Full-Length Paper

Molecular analysis of the *embCAB* locus and *embR* gene involved in resistance to ethambutol in clinical isolates of *Mycobacterium tuberculosis* in France

Running title: *embCAB*/*embR* in ethambutol-resistant *M. tuberculosis*

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ABSTRACT

Modification of codon 306 in *embB* is regarded as the main mechanism leading to ethambutol (ETB) resistance in clinical isolates of *Mycobacterium tuberculosis*. However, numerous mutations elsewhere in the *embCAB* locus and *embR*, a putative transcriptional activator of this locus, have been reported to be involved in ETB resistance. Here, we investigated the diversity of nucleotide variations observed in *embCAB* and *embR* in *M. tuberculosis* complex isolates from France. These regions were sequenced in 71 ETB resistant (ETB-R) and 60 susceptible (ETB-S) clinical isolates of known phylogenetic lineage. The 131 isolates had 12 mutations corresponding to phylogenetic markers. Among the 60 ETB-S isolates, only 3 (5%) had nonsynonymous mutations that were not phylogenetic markers. Among the 71 ETB-R isolates, 98% had mutations in *embCAB* that likely contribute to ETB resistance; 70% located in *embB* codon 306, 406, or 497, 13% outside these three positions between codons 296 and 426, and 15% corresponding to mutations in the *embC-embA* intergenic region. We found a strong association between resistance to ETB and the presence of mutations in *embB* and the *embC-embA* intergenic region ($P<0.001$). In contrast, the mutations detected in *embC* and *embA* were not involved in ETB resistance, and no mutation was detected in *embR*. These results strongly suggest that the sensitivity of the diagnostic assays for detecting ETB resistance based on testing *embB* codon 306 can be increased by testing the *embB* region between codons 296 and 497, and by including the *embC-embA* intergenic region between positions -8 and -21.

INTRODUCTION

The emergence of multidrug-resistant tuberculosis (MDR-TB), which is resistant to at least rifampicin (RIF) and isoniazid (INH), and more recently extensively drug-resistant TB (XDR-TB), which is MDR-TB resistant to any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin), is widely considered to be a serious threat to global TB control (1). Rapid detection of drug resistance is essential to designing appropriate treatment regimens, preventing treatment failure, and reducing the spread of drug-resistant isolates. Molecular assays for the detection of mutations that confer resistance (e.g., based on DNA sequencing, real-time PCR, and strip technologies such as line-probe assay GenoType® MTBDR) have been increasingly used and have the potential to shorten the time to detection of resistance to one working day (2-6). However, these molecular assays require precise knowledge of the genetic variations involved in the development of resistance to particular anti-TB drugs.

Ethambutol (ETB) [dextro-2,29-(ethylenediimino)-di-1-butanol] was introduced in 1961 and is a first-line anti-TB agent used in drug combinations to prevent the emergence of drug resistance. ETB is also included in second-line regimens for MDR-TB when susceptibility is demonstrated (7). ETB interferes with mycobacterial cell wall synthesis and integrity (8-13) by inhibiting arabinosyl transferases encoded by the *embCAB* locus (≈10 kb), which encompasses three contiguous genes: *embC*, *embA*, and *embB* (14). These enzymes are essential for the synthesis of arabinogalactan (EmbA, EmbB) and lipoarabinomannan (EmbC) in the cell wall of the *Mycobacterium tuberculosis* complex (MTBC) (10, 12). Although the *embCAB* locus is also called the *embCAB* operon, it is not a real operon because the promoter of *embA* and *embB* is thought to be in the *embC-embA* intergenic region (85 bp) (15, 16) and the promoter of *embC* is in the region upstream of *embC*, possibly in the Rv3792 gene (11, 17), while the *embR* gene of MTBC is located 2 Mb from the *embCAB* locus (39, 40).

Resistance to ETB in MTBC has been associated with chromosomal mutations in the *embCAB* locus, but mainly *embB* (5, 14, 16, 20). The majority of detected mutations are concentrated in a 576-bp region of *embB*, called the ETB resistance-determining region (ERDR). This region includes codons 306, 406, and 497 and is predicted to be the recognition site of the enzyme EmbB (21). Mutations in this region cause structural changes in the enzyme and alterations in the ETB-binding site and drug-protein interactions (22, 23), which result in the development of ETB resistance. Nucleotide changes in the ERDR of *embB* are found in approximately 50% to 70% of ETB-resistant isolates of MTBC, mainly in codon 306, with reports estimating that 18% to 78% of the isolates presenting with *embB* mutations have an EmbB 306 substitution (2-5, 13, 16, 17, 23-37). A meta-analysis of the line probe assay Genotype® MTBDRsl, which allows rapid diagnosis of ETB resistance by analyzing EmbB 306, showed that the sensitivity and specificity for ETB are 0.679 (0.652–0.706) and 0.799 (0.773–0.823), respectively (3). However, approximately 30% to 50% of ETB-resistant clinical isolates do not carry a mutation in EmbB 306 and are not detectable by molecular methods based only on the polymorphisms in EmbB 306 (2-5). Although other mutations in the *embCAB* locus have been suggested to confer resistance, limited data have been available until now, with most studies analyzing only a short fragment of the *embB* gene encompassing codon 306 (2-4, 24, 25, 28, 30, 34, 35, 38).

Our main objective was to study the mutations in the entire *embCAB* locus and *embR* that are involved in ETB resistance in clinical isolates of MTBC in France because the majority of previous studies have been based on partial sequencing of the 10-kb region containing the *embCAB* locus. The results were challenged with phenotypic drug susceptibility testing, phylogenetic analysis, data from the literature, and the PolyTB web-based tool. We also assessed the association between the presence of *embB* mutations and the number of first-line drugs resistance.

MATERIALS AND METHODS

Mycobacterium tuberculosis complex clinical isolates

A total of 131 MTBC clinical isolates collected throughout France and received by the French National Reference Center for Mycobacteria (NRC) between 2009 and 2014 were included in this study. Seventy-one isolates were resistant to ETB (ETB-R), among which 68 were MDR (including 7 XDR), 1 was resistant to RIF but susceptible to INH, and 2 were resistant to INH but susceptible to RIF. Among the 71 patients with ETB-R isolates, TB treatment history was positive for 31 (43%), negative for 36 (51%), and unknown for 4 (6%). Sixty-nine patients with ETB-R isolates had 26 different countries of birth and two had an unknown country of birth (Table S1). Sixty of the patients were susceptible to ETB (ETB-S), among which 16 were MDR, 5 were resistant to RIF, 9 were resistant to INH, 1 was resistant to streptomycin (STR), and 29 were susceptible to all anti-TB drugs. Among the 60 patients with ETB-S isolates, TB treatment history was positive for 13 (22%), negative for 26 (43%), and unknown for 21 (35%). Forty-nine of the patients with ETB-S isolates had 25 different countries of birth and 11 had an unknown country of birth (Table S1).

Phenotypic drug susceptibility testing

In vitro drug susceptibility testing for RIF, INH, STR, ofloxacin, amikacin, kanamycin, capreomycin, and ETB was performed on Löwenstein-Jensen medium following the proportion method with the following concentrations: 40 mg/l for RIF, 0.2 and 1 mg/l for INH, 4 mg/l for STR, 2 mg/l for ofloxacin, 20 mg/l for amikacin, 30 mg/l for kanamycin, 40 mg/l for capreomycin, and 2 mg/l for ETB (41).

DNA sequencing of ethambutol resistance-associated genes

Genomic DNA was isolated from bacteria grown on Löwenstein-Jensen medium. A loop of culture was suspended in water (500 µl) and heated at 95°C for 15 min. The DNA used for PCR amplification was obtained by heat shock extraction (1 min at 95°C and 1 min on ice,

repeated five times). PCR amplification of the *embCAB* locus (9,949 bp) and *embR* (1,167 bp) was performed in a volume of 5 µl using the 15 oligonucleotide primer pairs in Table S2. After amplification, unincorporated nucleotides and primers were removed by filtration with Microcon® 100 microconcentrators (Amicon Inc., Beverly, MA) and the amplicons sequenced using the Big Dye® Terminator cycle sequencing-ready kit following the manufacturer's instructions.

Determination of phylogenetic lineage

The phylogenetic lineages of our 131 MTBC isolates were determined using the MIRU-VNTR molecular typing method for all isolates. Spoligotyping was performed for some isolates for which the MIRU-VNTR results were ambiguous. Standard 24-locus-based MIRU-VNTR typing was performed as described previously (42) with the MIRU-VNTR Typing Kit® from GenoScreen. The amplified fragments were analyzed on a 16-capillary Applied Biosystems® 3130 Genetic Analyzer. To determine the lineages of the isolates, the 24 numerical values generated by MIRU-VNTR were compared to those in the MIRU-VNTRplus database (http://www.miru-vntrplus.org). Spoligotyping was performed as described by Abadia et al. using the Luminex microbead-based approach (43). Spoligotypes in binary format were converted to an octal code based on the signatures given by the 43 spacer-spoligotyping patterns for comparisons with the international spoligotype database SITVIT/SpolDB4 (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo), which contains all of the spoligotype international types (SIT) previously described in MTBC. The different phylogenetic lineages were described by Gagneux et al. (44).

PolyTB web-based tool

PolyTB is a web-based resource designed to explore MTBC genomic variation on a global scale (45, 46). Genomic polymorphisms and important meta-data, such as *in silico* inferred

strain types and locations, are presented in a browser, geographic map, and phylogenetic views.

Statistical analysis

EmbCAB mutations and ETB resistance on one hand, and *embB* mutations and the number of first-line drugs resistance on the other hand, were compared using the chi-square test. *P*-values were two-tailed, and $P \leq 0.05$ was considered significant. For statistical analysis, 67/71 ETB-R and 60/60 ETB-S isolates were compared (4 related ETB-R isolates were excluded).

Nucleotide sequence accession numbers

The nucleotide sequences determined for the mutant genes included in the present report were deposited into the GenBank database under the following accession numbers: GU323395 to GU323398, KJ571490 to KJ571499, KJ571510 to KJ571513, KJ571515 to KJ571520, KM189805 and KR092803 for the EmbB nonsynonymous mutants M306V, M306I (atc/ata/att), G406C, G406S, G406A, G406D, Q497R, L402V, F330I, E378A, N296H, N399I, Y334H, D354A, M423T, A19D+N296H, S426N, and N13S and the EmbB synonymous mutants D534D (c1602t), T1027T (g3081a), L986L (c2956t), P965P (g2895a), T44T (g132t), V117V (c351t), and N760N (t2280c), respectively; KJ571500, KJ571509, KR092801, KJ571523 to KJ571525, and KR092802 for the EmbC nonsynonymous mutants A426T, V981L, and V987A and the EmbC synonymous mutants G559G (t1677c), L121L (g363t), R345R (c1035g), and T1036T (c3108t), respectively; KJ571501 to KJ571506 for the *embC-embA* intergenic region nucleotides -c8a, -c8t, -c11a (+EmbC V981L), -c12t, -c16t, and cg deleted at -21 and -20, respectively; and KJ571507, KJ571508, KJ571514, and KJ571521 for the EmbA nonsynonymous mutant G5V and the EmbA synonymous mutants C76C (c228t), Q38Q (a114g), and H764H (c1995t), respectively.

RESULTS

Ethambutol-susceptible isolates

The 60 ETB-S strains had different spoligotypes and MIRU-VNTR codes, representing 60 unrelated clinical isolates. Twenty isolates had no mutation in the *embCAB* locus (Table 1). Among the other 40 isolates, one of the two type S isolates and one of the eight LAM isolates had undescribed synonymous mutations: *embC* g363t (L121L) and *embB* t2280c (N760N), respectively (Table 1). Eleven Beijing isolates harbored the synonymous mutation *embA* c228t (C76C), which is known to be associated with the Beijing lineage (20, 46), and one of the Beijing isolates carried an undescribed mutation, EmbC V987A (Table 1). Twenty-two ETB-S isolates with the nonsynonymous mutation V981L in EmbC belonged to the Haarlem (n=15), Ghana (n=2), T3 variant (n=3), T2 (n=1), or X (n=1) phylogenetic lineages (Table 1). This mutation was previously reported in the Haarlem lineage (16, 20) in all H1, X, and ambiguous T2T3 and T2X1 isolates, as well as one Manu2, one T5, some T1, and some T2 isolates (46). Among the ETB-S isolates with V981L, one T3 variant isolate carried a not yet described synonymous mutation, *embB* g132t (T44T), whereas an X isolate carried two mutations: g2895a (P965P) in *embB* and c1035g (R345R) in *embC*, which were previously reported in the X and X2 lineages, respectively (46). The last isolate carrying the EmbC V981L polymorphism belonged to the Haarlem family and harbored an uncharacterized mutation, EmbC A426T (Table 1). Among the five remaining ETB-S isolates, two Delhi/CAS isolates carried the mutation EmbC R738Q reported in the CAS lineage (20, 46); a Cameroon strain had a mutation in EmbB associated with ETB resistance, M306I; and a West africanum 2 strain and a *M. bovis* strain had both EmbB E378A and EmbC T270I, which were previously reported as phylogenetic markers of ancestral MTBC (in *M. bovis* and lineages 1, 5, and 6 of MTBC) (16, 46, 47, 48). The *M. bovis* strain also carried mutations EmbB N13S and *embC* c3108t (T1036T) previously reported in *M. bovis* (46), and

embB c351t (V117V) previously reported in *M. bovis* and AFRI-1 strains (46). Finally, no *embR* mutation was detected in ETB-S isolates.

Ethambutol-resistant isolates

Based on spoligotyping and MIRU-VTNR typing, the 71 ETB-R isolates represented 67 unrelated strains; in the Beijing family, two distinct subsets each included three isolates sharing the same MIRU code or spoligotype, respectively.

Regarding the mutations corresponding to phylogenetic markers, the 41 ETB-R isolates carrying synonymous mutation *embA* c228t (C76C) belonged to the Beijing phylogenetic lineage as expected. Among these isolates, three also harbored the *embA* a114g (Q38Q) mutation reported as a phylogenetic marker of a subgroup of Beijing strains (20, 46). The ETB-R isolates with the EmbC V981L mutation previously reported in several phylogenetic lineages (16, 20, 46) belonged to the Haarlem (n=5), T1 (n=1), Ghana (n=1), or X (n=1) strain families, with the latter also harboring the phylogenetic marker *embB* g2895a (P965P) that is specifically present in the X lineage (46). Furthermore, one LAM ETB-R isolate obtained from a patient born in Portugal had the EmbB M423T mutation commonly reported in LAM strains from Portugal (46), and one Delhi/CAS ETB-R isolate carried the EmbC R738Q mutation reported in the CAS lineage (20, 46) (Table 1).

Regarding mutations potentially associated with ETB resistance, 40/71 (56%) ETB-R isolates had mutations in codon M306 of EmbB, including 24 with M306V (34%) and 16 with M306I (22%). For the remaining ETB-R isolates, 30 (42%) had mutations in the *embCAB* locus outside EmbB 306 and one belonging to the NEW1 lineage had no mutation (Table 1). Among the 40 isolates with a mutation in codon M306 of EmbB, five had additional mutations in the *embC-embA* intergenic region (-c12t, n=3; -c16t, n=1; and -c11a, n=1), whereas two had undescribed synonymous mutations in *embB*: c1602t (D534D) and g3081a (T1027T) (Table 1).

Among the 30 isolates with mutations in the *embCAB* locus outside codon 306 of *embB*, 18 harbored nonsynonymous mutations in EmbB exclusively, two of which were the undescribed synonymous mutations c2956t (L986L) in *embB* and c1995t (H764H) in *embA* (Table 1). The repartition of EmbB mutations into 18 isolates was as follows: 8 G406A/C/D/S, 2 Q497R, 1 F330I, 1 D354A, 1 N399I, 1 L402V, 1 S426N, and 3 N296H. For the remaining 12 isolates with mutations in the *embCAB* locus, seven had mutations in the *embC-embA* intergenic region exclusively (-c8a, n=1; -c8t, n=1; -c12t, n=2; -c16t, n=2; and deletion cg -21/-20, n=1), two of them with the X and CAS phylogenetic markers *embB* g2895a (P965P) and EmbC R738Q, respectively. Four isolates had a –c12t mutation in the *embC-embA* intergenic region in association with nonsynonymous mutations in EmbB outside codon 306: Y334H (n=3) and D354A (n=1), with the latter also carrying the undescribed silent mutation t1677c (G559G) in *embC*. Finally, one Uganda isolate harbored three nonsynonymous mutations in EmbA (G5V) and EmbB (A19D and N296H) (Table 1). No *embR* mutation was detected in the 71 ETB-R isolates.

Notably, the synonymous mutation *embC* c2781t (R927R) was present in all ETB-S and ETB-R isolates included in the present study. This polymorphism has also been reported in all isolates in the PolyTB web-based tool (46). This single nucleotide polymorphism (SNP) could be the result of a sequencing discrepancy in the *M. tuberculosis* reference strain H37Rv deposited in GenBank (accession number AL123456.3).

DISCUSSION

Few publications have reported sequencing the entire *embCAB* locus (16, 20, 49), most previous studies investigated only part of the *embCAB* locus (5, 26), part of *embC*/*embB* and *embR* almost entirely (23), or the *embC-embA* intergenic region entirely and part of *embB* (15). In these studies, the percentage of ETB-R isolates with mutations in the *embCAB* locus

ranged from 65% (16) to 96% (49). In our study, 70/71 (98%) of ETB-R isolates harbored mutations in the *embCAB* locus that likely contribute to ETB resistance.

Regarding mutations that do not generate ETB resistance, we identified 12 mutations that are associated with phylogenetic lineages of MTBC (Table 2), four of which are well described in the literature: EmbC T270I and EmbB E378A in ancestral MTBC lineages 1, 5, and 6 and *M. bovis* (16, 46, 47, 48); *embA* c228t (C76C) in the Beijing lineage (20, 46); and EmbC V981L in the Haarlem lineage (16, 20). We found EmbC V981L not only in Haarlem strains, but also type X, T1, T3 variant, and Ghana isolates. In the PolyTB database, this mutation has been reported in all H1, all X, all ambiguous T2T3 and T2X1, in some T1, T2, and T5, and in one Manu2 (but not Manu1) strain. Taken together, these data suggest that EmbC V981L is not a phylogenetic marker restricted to Haarlem strains, but it could correspond to a SNP that occurred earlier in the evolutionary pathway of MTBC lineages at the level of a branch leading to a group encompassing the Haarlem, X, Ghana, and some T strains. Five other mutations found in our study are also associated with phylogenetic lineages of MTBC according to the PolyTB database. Several of the mutations were found in ETB-S strains, including *embC* c1035g (R345R) and *embB* g2895a (P965P) in the X lineage (46), *embB* c351t (V117V) in all AFRI-1 isolates and *M. bovis*, and *embC* c3108t (T1036T) and EmbB N13S in *M. bovis* (46), whereas others were detected in ETB-R isolates: *embA* a114g (Q38Q), specific to a subgroup of Beijing lineage and associated with EmbB N296H in three Beijing isolates in our study (20, 46); the CAS-specific EmbC R738Q polymorphism associated with *embCA* -c16t (20, 46); and the EmbB M423T polymorphism, which characterizes the LAM4 lineage from Portugal and was associated with EmbB M306V and *embCA* –c16t in our study (Table 2). Finally, eight SNPs that are probably not involved in ETB resistance were also identified from our strains, but they are unlikely to represent phylogenetic markers because they correspond mostly to synonymous SNPs found in single

isolates within a given phylogenetic group. Four of the SNPs were found in ETB-S isolates: *embB* g132t (T44T) and t2280c (N760N), *embC* g363t (L121L), and the nonsynonymous mutation EmbC A426T (Table 1). Five sporadic synonymous mutations, *embB* c1602t (D534D), g3081a (T1027T) and c2956t (L986L), *embC* t1677c (G559G), and *embA* c1995t (H764H), were also detected in ETB-R isolates in association with mutations known to confer ETB resistance (Table 1). These synonymous mutations are unlikely to participate in drug resistance.

In the literature, between 38 and 94% of ETB-R isolates are reported to have mutations in EmbB (5, 13, 15-17, 20, 23, 26, 28, 29, 31, 32, 34, 36, 49). In our study, 90% of ETB-R isolates had mutations in 18 EmbB codons, corresponding to 22 mutations, including the aforementioned phylogenetic markers (n=3) and synonymous mutations (n=4). Among the 15 remaining nonsynonymous EmbB mutations likely involved in ETB resistance, 7 (M306V/I, G406D/A/S/C, and Q497R) have been unequivocally proven by site-directed mutagenesis or allelic exchange to increase minimum inhibitory concentrations (MICs) and can be considered canonical EmbB mutations in ETB resistance (37, 51-54). Accordingly, in our study, only one isolate phenotypically susceptible to ETB harbored the M306I mutation, and a strong statistical association was found between ETB resistance and mutation 306 alone ($P<0.001$) or EmbB mutations 306-406-497 ($P<0.001$). Some authors (38, 54, 56, 57) have argued that mutations at 306 in EmbB do not cause ETB resistance, but predispose *M. tuberculosis* isolates to the development of drug resistance and increase the capacity of resistant isolates to be transmitted (38, 54, 56, 57). Accordingly, several reports have described isolates with mutations at codon 306 of EmbB that remain susceptible to ETB (2, 4, 8, 13, 17, 25, 29, 30, 34, 36, 49, 56, 57). There are two explanations for the presence of a mutation at codon 306 in EmbB in an ETB-S isolate: the ETB concentration recommended for drug susceptibility testing varies from 2 to 7.5 mg/l depending on the phenotypic method used for susceptibility

testing (41) and the ETB MICs induced by mutations in codon 306 could be close to the breakpoint defining ETB resistance (37, 54).

Other mutations in EmbB that are likely responsible for ETB resistance were identified in our study: D354A was previously reported in ETB-R strains selected *in vitro* (37). For F330I and N399I, other mutations (F330V/S/L and N399T/H/D) were reported in the same codons as ETB-R strains (n=9) in five studies (16, 17, 23, 36, 50) and a single ETB-S strain in one study (50). Similarly, mutations L402V and Y334H were previously described in respectively ETB-R (n=3) strains in three studies (16, 20, 35) and ETB-S (n=1) strain in one study (50). A total of three new EmbB mutations were identified in our study: S426N in an Ural isolate harboring no other mutation in *embCAB*, N296H in a Beijing isolate with three additional synonymous mutations in EmbA (C76C, Q38Q, and H764H), and A19D in an Uganda strain harboring N296H in EmbB and G5V in EmbA. Causal relationships between these three mutations and ETB resistance are yet to be experimentally verified. Overall, a strong statistical association was found between ETB resistance and all the *embB* mutations reported in our study ($P<0.001$).

It is worth to highlight here that ETB-R conferring mutations in *embB* could be regarded as sensitive markers for the prediction of MDR-TB. By performing trend analysis correlating any ETB-R conferring mutations in *embB* to the number of first-line drugs resistance, we found a statistically significant association between these mutations and resistance to INH plus RIF ($P<0.001$). However, among the 23 strains showing resistance to 2 to 3 first-line anti-TB drugs excluding ETB, of which 16 were MDR, only 1 was found to have an *embB* mutation, supporting the idea that resistance to ETB is not a prerequisite for the development of multidrug resistance.

According to the literature, the proportion of ETB-R isolates carrying a mutation in the *embC-embA* intergenic region varies from 0 to 27% (5, 15, 16, 20, 49, 50). In our study, 23%

of ETB-R isolates had mutations at five positions in the *embC-embA* intergenic region, with a total of six distinct mutations: -c8a/t, -c11a, -c12t, -c16t, and a deletion of two nucleotides at positions -21 and -20. None of these mutations was observed in ETB-S isolates and we found a strong statistical association between ETB resistance and mutations in the *embC-embA* intergenic region ($P<0.001$). Excluding -c11a, the mutations at positions -8, -12, and -16 and the deletion of cg in positions -21 and -20, which are located within/adjacent to a predicted TATA box (15, 16, 50), were found in ETB-R strains carrying no other mutations involved in ETB resistance, confirming their involvement in ETB resistance. The role of –c11a is less clear because this mutation was identified in a strain that also has the EmbB M306I mutation, but Cui et al demonstrated that mutations in the *embC-embA* intergenic region (including –c11a) increase ETB resistance by enhancing the transcription of *embA* and *embB*, the MICs of ETB for strains with both *embC-embA* intergenic region mutations and *embB* mutations being much higher than strains with only an *embB* mutation (50).

Notably, we detected no mutation that is clearly associated with ETB resistance in *embC* and *embA*, despite the percentage of ETB-R isolates harboring a mutation in these two genes varying in the literature from 0.6 to 77% and 0 to 12%, respectively (5, 16, 20, 23, 49). In our study, 13% (9/71) of ETB-R isolates and 47% (28/60) of ETB-S isolates had mutations in *embC,* affecting eight codons, but six of them corresponded to synonymous mutations (n=3) or markers of phylogenetic lineages (V981L, T270I, R738Q). Among these mutations, the absence of a role of EmbC T270I in ETB resistance was experimentally confirmed by site-directed mutagenesis (55). Regarding the last two mutations EmbC (A426T and V987A), a previous publication described a similar mutation in codon 426 but not with the same substitution (A426P) (46). In our study, A426T and V987A were found in clinical isolates susceptible to ETB, suggesting that these mutations play no significant role in ETB resistance. With respect to EmbA, 58% of ETB-R and 18% of ETB-S isolates included in our study had

mutations in *embA* in four codons, but all were synonymous mutations, except G5V. Codon 5 in *embA* was previously reported by two different groups to have a G5S mutation in ETB-R isolates (16, 17). In our study, this mutation was detected in association with two EmbB mutations, A19D and N296H, the latter being detected alone in another of our ETB-R isolates. Therefore, we cannot definitively state that ETB resistance is associated with the occurrence of G5V in EmbA.

Finally, we found no isolate with a mutation in *embR*, despite their presence in 1% to 36% of ETB-R isolates in the literature (5, 16, 23). Based on the data generated from the panel of clinical isolates included in the present study, *embR* plays no significant role in the development of ETB resistance in *M. tuberculosis*.

In conclusion, our results strongly suggest that molecular analysis of the *embCAB* locus should prioritize the search for mutations at the level of two "hot spots" for mutations in order to efficiently detect ETB resistance in clinical isolates of *M. tuberculosis*. These two regions correspond to (i) the ERDR in EmbB that could be tentatively extended to a 606 bp region spanning residues 296 to 497 and encompassing the canonical mutations at the level of M306, G406, and Q497, and (ii) the DNA segment extending from position -8 to -21 in the *embC-embA* intergenic region. According to our data and excluding synonymous mutations and those corresponding to phylogenetic markers, 70% of ETB-R isolates had amino acid substitutions affecting codons M306 (56%), G406 (11%), or Q497 (3%) of EmbB, and 13% EmbB mutations outside positions 306, 406, and 497 that are very likely involved in ETB resistance (i.e., N296H, F330I, D354A, N399I, L402V, and S426N), and 15% had nucleotide substitutions in the *embC-embA* intergenic region between positions -8 and -21 (without taking into account the isolates harboring a mutation in codons 306, 406, or 497). Importantly, the commercial DNA strip assay Genotype® MTBDRsl, which is widely used in routine laboratories to detect ETB resistance in clinical *M. tuberculosis* isolates, evaluates only the

EmbB M306 codon, resulting in its limited sensitivity (68%) in the detection of ETB resistance (3). Taken together the molecular results from testing the *embB* ERDR region and *embC-embA* intergenic region and the results obtained from the MTBDRsl line probe assay should markedly increase the percentage of ETB-R clinical isolates of *M. tuberculosis* detected by molecular testing: by 85% when testing codons 306, 406, and 497 and nucleotide positions -8, -12, and -16, and up to 98% by testing the entire *embB* gene and the *embC-embA* intergenic region from -8 to -21.

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TABLE 1 Distribution of the *embCAB* mutations in 60 ETB-S and 71 ETB-R isolates of MTBC

Resistance phenotype	**Nb of isolates**	**Phylogenetic lineage**	**Mutations in the *embCAB* locus[a]**		
			Markers of phylogenetic lineages	**Potentially associated with ETB resistance[b]**	**Synonymous mutations (excluding marker of phylogenetic lineage)**
	3	T	/	/	
	2	T1	/	/	
	3	Cameroon	/	/	
	7	LAM	/	/	
	1	Ural	/	/	
	1	S	/	/	
	3	Uganda	/	/	
	1	S	/	/	*embC* g363t (L121L)
	1	LAM	/	/	*embB* t2280c (N760N)
S	10	Beijing	*embA* c228t (C76C)	/	
(n=60)	1	Beijing	*embA* c228t (C76C)	EmbC V987A	
	14	Haarlem	EmbC V981L	/	
	2	Ghana	EmbC V981L	/	
	2	T3 variant	EmbC V981L	/	
	1	T3 variant	EmbC V981L	/	*embB* g132t (T44T)
	1	T2	EmbC V981L	/	
	1	X	EmbC V981L, *embB* g2895a (P965P), *embC* c1035g (R345R)	/	
	1	Haarlem	EmbC V981L	EmbC A426T	
	2	Delhi/CAS	EmbC R738Q	/	
	1	Cameroon	/	EmbB M306I[c]	
	1	West africanum 2	EmbB E378A, EmbC T270I	/	
	1	*M. bovis*	EmbB N13S, EmbB E378A, *embB* c351t (V117V), EmbC	/	

			T270I, *embC* c3108t (T1036T)		
R (n=71)	3	Beijing	*embA* c228t (C76C)	EmbB M306V[c], *embCA* -c12t	
	1	LAM	EmbB M423T	EmbB M306V[c], *embCA* -c16t	
	14	Beijing	*embA* c228t (C76C)	EmbB M306V[c]	
	1	Beijing	*embA* c228t (C76C)	EmbB M306V[c]	*embB* c1602t (D534D)
	3	LAM	/	EmbB M306V[c]	
	1	T1	EmbC V981L	EmbB M306V[c]	
	1	S	/	EmbB M306V[c]	
	5	Beijing	*embA* c228t (C76C)	EmbB M306I[c]	
	3	Haarlem	EmbC V981L	EmbB M306I[c]	
	1	T1	/	EmbB M306I[c]	
	1	T2	/	EmbB M306I[c]	*embB* g3081a (T1027T)
	5	LAM	/	EmbB M306I[c]	
	1	Ghana	EmbC V981L	EmbB M306I[c], *embCA* -c11a	
	1	Beijing	*embA* c228t (C76C)	EmbB G406C[c]	
	1	Beijing	*embA* c228t (C76C)	EmbB G406S[c]	
	1	LAM	/	EmbB G406A[c]	*embB* c2956t (L986L)
	1	Haarlem	EmbC V981L	EmbB G406A[c]	
	3	Beijing	*embA* c228t (C76C)	EmbB G406D[c]	
	1	LAM	/	EmbB G406D[c]	
	1	Beijing	*embA* c228t (C76C)	EmbB Q497R[c]	
	1	Cameroon	/	EmbB Q497R[c]	
	1	Ural	/	EmbB F330I	
	1	Beijing	*embA* c228t (C76C)	EmbB D354A[d]	
	1	Ural	/	EmbB N399I	
	1	Beijing	*embA* c228t (C76C)	EmbB L402V	
	1	Ural	/	EmbB S426N	
	3[e]	Beijing	*embA* c228t (C76C), *embA* a114g (Q38Q)	EmbB N296H	*embA* c1995t (H764H)
	1	X	EmbC V981L, *embB* g2895a (P965P)	*embCA* -c8a	

1	Beijing	*embA* c228t (C76C)	*embCA* -c8t	
1	Beijing	*embA* c228t (C76C)	*embCA* -c12t	
1	LAM	/	*embCA* -c12t	
1	Beijing	*embA* c228t (C76C)	*embCA* -c16t	
1	Delhi/CAS	EmbC R738Q	*embCA* -c16t	
1	Beijing	*embA* c228t (C76C)	*embCA* del cg -21-20	
3[e]	Beijing	*embA* c228t (C76C)	*embCA* -c12t, EmbB Y334H	
1	Haarlem	EmbC V981L	*embCA* -c12t, EmbB D354A[d]	*embC* t1677c (G559G)
1	Uganda	/	EmbB A19D+N296H, EmbA G5V	
1	NEW-1	/	/	

[a] Excluding the synonymous mutation *embC* c2781t (R927R) present in all ETB-S and ETB-R isolates

[b] *embCA* = *embC-embA* intergenic region

[c] Implication in ETB resistance proved by site-directed mutagenesis or allelic exchange

[d] Involvement in ETB resistance suggested by *in vitro* selected mutant

[e] Isolates sharing identical MIRU codes and spoligotypes

TABLE 2 Mutations in the *embCAB* locus that are markers of phylogenetic lineages or potentially associated with ETB resistance in 60 ETB-S and 71 ETB-R isolates of MTBC

		Mutations		**Phylogenetic lineage**
		Amino acid	**Nucleotide**[a]	
Mutations in the *embCAB* locus	**Markers of phylogenetic lineages**	EmbC R738Q		CAS
		EmbC V981L		Haarlem, all H1, all X, all ambiguous T2T3 and T2X1, in some T1, T2, and T5, and in one Manu2, Ghana, T3 variant
		EmbA C76C	*embA* c228t	Beijing
		EmbA Q38Q	*embA* a114g	subgroup of Beijing
		EmbC T1036T	*embC* c3108t	*M. bovis*
		EmbB N13S		*M. bovis*
		EmbB V117V	*embB* c351t	AFRI-1 and *M. bovis*
		EmbB E378A		ancestral MTBC lineages 1, 5, 6 and *M. bovis*
		EmbC T270I		ancestral MTBC lineages 1, 5, 6 and *M. bovis*
		EmbB M423T		LAM4 from Portugal
		EmbB P965P	*embB* g2895a	X
		EmbC R345R	*embC* c1035g	X2
	Potentially associated with ETB resistance[a]	EmbB M306V/I[b]		
		EmbB G406C/S/A/D[b]		
		EmbB Q497R[b]		
		EmbB F330I		
		EmbB Y334H		
		EmbB D354A[c]		
		EmbB N399I		
		EmbB L402V		
		EmbB S426N		
		EmbB N296H		
		EmbB A19D[d]		

	EmbA G5V[d]	
		embCA -c8a/t
		embCA -c12t
		embCA -c16t
		embCA del cg -21 -20

[a] *embCA* = *embC-embA* intergenic region

[b] Implication in ETB resistance proved by site-directed mutagenesis or allelic exchange

[c] Involvement in ETB resistance suggested by *in vitro* selected mutant

[d] Implication in ETB resistance remaining to be experimentally verified