Role of Disputed Mutations in the *rpoB* Gene in Interpretation of Automated Liquid MGIT Culture Results for Rifampin Susceptibility Testing of *Mycobacterium tuberculosis*

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ABSTRACT Low-level rifampin resistance associated with specific *rpoB* mutations (referred as “disputed”) in *Mycobacterium tuberculosis* is easily missed by some phenotypic methods. To understand the mechanism by which some mutations are systematically missed by MGIT phenotypic testing, we performed an *in silico* analysis of their effect on the structural interaction between the RpoB protein and rifampin. We also characterized 24 representative clinical isolates by determining MICs on 7H10 agar and testing them by an extended MGIT protocol. We analyzed 2,097 line probe assays, and 156 (7.4%) cases showed a hybridization pattern referred to here as “no wild type + no mutation.” Isolates harboring “disputed” mutations (L430P, D435Y, H445C/L/N/S, and L452P) tested susceptible in MGIT, with prevalence ranging from 15 to 57% (overall, 16 out of 55 isolates [29%]). Our *in silico* analysis did not highlight any difference between “disputed” and “undisputed” substitutions, indicating that all *rpoB* missense mutations affect the rifampin binding site. MIC testing showed that “undisputed” mutations are associated with higher MIC values (≥ 20 mg/liter) compared to “disputed” mutations (4 to >20 mg/liter). Whereas “undisputed” mutations didn’t show any delay (Δ) in time to positivity of the test tube compared to the control tube on extended MGIT protocol, “disputed” mutations showed a mean Δ of 7.2 days (95% confidence interval [CI], 4.2 to 10.2 days; $P < 0.05$), providing evidence that mutations conferring low-level resistance are associated with a delay in growth on MGIT. Considering the proved relevance of L430P, D435Y, H445C/L/N, and L452P mutations in determining clinical resistance, genotypic drug susceptibility testing (DST) should be used to replace phenotypic results (MGIT) when such mutations are found.

KEYWORDS *Mycobacterium tuberculosis*, diagnostics, drug susceptibility testing, multidrug resistance

Molecular approaches identifying mutations conferring drug resistance represent a revolution in the field of tuberculosis (TB), shortening the time to diagnosis from months to hours. Indeed, culture-based conventional phenotypic drug susceptibility testing (DST) has a time frame too long for proper patient management (1). The molecular diagnostics landscape offers a wide panel of new molecular assays, especially in high-income countries. Among them, the GenoType MTBDR^{plus} line probe assay (LiPA) (Hain, Nehren, Germany) and the Xpert MTB/RIF and Xpert MTB/RIF Ultra (Cepheid, Sunnyvale, CA) assays are valuable alternatives for the rapid detection of rifampin resistance in *Mycobacterium tuberculosis*, with rapid and highly specific results (2–4). However, whereas the presence of specific and well-known mutations in the rifampin resistance-determining region (RRDR) of the *rpoB* gene allows easy interpretation of

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molecular DST and consequent clinical decision, less common mutations often not specifically targeted by the current assays, such as those based on LiPA technology, are more difficult to interpret. Indeed, commercially available LiPAs specifically target only a few mutations (D435V, H445Y, H445D, and S450L), whereas the remaining mutations affecting the RRDR are inferred by the lack of hybridization of the wild-type probe. Hence, phenotypic DST methods for *M. tuberculosis* are still considered the “gold standard” for identification of rifampin resistance. Previous results indicated that low-level rifampin resistance associated with specific *rpoB* mutations (referred as “disputed”) is easily missed by some phenotypic methods, thus highlighting discrepancies between genotypic versus phenotypic testing and also between different testing media (5–16). While the relevant clinical implications of these findings have been described in several studies, the technical bases of these observations were not further elucidated. Given the urgent need for rapid and accurate DST methods, a more extensive understanding of discrepancies between phenotypic and genotypic approaches is needed. To this end, in this study we focused on discrepant cases between DST performed on the Bactec MGIT 960 (BD, Franklin Lakes, NJ) system and the “no-wild-type” pattern shown by some molecular assays. To understand the mechanism by which some mutations are systematically missed by MGIT phenotypic testing, we performed an *in silico* analysis of their effect on the structural interaction between the RpoB protein and rifampin. We also characterized a subset of representative clinical isolates by determining MICs on 7H10 agar and testing them by a modified (extended) MGIT protocol.

MATERIALS AND METHODS

Selection of strains. We included in the study isolates available in our strain collection and previously tested with line probe assays (LiPAs) for the rapid detection of rifampin and isoniazid resistance. We retrospectively identified 156 isolates tested with LiPAs (namely the GenoType MTBDR and the GenoType MTBDRplus version 1 or 2; Hain Lifescience, Nehren, Germany) showing a hybridization pattern for the *rpoB* gene in which at least one wild-type band was missing, without any further hybridization of bands identifying specific mutations (a hybridization pattern herewith referred to as “no wild type + no mutation”). From this starting data set, we selected 24 clinical isolates harboring specific mutations plus 10 control isolates with known mutations (Ser450Leu) or wild type (including the reference strain H37Rv) for the *rpoB* gene for further characterization based on a convenient sampling method.

DNA extraction. DNA from isolates was extracted by thermal lysis and sonication as described elsewhere (17).

Sequencing. All 156 isolates were sequenced by paired-end Sanger sequencing for the rifampin resistance-determining region (RRDR) of the *rpoB* gene for mutations responsible for rifampin resistance. The primers used and the detailed genomic region covered are available upon request. Direct sequencing of the PCR products was carried out with an ABI Prism 3100 capillary sequencer (Applied Biosystems, Foster City, CA) and an ABI Prism BigDye Terminator kit v.2.1 (Applied Biosystems) according to the instructions provided by the manufacturer. Sequencing results were analyzed using BioEdit v.7.1.3.0 (18), aligning sequences with the corresponding reference strain (*M. tuberculosis* H37Rv; GenBank accession no. [AL123456](#)), and results were reported according to the *M. tuberculosis* numbering system (19).

Drug susceptibility testing. Initial phenotypic drug susceptibility testing for rifampin was performed on the Bactec MGIT 960 (BD, Franklin Lakes, NJ) using 1 mg/liter as the critical concentration (CC), according to WHO recommendations (20). For this study, we further repeated phenotypic drug susceptibility testing on the Bactec MGIT 960 using one tube without rifampin as a control and another with 1 mg/liter rifampin added as a test tube. The protocol was set up with the BD EpiCenter TB extended individual susceptibility testing software (TB-eXiST, v.3.00c, 2011). The drug susceptibility testing (DST) was not considered over when the control tube reached 400 growth units (GU), but we followed up the results until the test tube with rifampin reached ≥ 100 GU, even if the control tube was already positive. The time to positivity (TTP) was defined as the number of days from sample inoculation to detection of mycobacterial growth. The TTPs of both the control and the test tubes were recorded in days, and the delay (Δ) between the growth observed in the control tube (≥ 400 GU) and the test tube (≥ 100 GU) was calculated as follows: $\Delta = \text{TTP}_{\text{test tube}} - \text{TTP}_{\text{control tube}}$. The protocol stopped at day 42: thus, the maximum TTP used for calculations was 42 days.

Isolates were also tested further for determination of the MIC by the 7H10 agar method. The MIC was determined as previously recommended (21). Briefly, testing concentrations included 0.5, 1.0, 4.0, 10.0, and 20.0 mg/liter. Testing plates were read at day 21 if the control plate showed a minimum number of 20 visible colonies; otherwise plates were incubated for another week and eventually read at day 28. If no growth appeared at day 28, the test was repeated. For our purposes, we considered results in terms of the MIC₉₉, the concentration of rifampin inhibiting 99% of the bacterial population. As a result, we considered 1% as a critical proportion. Results were reported both as MIC values and category (susceptible or resistant). Isolates were considered rifampin resistant on 7H10 agar medium when the MIC was found to be >1 mg/liter.

Data analysis. For data analysis, first of all, we correlated *rpoB* mutations and phenotypic resistance in both MGIT and 7H10 media. Then, we correlated the *rpoB* mutations with the delay (Δ) observed in MGIT. To provide an overall picture, we compared by *t* test mutations associated with Δ in MGIT-susceptible, 7H10-resistant cases versus MGIT-resistant, 7H10-resistant cases and also Δ in MGIT-susceptible, 7H10-resistant cases versus MGIT-susceptible, 7H10-susceptible cases. Analyses were performed using GraphPad Prism 5 v.5.04 (GraphPad Software, Inc., La Jolla, CA).

Structural analysis. The effect on the protein structure of the mutations associated with Δ were evaluated using visual and computational analyses. First, the crystal structures of *M. tuberculosis* transcription initiation complex bound to rifampin (PDB codes 5UH6, 5UHB, 5UHC, 5UHD, and 5UHG) (22) were visualized with PyMOL software (the PyMOL Molecular Graphics System, version 1.8; Schrödinger, LLC). Next, the effect on the protein stability of amino acid substitutions not directly in contact with the drug was calculated with the program FoldX, which uses a semiempirical energy function to evaluate the free-energy variation upon amino acid substitution (23). Briefly, the isolated complete RNA polymerase structure was subjected to an energy minimization to remove any steric clashes that involved side-chain atoms. Each *rpoB* mutation, individually or in combination, was introduced in the protein structure using the BuildModel module in FoldX, and the variation of the folding free energy ($\Delta\Delta G$) compared to the wild-type protein was then computed. The procedure was repeated 5 times to ensure that the mutant structure was not trapped in local minima. Energy values were considered significantly destabilizing if greater than 2 times the standard deviation of the program. The variation in rifampin affinity toward RpoB mutants was calculated using the program mCSM-lig, which takes advantage of graph-based signatures of the environment of the mutations and does not require the direct modeling of the mutation (24).

RESULTS

A retrospective analysis of isolates tested on LiPAs in our laboratory showed 156 out of 2,097 (7.4%) cases harboring a hybridization pattern designated here “no wild type + no mutation” (details available upon request), and sequencing results are summarized in Table 1. The most frequently mutated codons were 445 (26.9%), 450 (19.2%), and 452 and 432 (8.3% each), followed by codons 435 (7.1%), 430 and 441 (3.2% each), and 431 (0.6%). Multiple mutations (single nucleotide polymorphisms) were found in 16.0% of cases, whereas nucleotide insertions and deletions (indels) were observed in 3.2% of samples. The presence of silent mutations accounted for 1.3% of “no wild type + no mutation” cases (two cases harboring the Q432Q and D435D mutations, respectively).

In order to ascertain whether the “disputed” mutations (L430P, D435Y, H445C, H445L, H445N, H445S, and L452P) could differentially affect rifampin binding to the RpoB protein compared to the “undisputed” ones, we analyzed *in silico* the effects of all amino acid substitutions presented here. Visual inspection of the structure of *M. tuberculosis* RNA polymerase in complex with DNA, nascent RNA, and rifampin (22) reveals that all missense mutations in the *rpoB* gene affect the rifampin binding site (Fig. 1) and define a contiguous molecular surface in direct contact with the drug. In order to investigate the effect of the observed mutations on the RpoB protein structure, we performed an *in silico* analysis using the program FoldX, which computes the variation in folding energy ($\Delta\Delta G$) following amino acid replacements (details available upon request). Of all mutations considered, only five (S431R, S441Q, S441W, S450F, and S450Y) had a significantly destabilizing effect (at least 0.92 kcal/mol, corresponding to twice the standard deviation of the computed energies by the program compared to the experimental values). We used the program mCSM-lig to evaluate the effect of each mutation on the binding affinity toward rifampin (details available upon request). The computed variations in affinity of the “disputed” and “undisputed” mutations were not statistically significant (see Fig. S1 in the supplemental material).

We selected 24 isolates harboring 14 mutations (tagged in Table 1) representative of the hybridization pattern “no wild-type + no mutation” for further characterization by extended phenotypic DST in MGIT and MIC determination on 7H10 agar medium. This selection included isolates harboring “disputed” mutations sometimes found to be associated with rifampin susceptibility on MGIT testing (D435Y, H445C, H445N, H445S, and L452P), as well as “undisputed” mutations found in rifampin-resistant isolates (S431R, Q432L, H445Q, S450F, M434I + H445N, deletion at codons 434 to 437, deletion at codon 435 + E460G, L449M + S450P, and M434T + H445S). In addition, as controls we included 2 rifampin-resistant isolates harboring an in-frame mutation affecting codon 445 (insertion of AAC at nucleotide position 1334), and the S450L mutation, respectively. Seven rifampin-susceptible wild-type isolates and one rifampin-susceptible isolate harbor-

TABLE 1 Isolates showing the pattern “no wild type + no mutation” on LiPA

<i>rpoB</i> RRDR sequencing type ^a	No. of isolates with phenotypic DST result ^b :		Total no. (%)
	R	S	
D435D		1	1 (0.6)
D435L	1		1 (0.6)
D435V	1		1 (0.6)
D435Y ^c	5	3	8 (5.1)
D435Y + L452P + G456V	1		1 (0.6)
del nt 1302–1310→GGACCAGAA ^d	1		1 (0.6)
del D435	1		1 (0.6)
del D435 + E460G ^d	1		1 (0.6)
del 435-L443F	1		1 (0.6)
S431R ^d	1		1 (0.6)
dupl F433	1		1 (0.6)
H445C ^d	3		3 (1.9)
H445D	3		3 (1.9)
H445L	7	2	9 (5.8)
H445N ^c	4	3	7 (4.5)
H445P	2		2 (1.3)
H445Q ^d	5		5 (3.2)
H445R	5		5 (3.2)
H445S ^c	3	4	7 (4.5)
H445S + L452P	1		1 (0.6)
H445Y	1		1 (0.6)
del nt 1295–1303→AATTCATGG	1		1 (0.6)
L430P	3	1	4 (2.6)
L430P + D435G	2		2 (1.3)
L430P + D435Y	1		1 (0.6)
L430Q	1		1 (0.6)
L430R + D435Y	2		2 (1.3)
L449M + S450P ^d	1		1 (0.6)
L452P ^c	11	2	13 (8.3)
M434I + D435Y	2		2 (1.3)
M434I + H445N ^c		1	1 (0.6)
M434T + H445S ^d	1		1 (0.6)
M434V + H445N	1		1 (0.6)
N437D + L452P	1		1 (0.6)
Q429V + D435Y	1		1 (0.6)
Q432E	1		1 (0.6)
Q432H + L452P	1		1 (0.6)
Q432K	1		1 (0.6)
Q432L ^d	4		4 (2.6)
Q432P	6		6 (3.8)
Q432P + H445S	1		1 (0.6)
Q432Q	1		1 (0.6)
S431R + H445N	1		1 (0.6)
S431T + M434I + H445N	3		3 (1.9)
S441L + H445Q	1		1 (0.6)
S441L + S450A	1		1 (0.6)
S441Q	3		3 (1.9)
S441W	2		2 (1.3)
S450F ^d	2		2 (1.3)
S450L	4		4 (2.6)
S450Q	1		1 (0.6)
S450Y	1		1 (0.6)
S450W	22		22 (14.1)
T444T + H445P + K446Q	1		1 (0.6)
Wild type	6		6 (3.8)
Total	139	17	156

^adel, deletion; nt, nucleotide; dupl, duplication.^bR, resistant; S, susceptible.^cMutations selected among rifampin-susceptible isolates for further characterization.^dMutations selected among rifampin-resistant isolates for further characterization.

FIG 1 Structural representation of the residues involved in the RpoB and rifampin interaction. (Left) Molecular surface of the RpoB protein (shown in white) with the bound rifampin depicted as sticks. The mutated amino acids formed are all located within 5 Å of the drug and define a contiguous surface in the binding cavity. Both “undisputed” (colored cyan) and “disputed” (yellow) mutations affect amino acids directly involved in shaping the rifampin binding site. The coordinates of RpoB and rifampin were taken from the PDB deposition 5UHB. (Right) The side chains of the mutated amino acids are shown as sticks. Since the resolution of the X-ray data used in the structure determination was lower than 3.5 Å, hydrogen bonds between RpoB and rifampin could not be unequivocally assigned. (The PDB numbering system = the MTB numbering system + 6).

ing a silent mutation (P454P) were also included. All the details for the selected strains are available upon request.

MIC testing on 7H10 agar medium showed that any amino acidic substitution or insertion/deletion in the RRDR was associated with rifampin resistance (CC, 1 mg/liter), with MICs ranging from 4 to >20 mg/liter (Fig. 2A). In general, “undisputed” mutations showed higher minimum inhibitory values (≥ 20 mg/liter) compared to “disputed” mutations (from 4 to >20 mg/liter). All wild-type isolates (including the one harboring the P454P silent mutation) showed MICs of ≤ 0.5 mg/liter. The same isolates tested for MIC were retested on MGIT using the TB-eXiST protocol. As shown in Fig. 2B, “undisputed” mutations did not show any delay (Δ) in the TTP of the test tube compared to the control tube (mean Δ of 0 days), whereas “disputed” mutations showed different degrees of Δ ; six rifampin-susceptible cases according to standard MGIT phenotypic DST showing a Δ in the TB-eXiST protocol were tested twice to confirm our findings (1 H445C, 1 H445N, 2 D435Y, and 2 H445S). Similarly, one rifampin-resistant case according to standard MGIT phenotypic DST showing a Δ in the TB-eXiST protocol was tested twice (H445S). As shown in Fig. 3, “disputed” mutations showed a mean Δ of 7.2 days (95% confidence interval [CI], 4.2 to 10.2 days). As expected, wild-type isolates showed the highest Δ (mean Δ , 30.8 days; 95% CI, 22.4 to 39.3 days). The differences observed in the Δ among the 3 categories of isolates (namely harboring “undisputed” mutations, “disputed” mutations, or no mutations) were found to be statistically significant ($P < 0.05$) (Fig. 3).

DISCUSSION

In this study, we provide further insights in understanding discrepancies between genotypic and phenotypic results for rifampin DST. In particular, we decided to focus our attention on mutations (i) associated with the hybridization pattern “no wild type + no mutation” on line probe assays and (ii) testing susceptible on conventional MGIT phenotypic DST. The majority of such mutations were associated with rifampin resistance (133 out of 150 [88.7%]), whereas more than 10% were found in isolates that tested phenotypically susceptible on conventional MGIT (CC, 1 mg/liter).

Notably, we found that isolates harboring “disputed” L430P, D435Y, H445L/N, or L452P mutations tested susceptible on MGIT with prevalences ranging from 15 to 43% (overall, 12 out of 48 isolates [25%]). Previous studies described similar findings, despite a resistant phenotype being observed when other testing media were used (7). All these mutations have also been proven to be associated with relapse or treatment

FIG 2 (A) MIC (MIC₉₉) values by 7H10 agar proportion phenotypic drug susceptibility testing for selected mutations. (B) Delay (Δ) in the time to positivity (TTP) results between the control and the test tube using the TB-eXiST protocol on MGIT for selected mutations. Note that in 1 case of H445C, 1 case of H445N, 2 cases of D435Y, and 2 cases of H445S, isolates for which rifampin susceptibility was found were tested twice. Similarly, 1 case of rifampin resistance (H445S) was tested twice. Squares represent mutations associated with a Δ of >0 using the MGIT TB-eXiST protocol, and circles represent mutations not associated with any delay by the MGIT TB-eXiST protocol. Black indicates phenotypically resistant to rifampin according to standard MGIT drug susceptibility testing, and gray indicates phenotypically susceptible to rifampin according to standard MGIT drug susceptibility testing. CC, critical concentration. $\Delta = \text{TTP}_{\text{test tube}} - \text{TTP}_{\text{control tube}}$. In panel B, mean values are also indicated (horizontal bars).

failure in clinical settings (8). Our analysis added the H445S variant (associated with a susceptible phenotype in MGIT in 57% of cases) to the list of “disputed” mutations, thus making codon 445 the position most affected by debatable mutations in the RRDR (9 out of 42 cases [21.4%]) (25). In order to unveil the role of these mutations in determining a susceptible or resistant phenotypic result, we provided further characterization by MIC testing on 7H10 agar and using an extended protocol for MGIT with the TB-eXiST software on a subset of *M. tuberculosis* strains. The “disputed” mutations tested resistant on 7H10 agar medium (CC of 1 mg/liter) like the control isolates harboring mutations associated with rifampin resistance (26).

Interestingly, the extended protocol used on the MGIT system highlighted a difference in the TTPs of strains affected by “disputed” mutations. Indeed, isolates harboring D435Y, H445C/N/S, or L452P showed a mean delay (Δ) of 7.2 days (95% CI, 4.2 to 10.2

FIG 3 Comparison between the delay (Δ) in the time to positivity (TTP) results using the TB-eXiST protocol on MGIT for “undisputed” and “disputed” mutations and wild-type isolates. The wild-type results include as well the P454P silent mutation. Δ values are reported together with mean and 95% confidence intervals. Squares represent mutations associated with a Δ of >0 on the MGIT TB-eXiST protocol, and circles represent mutations not associated with any delay on MGIT TB-eXiST protocol. Black indicates phenotypically resistant to rifampin according to standard MGIT drug susceptibility testing, and gray indicates phenotypically susceptible to rifampin according to standard MGIT drug susceptibility testing. $\Delta = \text{TTP}_{\text{test tube}} - \text{TTP}_{\text{control tube}}$. Mean values are indicated by horizontal bars.

days), suggesting that these mutations might somehow cause a defective growth rate during rifampin exposure. Susceptible cases showed a significantly higher delay in TTP (mean Δ , 30.8 days; 95% CI, 22.4 to 39.3 days) compared to “disputed” ones: note that the protocol was stopped at day 42, thus for such strains, the Δ is likely underestimated. This confirms our findings that isolates with “disputed” mutations are associated with growth impairment in MGIT rifampin DST.

It was hypothesized that such mutations were somehow responsible for a limited (or absent) effect on the RpoB-rifampin interaction, thus justifying their susceptible phenotype on MGIT. Rifampin inhibits bacterial RNA polymerases through the steric occlusion of the exit tunnel of the nascent oligonucleotide chain, and resistance to rifampin is caused by mutations in the RRDR affecting directly the rifampin binding site (Fig. 1) (25). These substitutions are all located within 5.0 Å of the bound rifampin in the crystal structures and result in either removal of direct contacts between RpoB and the drug or disruption of interactions that shape the binding site (Fig. 1). Neither physico-chemical characteristic of the amino acid substitutions (e.g., introduction of a charge or variations of the size of the mutated residue) was associated with the “disputed” mutations, nor could a stabilizing/destabilizing effect be associated with a unique phenotype: our analysis using mCSM-lig showed that apparently the different behavior of mutants in liquid culture cannot be directly ascribed to specific effects on the binding affinity (Fig. S1). However, since the program does not account for variations in the binding mode between RpoB and rifampin, it is possible that the “disputed” mutations leading to a delay in growth reduce the affinity but do not completely prevent rifampin from binding to RpoB. This partial inhibitory effect is then overcome in the liquid conditions, possibly due to differences in metabolic profile of the bacteria

compared to the growth on solid media. This hypothesis is supported by a molecular dynamics analysis that showed how specific mutations at residue H445 could retain drug binding to the RpoB protein, albeit in a different conformation that may allow RNA synthesis to proceed (27). Studies on the affinity and kinetics of rifampin binding to these mutants could provide further insights into a molecular basis for the observed delay in growth. Furthermore, we performed a preliminary analysis of the sequence of *rpoA* and *rpoC* genes looking for compensatory mutations; however, we did not detect any of the mutations previously described for rifampin-resistant isolates (28–31) (details available upon request). In addition, the mutations we found were observed in both isolates showing a Δ of >0 and those not showing any delay: thus, further studies are needed to rule out any role in fitness costs for mutations associated with a delayed growth in MGIT.

For the first time, our data provide further details on the basics of the discrepancies between genotypic and phenotypic DST for rifampin, suggesting that mutations conferring low-level resistance are associated with a delay in growth on MGIT phenotypic testing. Mutations frequently missed by MGIT are L430P, D435Y, S441Q, H445L, H445C, H445N, H445S, and L452P; accordingly, between 2 and 3% of rifampin-resistant cases could be missed by MGIT testing. Moreover, the prevalence of such mutations can vary across different geographical regions. Considering the proven relevance of L430P, D435Y, H445C, H445L, H445N, and L452P mutations in determining clinical resistance, as documented by different studies (6, 8, 10, 13–15, 32), genotypic DST should be used to replace phenotypic MGIT results when such specific mutations are found.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.01599-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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