variation of intrinsic MICs are unknown, as are the possible relationships between the innate MIC and the propensity to develop higher-level resistance, but the level of resistance conferred by a specific QRDR mutation can vary depending upon the strain background into which it is inserted (45). It is possible that FQ resistance in M. tuberculosis can be caused by mutations activating alternative resistance mechanisms, as described in other bacteria and discussed below, and perhaps some of these additional mechanisms may be more inducible in particular strains, accounting for the variation in baseline MICs. At this time, however, neither the putative nongyrase mutations, which would likely confer only low-level resistance, nor genes causing inducible resistance or tolerance, have been identified.

The success at finding gyrase mutations in FQ-resistant isolates can also depend upon the technique used and the definition of resistance. Clinical resistance is best defined by phenotypic resistance, tested by growing the bacteria in culture media in the presence of an FQ. The World Health Organization (WHO) recently revised its cutoff concentrations upward from 2 μg/ml to 4 μg/ml for ofloxacin and to 1 μg/ml for levofloxacin in 7H10 and 1.5 μg/ml with the MGIT 960 method (60). Two cutoffs were recommended for moxifloxacin, 0.5 and 2.0 μg/ml. The most accepted technique, the proportion method, takes 4 to 6 weeks to obtain results, but there are quicker methods on thin-layer agar, or using the nitrate reductase method, or in liquid media with the BACTEC-MGIT system, the Microscopic Observation Drug Susceptibility assay (MODS), or the Microplate Alamar Blue method (MAB), that require as few as 5 to 7 days (61, 62). Several of these methods require primary cultures and cannot be used directly on clinical material, although the green fluorescent protein phage system shows promise for identifying resistant strains from sputum specimens in less than 48 hours (63).

**Heteroresistance**

All phenotypic drug sensitivity methods currently take more than a day to perform and therefore require a second visit by the patient to adjust therapy if resistance is found, which can result in critical delays before the patient receives an appropriate drug regimen. The goal for optimal treatment of resistant strains of MDR- or XDR-TB would be a point-of-care method that could both diagnose the presence of M. tuberculosis and also
identify FQ resistance (in addition to resistance to other first- and second-line anti-TB drugs) within a couple of hours, something that the Xpert system has effectively done for rifampin resistance (64). To achieve this, several molecular techniques, such as the recently WHO-endorsed MTBDRs/ line probe assay (42, 60), have been developed to rapidly detect the mutations most commonly associated with FQ resistance (65). An inherent problem with most molecular methods is that they only detect the resistance-conferring mutations if the mutations are present in the majority of the bacteria probed. Current methods do not have sufficient sensitivity to detect the 1% of resistant bacteria that defines resistance by the proportion method (66–70), although a couple of recent techniques can detect mutations in 10% or less of the bacteria probed (71, 72).

Primary resistance to the FQs appears to develop when FQ therapy selects for a resistant bacterium that then expands into a subpopulation that eventually becomes the dominant strain within a patient. During the course of this process, bacilli isolated from the patient may show “heteroresistance”—mixed populations of drug-resistant and drug-sensitive bacteria that are otherwise genetically homogenous (67, 73–77). Alternatively, patients may be infected with two genotypically different strains, only one carrying the resistance mutation (68), but this has been described less frequently. Heteroresistance has been found in 20% or more of FQ-resistant isolates (68, 78, 79). In such cases, isolates with FQ heteroresistance are designated as resistant by the proportion method (which determines a strain to be resistant when ≥1% of the bacterial population grows in the presence of the antibiotic) but may be characterized as sensitive by rapid molecular techniques and thereby produce confusing or “indeterminate” results. However, this depends upon the percentage of mutant bacteria present in the population. As a comparison, for the Xpert MTB/RIF assay to detect a mutation conferring rifampin resistance with a high degree of certainty, the mutation must be present in at least 65% of the bacteria tested (80).

A recent study that methodically searched for heteroresistance (78) by sequencing the QRDR of 171 FQ strains found that 21% had several sequencing peaks of either the wild-type and mutant nucleotides or more than one mutation present in the same codon (78) (Fig. 6). Spoligotyping (81) confirmed that only one genotype was present in all patients except for one who appeared to be infected with two genotypically different

FIGURE 6 The sequence of the QRDR-A region from the initial patient isolate shows a wild-type GAC encoding aspartic acid at codon 94. In an isolate taken after 7 months of FQ therapy, the QRDR-A sequence shows that two mutant bacilli populations were present, one with a GCC encoding an alanine at codon 94 and one with GGC encoding glycine at codon 94. By month 10 the bacteria containing the D94G substitution predominated, and the population with the D94A substitution was no longer detected by sequencing. Figure modified from reference 78. doi:10.1128/microbiolspec.MGM2-0009-2013.f6
strains. In follow-up isolates taken during treatment with an FQ from the patients showing heteroresistance, one resistant genotype became dominant and some developed additional resistance mutations. In one patient whose FQ treatment was discontinued due to the detection of resistance, the pattern returned to wild-type, suggesting the reemergence of an underlying FQ-susceptible population. In 5% of the resistant isolates only wild-type sequence was detected in the first isolate, but when this isolate was cultured in media containing 2 μg/ml of ofloxacin, the colonies that appeared were found to contain QRDR mutations. This suggests that resistant bacteria were present in the original isolate but their proportion was too low for the mutation to be detected by sequencing. In two resistant isolates, no QRDR mutation was ever found. The results suggest that most of the phenotypically FQ-resistant strains without gyrase mutations are really heteroresistant and that although the resistant subpopulation is greater than 1% and can be detected by the proportion method, its percentage of the population is not enough to be detected by sequencing or rapid molecular methods.

It is thought that FQ resistance develops due to poor adherence when the patient does not take or does not absorb the antibiotics at the appropriate frequency or dosage, or when it is given inadvertently as monotherapy, such as when an FQ is added to a failing drug regimen to which the strain is resistant (84–86). However, there are reports of resistance developing despite excellent adherence (84, 85). This might be explained if the patient initially harbored a small subpopulation of resistant bacteria with a QRDR-A mutation prior to exposure to the antibiotic, which then had a growth advantage when the FQ was administered (86). One possibility for how resistance can develop was suggested by a recent study that found that serum concentrations of ofloxacin patients taking the prescribed dose of 800 mg/day did not appear to be sustained, even at the minimal therapeutic level of 1 μg/ml, for sufficient time to kill even some strains considered FQ “susceptible” (87).

Another possibility is that the mutation may have occurred if the patient received FQ therapy prior to the diagnosis of TB. FQs are used to treat infections at many sites (urinary and gastrointestinal tracts, paranasal sinuses, wounds, and sexually transmitted diseases [88]), and in some countries ciprofloxacin is freely available without a prescription. A patient in the early phases of incipient TB could have taken an FQ, which would have constituted FQ monotherapy for their TB. Resistance has been reported to develop after taking FQs for only 7 days (89). Also, FQs are commonly prescribed for community-acquired pneumonia, so when a patient initially presents with nonspecific respiratory symptoms that are due to undiagnosed TB, they may be given a course of an FQ. This may kill off some of the bacilli and lead to a transient improvement, but when the patient returns with a recrudescence of the symptoms there may be fewer bacilli, which make the diagnosis more difficult. Several studies have shown that taking an FQ for >5 days in the months before TB is diagnosed results in delays of 2 to 5 weeks in initiating anti-TB therapy and has been associated with increased mortality (5, 89).

Given the lack of sensitivity of current molecular tests to detect mutations in FQ heteroresistant isolates, the current WHO recommendation is that these tests, especially MTBDRsl, which has high specificity, should only be used to “rule in” a mutation: if a mutation is detected, the result should be believed (60). However, when no mutation is detected, the strain should be tested for FQ resistance with a phenotypic assay, such as the proportion method.

In the study cited above (78) that sequenced the QRDRs from several isolates of FQ-resistant strains and documented heteroresistance in a large percentage, there were two FQ-resistant strains in which no mutations were ever found. This suggests that there must be other sites for mutations that confer FQ resistance, although they may be responsible for only a small percentage of resistance.

The rest of this article describes possible nongyrase mechanisms of FQ resistance that have been identified in other bacteria and the evidence that these might be related to resistance in M. tuberculosis.

**Efflux Pumps**

Most bacteria have a variety of efflux pumps belonging to different transporter families (90), and many antibiotics are substrates for one or more pumps in a given bacteria (91). However, not all pumps are always expressed at high levels, because some require their expression to be induced, and even if a pump is constitutively expressed and may partially determine the innate baseline MIC for a drug, it does not necessarily mean that the pump is involved in the development of acquired resistance to that drug.

For a pump to be involved as a mechanism of genetic drug resistance there should be a mutation that increases the expression of the pump and thereby reduces the drug concentration within the bacteria. This can occur through simple up-mutations in the promoter, such as is seen with mutations in the promoter region of inhA that
increase the strength of the promoter and the expression of the isoniazid drug target (92) and isoniazid resistance. The expression of many efflux pumps is regulated, and the expression can be increased, or “induced,” either by the presence of some of their substrates or as part of a global stress response. The classic example of a regulated efflux pump is TetA, which presumably originated as a self-protection mechanism in a Streptomyces species that produced tetracycline. Upstream of the tetA gene and divergently transcribed is the gene encoding its transcriptional repressor TetR, which binds to the DNA in the promoter region of tetA and blocks its transcription. When tetracycline binds to the TetR repressor, it induces a conformational change such that the repressor no longer binds and blocks the promoter region of tetA, leading to the expression of TetA and consequent extrusion of tetracycline out of the bacterium (93). In contrast, MarA is a transcriptional activator regulated by MarR in E. coli and an example of a global transcriptional regulator that induces the expression of different efflux pumps and confers resistance to several antibiotics (94). In E. coli, FQ efflux pumps are often part of the MarA regulon (82).

Mutations in the regulatory mechanisms involved with induction can occur in the repressor proteins or in their operators—the DNA sites to which they bind upstream of the genes they repress—and cause increased or unregulated, constitutive expression of the efflux pump (95). The increased number of pump proteins confers resistance by transporting more of the antibiotic out of the bacterium, thereby reducing the antibiotic concentration within the cytoplasm to levels below what is required to completely inhibit its target. Although efflux-mediated resistance generally has a smaller effect on MICs than mutations in the drug target, mutations increasing efflux can also occur subsequent to gyrase mutations and cause higher levels of resistance than the gyrase mutations alone (94).

In Gram-positive bacteria the most common efflux pumps associated with resistance to ciprofloxacin belong to the major facilitator family of proton antiporters (96) and are similar to the NorA efflux pump of S. aureus (82). Although they often have other substrates, such as ethidium bromide, acriflavine, or quaternary ammonium compounds, the pumps that confer FQ resistance often do not confer resistance to other antibiotics. When they are expressed, through either induction, a regulatory mutation in the promoter region, an inactivating mutation in a transcriptional regulator, or a mutation in the DNA where the regulator binds, these pumps generally confer low-level resistance to ciprofloxacin and ofloxacin, but not to the later-generation, more hydrophobic FQs such as moxifloxacin and gatifloxacin (97). Moxifloxacin and gatifloxacin (98) (and a few others that have been withdrawn because of toxicity) have better activity against Gram-positive bacteria, especially respiratory pathogens such as S. pneumoniae and mycobacteria, and have been called the “respiratory FQs” (10). The specificity of pumps such as the S. aureus pump NorA for different FQs may be more complex than simple hydrophobicity and may include elements of FQ structure and side groups (99). There are other similar pumps, such as the S. aureus NorB, which recognize moxifloxacin as a substrate and, when induced under stress conditions such as low oxygen, can increase the MIC for moxifloxacin and sparflaxin as part of a generalized response involving several genes (100, 101).

Increased expression of ABC transporters, such as PatA and PatB in S. pneumoniae, has also been found in FQ-resistant strains (102). Their expression is induced by all FQs as well as DNA-damaging agents such as mitomycin, presumably as part of a global stress response inducing the competence system (102). These ABC transporters, like most of the major facilitator transporters, have a preference for hydrophilic FQs as substrates, so while all FQs may induce their expression, their effect on MICs varies with the FQ. They confer the most resistance to norfloxacin and ciprofloxacin but have less activity on ofloxacin and levofloxacin, much less on sparflaxin, and almost no effect on moxifloxacin (102), which may contribute to making moxifloxacin a very effective drug (5, 103).

There is a growing notion that the first step in the development of resistance to antibiotics may be the reversible induction of an efflux pump that makes the bacterium relatively tolerant to the antibiotic, which then increases the frequency for high-level resistance mutations in the drug target (104–106). In S. pneumoniae it has been shown that ciprofloxacin is an excellent inducer of an efflux pump that confers low-level resistance to ciprofloxacin and increases the frequency of parC mutations. Surprisingly, although induction of this pump only slightly increases the MIC for levofloxacin, from 0.6 μg/ml to 0.8 μg/ml, its overexpression dramatically increases the frequency at which parC mutations appear (107) in the presence of levofloxacin. Perhaps the efflux pump permits increased survival in the presence of the FQ, which allows chance mutations to appear. Additionally, it has been suggested that the FQs increase the mutation frequency, although this has only been demonstrated in Mycobacterium fortuitum (108).
The antiporter (90) LfrA of *M. smegmatis* was the first FQ-transporting mycobacterial efflux pump identified (109, 110). Like NorA, it confers low-level resistance to the hydrophilic FQs such as ciprofloxacin and levofloxacin, increasing the MIC by >4-fold, but has little effect on sensitivity to sparfloxacin or moxifloxacin. Upstream of LfrA is LfrR, a repressor of the TetR family, which normally represses the expression of LfrA to a low-baseline level (111). The repression is relieved and LfrA expression increased when the repressor LfrR binds to some of its substrates such as ethidium bromide or acriflavine but not the FQ (112, 113). FQ-resistant mutant strains of *M. smegmatis* selected on ciprofloxacin have increased constitutive expression of LfrA, presumably due to mutations that impede the repression by LfrR. In strains with a disrupted lfrA gene, the MICs increase significantly for ethidium bromide and acriflavine, but only 2-fold for ciprofloxacin, probably due to its low baseline expression (114). Resistant strains of *M. smegmatis* can also be selected that show low uptake of sparfloxacin, presumably due to a second, as yet unidentified, efflux pump (R. Rodriguez and H. Takiff, unpublished results). LfrA is a good example of an FQ resistance pump: its expression can be induced, although not by the FQs, and while it does not seem to significantly affect intrinsic MICs for ciprofloxacin, mutations altering its regulation cause low-level resistance to the hydrophilic FQs. However, it is not broadly conserved in mycobacteria and is not found in *M. tuberculosis* (115).

In contrast, the TAP efflux pump, originally found in *M. fortuitum*, is broadly conserved in mycobacteria (115) and has a homologue in *M. tuberculosis*: Rv1258c. When the *M. fortuitum* gene was overexpressed in *M. smegmatis*, it conferred resistance to tetracycline and some aminoglycosides. Its expression was induced in the presence of rifampin and isoniazid in one study, and of rifampin and ofloxacin but not isoniazid in another study (116, 117). It has also been suggested that the TAP efflux pump may play a role in the increase in the MICs for ofloxacin when *M. tuberculosis* is exposed to rifampin (106) and also when *Mycobacterium marinum* is taken up by macrophages (105). However, neither the overexpression of Rv1258c in *M. smegmatis* nor elimination of the gene from *Mycobacterium bovis* BCG had any effect on FQ MICs, although the MICs for tetracycline and aminoglycosides were altered (118). So while Rv1258c seems clearly to be inducible in *M. tuberculosis*, there is no evidence that the FQs are its substrates.

Much of the work on efflux pumps in mycobacteria has been done in rapid growers, especially *M. smegmatis*, and many of the putative pumps of *M. tuberculosis* have been expressed and studied in this genetically tractable avirulent bacterium (119, 120). Four *M. tuberculosis* efflux pumps have been found to confer at least low-level resistance to FQs when expressed in *M. smegmatis*: one from the major facilitator family and three ATPases. Rv1634 belongs to the major facilitator family and, when expressed in *M. smegmatis*, increases the MICs 2- to 4-fold for ciprofloxacin, norfloxacin, and ofloxacin (121). When the ABC efflux pump complex Rv2862c-Rv2867c-Rv2688c was cloned into *M. smegmatis*, the MICs increased 8-fold for ciprofloxacin and 2-fold for norfloxacin, sparfloxacin, and moxifloxacin but had no effect on the MICs for levofloxacin or ofloxacin (122). Expression of the *M. tuberculosis* DrrAB ATPase in *M. smegmatis* increased the MIC for norfloxacin by 4-fold, which was returned to normal by verapamil, an ATPase inhibitor (123). Finally, the phosphate-specific transporter *pst* is an ABC efflux pump that has been reported both as involved in ciprofloxacin resistance and as a determinant of baseline FQ sensitivity in *M. smegmatis* (124–126). A similar system exists in *M. tuberculosis* but has not been related to FQ resistance or baseline FQ MICs.

Are any *M. tuberculosis* efflux pumps involved in FQ resistance? Although no mutations affecting efflux pumps have been shown to play a role in FQ resistance in *M. tuberculosis*, when *M. tuberculosis* was exposed to the efflux pump inhibitors reserpine and MC 207.110, there was a 2- to 6-fold decrease in the MICs for ciprofloxacin, levofloxacin, ofloxacin, gatifloxacin, and moxifloxacin in both FQ-sensitive and FQ-resistant strains, but the responsible efflux pumps were not identified (127). This suggests that *M. tuberculosis* expresses efflux pumps that extrude FQs and play a role in determining the baseline FQ MICs, but there is still no evidence that mutations involving efflux pumps are involved in the development of acquired FQ resistance. It is also probable that the expression of FQ pumping transporters is induced under certain conditions (105, 106) and that this can lead to a relative tolerance to FQs that may increase the frequency of a gyrase mutation, but an inducible FQ efflux pump has yet to be identified in *M. tuberculosis*.

A study looking at the effect of pump inhibitors on nonreplicating bacteria found that decreased drug permeability contributes to the phenotypic drug resistance of dormant *M. tuberculosis*, but the differences were independent of efflux processes (128). Studies in an
M. smegmatis strain lacking porins, MspA, and MspC showed 2- to 4-fold increases in the MIC for norfloxacin, moxifloxacin, sitafloxacin, levofloxacin, and ofloxacin. While the porins may be important for entry of hydrophilic FQs into the bacteria, it is not clear if these are the primary entry pathway for FQs such as moxifloxacin, whose hydrophobicity may permit them to directly diffuse across the cell membrane (129). The Msp porins are not conserved in M. tuberculosis, and although channel-forming outer membrane proteins have been reported (130), true porin proteins have not yet been described. It seems probable that hydrophilic FQs such as ciprofloxacin require an entry vehicle to pass the hydrophobic, impermeable M. tuberculosis membrane and cell envelope (131), and the slight increases in MICs for moxifloxacin and sparfloxacin in M. smegmatis strains lacking the porins suggest that channels could also be an important entry vehicle for these agents.

Pentapeptide Repeat Proteins

MfpA was discovered by screening a plasmid genomic library for genes that would increase resistance to the FQ (132). Originally found in M. smegmatis, it appears to be present in all mycobacteria as well as in some other actinobacteria (133) but is likely a pseudogene in M. leprae. When MfpA was expressed on a plasmid in M. smegmatis or M. bovis BCG, the MICs for all the FQs increased by 2- to 8-fold, and when it was eliminated from the chromosome of M. smegmatis, the MICs decreased by 2- to 4-fold, suggesting a role in determining the innate bacterial MICs. MfpA belongs to a protein family termed pentapeptide repeat proteins (PRPs) (134) that are composed of 5-amino-acid units, where every fifth amino acid is either a leucine or a phenylalanine and can be described as A(D/N)(L/F)XX (where X can be any amino acid) (135). The repeats form a regular right-handed quadrilateral β-helix structure known as the Rfr-fold (136) (see below).

Shortly after MfpA was described, it was realized that the Qnr proteins, which were found on transmissible plasmids that confer low-level FQ resistance, are also pentapeptides. Members of the Qnr family of pentapeptide proteins have been found in several pathogenic Gram-negative bacteria on a variety of different transferrable plasmids (137), often within integrons or other mobile elements. Many of the Qnr proteins seem to originate from the chromosomes of environmental aquatic bacteria and were perhaps transferred onto plasmids and into pathogenic bacteria due to the selective pressure from extensive use of FQs in commercial fish farming. PRPs are also present in the chromosomes of several Gram-positive bacteria, including pathogenic species such as Enterococcus faecalis (138), Enterococcus faecium, Listeria monocytogenes, Clostridium perfringens, and Clostridium difficile (137), but these have not been found on plasmids, and their function is unknown. While the Qnr proteins do not confer high-level resistance, they may increase the mutant prevention concentration up to 10-fold, making it more likely that mutations conferring higher-level resistance mutations in the gyrase or topoisomerase IV are selected in the course of FQ therapy (139).

Today the PRP family includes more than 1,000 members (140). While the function of these repeats in their original chromosomal locations is uncertain for most proteins, the two bacterial PRPs, Qnr and MfpA, have been reported to interact with DNA gyrase or topoisomerase IV (141, 142), conferring a new mechanism of quinolone resistance by protecting DNA gyrase and topoisomerase IV from the inhibitory effect of quinolones. MfpA is dimeric in solution and in the crystal and forms as a right-handed quadrilateral β-helix with a size, shape, and negative charge that mimic 30 base pairs of B-form DNA (141). Each monomer has eight complete coils of square quadrilateral shape, with the conserved L/F residue in the middle of each side, pointing inward. The C-terminal α-helices interact to form the dimer. A model of interaction was proposed in which the MfpA dimer was docked into the gyrase groove formed by the dimer of the breakage-reunion domains (141) (Fig. 7). In this model, the MfpA protein represents a form of DNA mimicry and competes with DNA for binding to the breakage-reunion domain of DNA gyrase. Exactly what role MfpA plays in gyrase function and how it decreases the susceptibility to FQ in mycobacteria is still a puzzle. One idea is that its interaction with the gyrase could regulate the effective concentration of free gyrase available for binding to DNA, and thus also to the FQ, which associates with a DNA-gyrase complex. Another possibility is that MfpA may destabilize the FQ-DNA-gyrase complex and thus relieve FQ inhibition (143). Whether all bacterial PRPs have the same kind of interactions with DNA gyrase is still unknown. However, structural differences observed between other Qnr structures and MfpA could explain their possible differences in function, notably a loop that is essential for the protective effect of Qnr (4, 32, 143).

Although it has never been described, it is conceivable that any mutation that increases the expression of the chromosomal MfpA in M. tuberculosis could confer low-level FQ resistance, as is seen when mfpA is present...
on a multicopy plasmid (132, 144). The exact mechanism of the regulation of the mfpA expression and the signals that modulate its expression are unknown, but it seems likely that its expression is regulated, because in all mycobacteria it is found as the fifth gene in an operon, preceded by four genes that occur together in many actinomycetes as a unit termed a “conservon” (156). Although there is only one conservon in mycobacteria, this four-gene complex is present 13 times in the chromosome of Streptomyces coelicolor A3(2), 10 times in Streptomyces avermitilis, 3 times in different Frankia species, 5 times in Nocardia farcinica, 5 times in Thermobifida fusca, and 2 times in Kinococcus radiotolerans. In Streptomyces venezuelensis it appears to regulate the expression of the genes encoding the proteins that synthesize the antibiotic jadomycin B (145). The conservon units precede different proteins in the other bacteria, and only in mycobacteria, where they are named MfpB to MfpE, are they followed by a gene encoding a pentapeptide protein.

The first gene in the conservon, Rv3365c, encodes MfpE, a protein similar to other bacterial histidine kinases, which is likely membrane-bound and may serve as a sensor, although it is distinct from the histidine kinases in most two-component systems. It has the histidine residue that is phosphorylated in the classic histidine kinases, but the surrounding amino acids in the “H-box” are different. The next gene, Rv3364c, encodes MfpD, a protein of 130 amino acids belonging to the Roadblock/LC7 family. A recent study found that a protein belonging to this family, MglB, served as a GAP (GTPase activating protein) for an adjacent small GTP binding protein, MglA (146). Rv3363c encodes MfpC, a 122-amino-acid peptide that belongs to the DUF 742 family (pfam05331.4) of proteins, whose function is unknown.

The fourth gene, Rv3362c, encodes MfpB, a small GTP binding protein that has been found to interact with MfpA, encoded by Rv3361c, the fifth and last gene in the operon (144). Overexpression of both MfpA and MfpB confers higher levels of resistance to ciprofloxacin than MfpA alone (132), and knock-out strains lacking either gene have reduced MICs for ciprofloxacin. Putting this together with what is known about the other proteins in the conservon, it is possible to imagine that the histidine kinase senses some unknown signal, which then is transmitted to the MfpA effector through the GAP protein (MfpD) that accelerates the GTPase activity of MfpB. This would then lead to an alteration of MfpA activity, perhaps changing its affinity for the gyrase and thereby regulating its inhibition of gyrase function, or toggling its function between supercoiling and decatenation, usually carried out by the topoisomerase IV that is lacking in mycobacteria. The protection of the gyrase from FQs and other poisons by MfpA (141) may be merely a side effect, or alternatively, the PRPs could have
evolved as protection from environmental gyrase poisons produced by other bacteria, as the pentapeptides McbG and AlbG serve as protection for bacteria producing the gyrase toxins B17 (147) and albicidin (148, 149), respectively. Whatever the true function of MfpA in mycobacteria may be, any mutation that increases its expression or alters its interaction with the gyrase might confere low-level FQ resistance or increase the level of resistance in strains with QRDR mutations. However, this has never been reported, neither in vitro nor in clinical isolates, although it is likely that the relevant mutations have not been sought in resistant isolates.

In Gram-negative bacteria, there are other plasmid-based mechanisms of resistance (137)—such as variants of aminoglycoside acetyltransferase AAC(6′)-Ib-cr that can acetylate ciprofloxacin, or efflux pumps of the major facilitator family such as QepA, or the RND family such as OqxAB—but neither transmissible FQ-resistance determinants nor FQ-inactivating enzymes have been described in mycobacteria.

**CONCLUSIONS**

The FQs are an important component in the treatment of MDR-TB, of which there are over 400,000 cases a year (150), and clinical trials are currently in progress to see if the use of FQs as first-line therapy can reduce the duration of treatment of drug-sensitive TB (5), so FQs are likely to be used extensively in the treatment of TB for at least the next several years. Their use, however, will likely be accompanied by the development of resistance, which must be detected early so that effective alternative drugs can be prescribed. Although there are some newer, more rapid phenotypic tests, the proportion method is still used because of its reliability and high sensitivity for identifying resistant strains, even if the resistant bacteria represent only 1% of the total bacilli population. However, even if performed directly on clinical specimens, results take 4 to 6 weeks (61). Rapid molecular tests to detect resistance-conferring mutations can be performed in a few hours, but their sensitivity has been limited because in a variable percentage of resistant strains (5 to 50%) no gyrA mutations are found, or results are equivocal. Finding out why these rapid molecular tests have less-than-optimal sensitivity should help improve their sensitivity. Expanding the tests to include mutations in the QRDR of gyrB might increase the sensitivity, but only by a few percent. The intriguing, and still unanswered, question is whether there are mutations in additional sites, other than the genes encoding the gyrase, that can confer resistance.

Mechanisms present in other bacteria include increased expression of efflux pumps that decreases the cytoplasmic FQ concentration, the presence of a plasmid with a pentapeptide that protects the gyrase or an enzyme that inactivates the FQ, and other as yet uncharacterized mechanisms. If these alternative non-gyrase mutations are causing resistance, improving the sensitivity of the rapid tests will require identification of these other sites for resistance-conferring mutations. Alternatively, if the explanation is heteroresistance—where resistance is caused by gyrase mutations that are initially present only in a minority of bacilli too small to be detected by the molecular tests in the initial isolates—improvements in the sensitivity of molecular tests will require techniques that can detect mutations present in only 1% or less of the bacterial population.

Because of the many FQ efflux pumps and Qnr proteins identified in other bacteria, it was thought that these non-gyrase mechanisms would eventually be found, but there are still no reports of non-gyrase mutations causing FQ resistance in M. tuberculosis, either in clinical isolates or in in vitro–selected resistant strains. Heteroresistance seems the more likely explanation, as has been well documented in several careful studies where it was shown to explain all, or almost all, instances where gyrase mutations were not detected in the initial isolates of strains that were determined to be resistant by the proportion method (78).

Alternative resistance mechanisms might be involved in FQ resistance, particularly if selected with ciprofloxacin, but are likely to cause only low-level resistance (151). They may play a role in the variation in baseline FQ MICs for different strains (21, 52) or the different levels of resistance in strains carrying the same gyrase mutations. These other mechanisms, and perhaps also the reversible induction of an FQ efflux pump yet to be identified, could cause a relative FQ tolerance that could increase the frequency at which higher-level gyrase mutations appear (104–106). Reports that efflux pump inhibitors reduce FQ MICs in both FQ-sensitive and -resistant strains suggest that an FQ efflux pump exists and could play a role in determining the baseline FQ MICs (105, 106, 127).

It is thought that FQ resistance develops in a stepwise fashion, at least in other bacteria, such that the level of resistance increases with each additional mutation, in the gyrase and the topoisomerase IV, or together with increased efflux pump expression and plasmid-based Qnr or inactivating genes. However, if the first-level mutation does not raise the level of resistance above the MIC for the FQ being used, it may still be effective. This
may explain why ciprofloxacin remained effective against *E. coli*, because even with a first-level mutation, the MICs were perhaps still below the maximal serum level for ciprofloxacin (109, 151). This may also explain why ciprofloxacin is a useless drug for *M. tuberculosis*, and moxifloxacin is a very good drug.

With the usual doses, the maximum serum level for ciprofloxacin is only about 1.5 µg/ml, for ofloxacin 4.0, for levofloxacin 6, and for moxifloxacin 4. The half-life of ciprofloxacin is 4 hours, ofloxacin 4 to 5 hours, levofloxacin 6 to 8 hours, and moxifloxacin 10 to 13 hours (16). At the maximum ciprofloxacin serum concentration of ∼1.5 µg/ml, resistant colonies can be selected in vitro at a frequency of ∼10^{-6}-7 (20, 151), and a tuberculous lung cavity contains about 10^{8} bacteria (16). Early studies using ciprofloxacin to treat TB found that the high percentage of treatment failures or relapses was generally in patients carrying strains that were ciprofloxacin resistant (16). Although many of the mutations selected in vitro on this concentration of ciprofloxacin may not have substitutions in GyrA but only in GyrB or in neither gyrase subunit (151), even most of these with low-level resistance will not be effectively killed at the 1.5 µg/ml maximum ciprofloxacin serum concentration.

The baseline MICs for ofloxacin in wild-type strains range from 0.05 to 2.0 µg/ml (52), and strains with QRDR-A mutations can be selected at frequencies of 10^{-7} to 10^{-8} on ofloxacin concentrations of 1 to 2 µg/ml, just 2 to 4 times above the MIC. This drug level is probably close to the serum concentration at the end of a dosing interval, but it could be even lower if the patient misses or delays a dose. Almost all QRDR-A substitutions have MICs for ofloxacin and levofloxacin of at least 4 µg/ml, so these strains will not be effectively killed by ofloxacin (52, 151). Levofloxacin is probably better because of its advantageous pharmacokinetics—a longer half-life and a higher maximum serum concentration (16)—which will lower the frequency of resistance mutations and may be adequate to kill most strains with QRDR-B mutations and even some with QRDR-A mutations.

The moxifloxacin MIC for most strains of wild-type *M. tuberculosis* is ≤0.125 µg/ml, while the maximum moxifloxacin serum concentration is 4 µg/ml (16). At even half the maximum serum concentration, 2 µg/ml, the in vitro frequency for selecting resistant strains is less than 10^{-9} (151). While almost all the resistant strains selected on moxifloxacin have GyrA substitutions (151), more than 90% have MICs of ≤2 µg/ml (152). There is a variation in the level of resistance in strains carrying the same mutation, and substitutions in amino acids 94 and 88 seem prone to have higher MICs (52). It has been previously observed that strains with both QRDR-A and QRDR-B substitutions or two QRDR-A substitutions have higher MICs than strains with a single mutation (43). Therefore, while moxifloxacin might be effective against most strains with a single mutation, it could be expected that during moxifloxacin therapy some of these strains would develop additional mutations that would raise their MICs above the moxifloxacin serum concentration. Surprisingly, though, it was found that two clinical isolates with both the A90V and D94G mutations had MICs of 1 and 2 µg/ml for moxifloxacin, suggesting that even these would be effectively killed.

Despite the efforts to find and detect FQ-resistance-conferring mutations, the thinking about the clinical management of these patients is changing. Moxifloxacin is an excellent drug because it has good pharmacokinetics (16), a long half-life, and high affinity for the gyrase (38); is a poor substrate for most efflux pumps (97, 102); and has a low frequency of toxic side effects (tendon rupture and worsening of symptoms in patients with myasthenia gravis), so patient adherence is good. Moxifloxacin is such an effective drug that it may contribute to curing many patients whose *M. tuberculosis* strains contain gyrase mutations. Studies in mice suggested that moxifloxacin can be effective against strains with gyrase mutations if the MIC is ≤2 µg/ml; it was effective against a strain with GyrB D500N (MIC 0.5 µg/ml), reduced mortality in strains with GyrA A09V (MIC 2 µg/ml), but had no effect on infections with a strain carrying the GyrA D94G substitution (MIC 4 µg/ml) (103). A meta-analysis of XDR-TB treatment outcomes found that despite the supposed FQ resistance, treatment with a later-generation FQ (levofloxacin, sparflaxacin, or moxifloxacin) was significantly associated with a better outcome (8). A WHO panel of experts recently recommended that resistance to moxifloxacin be tested at two cutoff levels: 0.5 µg/ml and 2.0 µg/ml (60). Given the very limited choices for treating XDR-TB, it may be advisable to give moxifloxacin to patients with XDR strains with moxifloxacin MICs less than 2 µg/ml.

Is it worthwhile to use molecular tests to find “FQ-resistance” mutations? Strains with GyrB substitutions, present in a small minority of “resistant” isolates, probably have MICs that are treatable with moxifloxacin. Although most GyrA substitutions might be susceptible to serum levels of moxifloxacin, rapid molecular tests could detect substitutions in amino acids 94 or 88 that, at least in some strains, have MICs above 2 µg/ml. However, not all strains with mutations in this codon...
have the higher moxifloxacin MICs (52). In a study of 51 clinical isolates with QRDR-A mutations and resistance to at least 2 μg/ml ofloxacin, only two had moxifloxacin MICs >2 μg/ml; the only G88C substitution had an MIC for moxifloxacin of 4 μg/ml, as did only one of six isolates with D94N. So even if these specific mutations are detected, withholding moxifloxacin might significantly reduce the patient’s chance to be cured (8).

Price, however, is an important consideration in the low- and medium-resource countries where most MDR and XDR-TB is found. Moxifloxacin is expensive, while levofloxacin is more economical. So if levofloxacin is to be used, a rapid test for resistance seems more useful. If a gyrase mutation is detected, the patient might be given moxifloxacin, but if the result is equivocal or no mutation is detected, phenotypic drug sensitivity testing is indicated. If there is a single mutation detected that results in an amino acid substitution at codons 89, 90, or 91, moxifloxacin will probably be effective. If there is a mutation affecting codons 88 or 94, the effectiveness of moxifloxacin is less certain, and phenotypic FQ sensitivity testing is probably indicated, but maybe moxifloxacin should be given while awaiting these results. The Bayer patents for moxifloxacin expire in March 2014, which should make less costly generic versions available (153). This is an evolving therapeutic topic, and the purpose here is not to make clinical recommendations, but merely to review the literature related to resistance mechanisms and their significance. Definitive recommendations may await further follow-up outcome studies of patients with characterized “resistance” mutations who receive moxifloxacin.

New FQs or other modified quinolones continue to appear (154), and a super-agent may eventually be discovered, but for now moxifloxacin still seems to be the most effective and least toxic. Levofloxacin is fairly effective against M. tuberculosis, but resistance seems to develop more readily than resistance to moxifloxacin, at least in vitro. Clinical trials are currently in progress to determine whether and how moxifloxacin can best be used in combination with recently developed drugs to optimize and reduce the duration of therapy for both drug-sensitive and drug-resistant TB (4). The quinolones currently used for TB treatment were designed before structural data on the M. tuberculosis DNA gyrase catalytic core were available. Extensive 3D-QSAR analyses of existing structural data and new crystallographic data on the protein-DNA-FQ ternary complex will allow a more complete understanding of the relationships between the sequence, the structure, and the resistance phenotypes of this enzyme and should help to design more effective FQs whose affinity for the gyrase would be less susceptible to mutations and therefore less likely to generate resistance.

ACKNOWLEDGMENT
This work was supported by FONACIT proyecto G-2005-000393, LOCTI project, Nuevos Fármacos contra la Tuberculosis, the visiting professor program of the Université de Montpellier 2, France, and an Ecos Nord project between the Unité de Microbiologie Structurale, Institut Pasteur, Paris, and the Laboratorio de Genética Molecular, IVIC, Caracas, Venezuela.

We thank Laurent Kremer, Mathew Vetting, and especially Robin Warren for critically reading the manuscript.

REFERENCES
The Molecular Genetics of Fluoroquinolone Resistance in *M. tuberculosis*


83. Chen TC, Lu PL, Lin CY, Lin WR, Chen YH. 2011. Fluoroquinolones are associated with delayed treatment and resistance in tubercu-
The Molecular Genetics of Fluoroquinolone Resistance in M. tuberculosis


