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Molecular Analysis of the *embCAB* Locus and *embR* Gene Involved in Ethambutol Resistance in Clinical Isolates of *Mycobacterium tuberculosis* in France

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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 in *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 ETB-susceptible (ETB-S) clinical isolates of known phylogenetic lineages. 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% had mutations located in *embB* codon 306, 406, or 497; 13% had mutations located outside these three positions between codons 296 and 426; and 15% had mutations 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 diagnostic assays for detecting ETB resistance based on testing of *embB* codon 306 can be increased by testing of the *embB* region between codons 296 and 497 and by including the *embC-embA* intergenic region between positions −8 and −21.

The emergence of multidrug-resistant tuberculosis (MDR-TB), which is resistant to at least rifampin (RIF) and isoniazid (INH), and, more recently, extensively drug-resistant tuberculosis (XDR-TB), which is 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 the GenoType MTBDR line probe assay) 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 and EmbB) and lipoarabinomannan (EmbC) in the cell wall of *Mycobacterium tuberculosis* complex (MTBC) isolates (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 located 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 isolates is located 2 Mb from the *embCAB* locus (18, 19).

Resistance to ETB in MTBC isolates has been associated with chromosomal mutations in the *embCAB* locus, mainly *embB* (5, 14, 16, 20). The majority of the 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

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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 ~50% to 70% of ETB-resistant (ETB-R) isolates of the MTBC, mainly in codon 306, with previous reports estimating that 18% to 78% of isolates presenting with *embB* mutations have an EmbB codon 306 substitution (2–5, 13, 16, 17, 23–37). A meta-analysis of the Genotype MTBDRsl line probe assay, which allows rapid diagnosis of ETB resistance by analyzing EmbB codon 306, showed that the sensitivity and specificity (with 95% confidence interval) for ETB are 0.679 (0.652 to 0.706) and 0.799 (0.773 to 0.823), respectively (3). However, ~30% to 50% of ETB-resistant clinical isolates do not carry a mutation in EmbB codon 306 and are not detectable by molecular methods based only on the polymorphisms in EmbB codon 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 the 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 resistance to first-line drugs.

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 ETB-R, of 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%) patients. Sixty-nine patients with ETB-R isolates had 26 different countries of birth, and two had an unknown country of birth (see Table S1 in the supplemental material). Sixty of the patients had isolates that were susceptible to ETB (ETB-S), of 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%) patients. Forty-nine of the patients with ETB-S isolates had 25 different countries of birth, and 11 had an unknown country of birth (see Table S1 in the supplemental material).

Phenotypic drug susceptibility testing. *In vitro* drug susceptibility testing for RIF, INH, STR, ofloxacin, amikacin, kanamycin, capreomycin, and ETB was performed on Lowenstein-Jensen medium according to the proportion method, with the following concentrations: 40 mg/liter RIF, 0.2 and 1 mg/liter INH, 4 mg/liter STR, 2 mg/liter ofloxacin, 20 mg/liter amikacin, 30 mg/liter kanamycin, 40 mg/liter capreomycin, and 2 mg/liter ETB (39).

DNA sequencing of ethambutol resistance-associated genes. Genomic DNA was isolated from bacteria grown on Lowenstein-Jensen medium. A loopful 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 the *embR* gene (1,167 bp) was performed with a volume of 5 μl, using the 15 oligonucleotide primer pairs listed in Table S2 in the supplemental material. After amplification, unincorporated nucleotides and primers were removed by filtration with Microcon 100 microconcentrators (Amicon Inc., Beverly, MA), and the amplicons were sequenced by using the BigDye Terminator cycle sequencing-ready kit according to the manufacturer's instructions.

Determination of phylogenetic lineage. The phylogenetic lineages of our 131 MTBC isolates were determined by using the mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (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 (40), 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 analysis were compared to those in the MIRU-VNTRplus database (http://www.miru-vntrplus.org/). Spoligotyping was performed as described previously by Abadia et al., using the Luminex microbead-based approach (41). 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 SITVIT/SpolDB4 international spoligotype database (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo), which contains all of the spoligotype international types (SITs) described previously for MTBC isolates. The different phylogenetic lineages were described previously by Gagneux and Small (42).

PolyTB Web-based tool. PolyTB is a Web-based resource designed to explore MTBC genomic variation on a global scale (http://pathogenseq.lshtm.ac.uk/polytb) (43). Genomic polymorphisms and important metadata, such as *in silico*-inferred strain types and locations, are presented in browser, geographic map, and phylogenetic views.

Statistical analysis. *embCAB* mutations and ETB resistance on one hand, and *embB* mutations and resistance to first-line drugs on the other hand, were compared by using the chi-square test. *P* values were two tailed, and a *P* value of ≤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 KJ571519, KM189805, and KR092803 for the nonsynonymous M306V, M306I (ATC/ATA/ATT), G406C, G406S, G406A, G406D, Q497R, L402V, F330I, E378A, N296H, N399I, Y334H, D354A, M423T, A19D plus N296H, S426N, and N13S EmbB mutants and the synonymous D534D (c1602t; nucleotide c replaced with nucleotide t at position 1602), T1027T (g3081a), L986L (c2956t), P965P (g2895a), T44T (g132t), V117V (c351t), and N760N (t2280c) EmbB mutants, respectively; KJ571500, KJ571509, KR092801, KJ571523 to KJ571525, and KR092802 for the nonsynonymous A426T, V981L, and V987A EmbC mutants and the synonymous G559G (t1677c), L121L (g363t), R345R (c1035g), and T1036T (c3108t) EmbC mutants, respectively; KJ571501 to KJ571506 for the *embC-embA* intergenic region nucleotides -c8a (nucleotide c replaced with nucleotide a at position −8), -c8t, -c11a (plus EmbC V981L), -c12t, -c16t, and nucleotides cg deleted at positions −21 and −20, respectively; and KJ571507, KJ571508, KJ571514, and KJ571521 for the nonsynonymous EmbA G5V mutant and the synonymous C76C (c228t), Q38Q (a114g), and H764H (c1995t) EmbA mutants, 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 40 other isolates, 1 of the 2

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

Resistance phenotype (total no. of isolates)	Phylogenetic lineage	No. of isolates	Mutation(s) in the *embCAB* locus[a]		
			Marker(s) of phylogenetic lineages	Mutation(s) potentially associated with ETB resistance[b]	Synonymous mutation (excluding marker of phylogenetic lineage)
Susceptible (60)	T	3			
	T1	2			
	Cameroon	3			
	LAM	7			
	Ural	1			
	S	1			
	Uganda	3			
	S	1			*embC* g363t (L121L)
	LAM	1			*embB* t2280c (N760N)
	Beijing	10	*embA* c228t (C76C)		
	Beijing	1	*embA* c228t (C76C)	EmbC V987A	
	Haarlem	14	EmbC V981L		
	Ghana	2	EmbC V981L		
	T3 variant	2	EmbC V981L		
	T3 variant	1	EmbC V981L		*embB* g132t (T44T)
	T2	1	EmbC V981L		
	X	1	EmbC V981L, *embB* g2895a (P965P), *embC* c1035g (R345R)		
	Haarlem	1	EmbC V981L	EmbC A426T	
	Delhi/CAS	2	EmbC R738Q		
	Cameroon	1		EmbB M306I[c]	
	West africanum 2	1	EmbB E378A, EmbC T270I		
	M. bovis	1	EmbB N13S, EmbB E378A, *embB* c351t (V117V), EmbC T270I, *embC* c3108t (T1036T)		
Resistant (71)	Beijing	3	*embA* c228t (C76C)	EmbB M306V,[c] *embCA* -c12t	
	LAM	1	EmbB M423T	EmbB M306V,[c] *embCA* -c16t	
	Beijing	14	*embA* c228t (C76C)	EmbB M306V[c]	
	Beijing	1	*embA* c228t (C76C)	EmbB M306V[c]	*embB* c1602t (D534D)
	LAM	3		EmbB M306V[c]	
	T1	1	EmbC V981L	EmbB M306V[c]	
	S	1		EmbB M306V[c]	
	Beijing	5	*embA* c228t (C76C)	EmbB M306I[c]	
	Haarlem	3	EmbC V981L	EmbB M306I[c]	
	T1	1		EmbB M306I[c]	
	T2	1		EmbB M306I[c]	*embB* g3081a (T1027T)
	LAM	5		EmbB M306I[c]	
	Ghana	1	EmbC V981L	EmbB M306I,[c] *embCA* -c11a	
	Beijing	1	*embA* c228t (C76C)	EmbB G406C[c]	
	Beijing	1	*embA* c228t (C76C)	EmbB G406S[c]	
	LAM	1		EmbB G406A[c]	*embB* c2956t (L986L)
	Haarlem	1	EmbC V981L	EmbB G406A[c]	
	Beijing	3	*embA* c228t (C76C)	EmbB G406D[c]	
	LAM	1		EmbB G406D[c]	
	Beijing	1	*embA* c228t (C76C)	EmbB Q497R[c]	
	Cameroon	1		EmbB Q497R[c]	
	Ural	1		EmbB F330I	
	Beijing	1	*embA* c228t (C76C)	EmbB D354A[d]	
	Ural	1		EmbB N399I	
	Beijing	1	*embA* c228t (C76C)	EmbB L402V	
	Ural	1		EmbB S426N	
	Beijing	3[e]	*embA* c228t (C76C), *embA* a114g (Q38Q)	EmbB N296H	*embA* c1995t (H764H)
	X	1	EmbC V981L, *embB* g2895a (P965P)	*embCA*-c8a	
	Beijing	1	*embA* c228t (C76C)	*embCA* -c8t	
	Beijing	1	*embA* c228t (C76C)	*embCA* -c12t	
	LAM	1		*embCA* -c12t	

(Continued on following page)

TABLE 1 (Continued)

Resistance phenotype (total no. of isolates)	Phylogenetic lineage	No. of isolates	Mutation(s) in the *embCAB* locus[a]		
			Marker(s) of phylogenetic lineages	Mutation(s) potentially associated with ETB resistance[b]	Synonymous mutation (excluding marker of phylogenetic lineage)
	Beijing	1	*embA* c228t (C76C)	*embCA* -c16t	
	Delhi/CAS	1	EmbC R738Q	*embCA* -c16t	
	Beijing	1	*embA* c228t (C76C)	*embCA* with deletion of cg at positions −21 and −20	
	Beijing	3[e]	*embA* c228t (C76C)	*embCA* -c12t, EmbB Y334H	
	Haarlem	1	EmbC V981L	*embCA* -c12t, EmbB D354A[d]	*embC* t1677c (G559G)
	Uganda	1		EmbB A19D plus N296H, EmbA G5V	
	NEW-1	1			

[a] Excluding the synonymous *embC* c2781t mutation (R927R) present in all ETB-S and ETB-R isolates.
[b] *embCA* is the *embC-embA* intergenic region.
[c] Implication in ETB resistance proven by site-directed mutagenesis or allelic exchange.
[d] Involvement in ETB resistance suggested by the *in vitro*-selected mutant.
[e] Isolates sharing identical MIRU codes and spoligotypes.

type S isolates and 1 of the 8 LAM isolates had undescribed synonymous mutations: *embC* g363t (L121L) and *embB* t2280c (N760N), respectively (Table 1). Eleven Beijing isolates harbored the synonymous *embA* c228t (C76C) mutation, which is known to be associated with the Beijing lineage (PolyTB database [http://pathogenseq.lshtm.ac.uk/polytb]) (20), and one of the Beijing isolates carried an undescribed mutation, EmbC V987A (Table 1). Twenty-two ETB-S isolates with the nonsynonymous V981L mutation in EmbC belonged to the Haarlem ($n = 15$), Ghana ($n = 2$), T3 variant ($n = 3$), T2 ($n = 1$), or X ($n = 1$) phylogenetic lineage (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 in one Manu2, one T5, some T1, and some T2 isolates (PolyTB database). Among the ETB-S isolates with the V981L mutation, 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 (PolyTB database). 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 EmbC R738Q mutation reported for the CAS lineage (PolyTB database) (20); a Cameroon strain had a mutation in EmbB associated with ETB resistance, M306I; and a West africanum 2 strain and a *Mycobacterium bovis* strain had both the EmbB E378A and EmbC T270I mutations, which were previously reported to be phylogenetic markers of ancestral MTBC isolates (in *M. bovis* and lineages 1, 5, and 6 of the MTBC) (PolyTB database) (16, 44, 45). The *M. bovis* strain also carried the EmbB N13S and *embC* c3108t (T1036T) mutations previously reported for *M. bovis* (PolyTB database) and the *embB* c351t (V117V) mutation previously reported for *M. bovis* and AFRI-1 strains (PolyTB database). 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 the synonymous *embA* c228t (C76C) mutation belonged to the Beijing phylogenetic lineage, as expected. Among these isolates, three also harbored the *embA* a114g (Q38Q) mutation reported to be phylogenetic marker of a subgroup of Beijing strains (20) (PolyTB database [http://pathogenseq.lshtm.ac.uk/polytb]). The ETB-R isolates with the EmbC V981L mutation reported previously in several phylogenetic lineages (PolyTB database) (16, 20) belonged to the Haarlem ($n = 5$), T1 ($n = 1$), Ghana ($n = 1$), or X ($n = 1$) strain family, with the latter also harboring the *embB* g2895a (P965P) phylogenetic marker that is specifically present in the X lineage (PolyTB database). Furthermore, one LAM ETB-R isolate obtained from a patient born in Portugal had the EmbB M423T mutation commonly reported for LAM strains from Portugal (PolyTB database), and one Delhi/CAS ETB-R isolate carried the EmbC R738Q mutation reported for the CAS lineage (PolyTB database) (20) (Table 1).

Regarding mutations potentially associated with ETB resistance, 40/71 (56%) ETB-R isolates had mutations in codon M306 of EmbB, including 24 isolates with the M306V mutation (34%) and 16 with the M306I mutation (22%). For the remaining ETB-R isolates, 30 (42%) had mutations in the *embCAB* locus outside EmbB codon 306, and 1 isolate belonging to the NEW-1 lineage had no mutation (Table 1). Among the 40 isolates with a mutation in codon M306 of EmbB, 5 had additional mutations in the *embC-embA* intergenic region ($n = 3$ for -c12t, $n = 1$ for -c16t, and $n = 1$ for -c11a), whereas 2 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, 2 of which harbored the undescribed synonymous mutations c2956t (L986L) in *embB* and c1995t (H764H) in *embA* (Table 1). The distribution 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, 7 had mutations in the *embC-embA* intergenic region exclusively ($n = 1$ for -c8a, $n = 1$ for -c8t, $n = 2$ for -c12t, $n = 2$ for -c16t, and $n = 1$ for the cg deletion at positions −21 and −20), with 2 of them harboring the X and CAS phylogenetic markers *embB* g2895a

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 the MTBC

Mutation in the *embCAB* locus	Mutation		Phylogenetic lineage(s)
	Amino acid	Nucleotide[a]	
Markers of phylogenetic lineages	EmbC R738Q		CAS
	EmbC V981L		Haarlem; all H1; all X; all ambiguous T2T3 and T2X1; some T1, T2, and T5; and one each of Manu2, Ghana, and 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, and 6 and *M. bovis*
	EmbC T270I		Ancestral MTBC lineages 1, 5, and 6 and *M. bovis*
	EmbB M423T		LAM4 from Portugal
	EmbB P965P	*embB* g2895a	X
	EmbC R345R	*embC* c1035g	X2
Mutations 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 with deletion of cg at positions −21 and −20	

[a] *embCA* is the *embC-embA* intergenic region.
[b] Implication in ETB resistance proven by site-directed mutagenesis or allelic exchange.
[c] Involvement in ETB resistance suggested by the *in vitro*-selected mutant.
[d] Implication in ETB resistance remains to be experimentally verified.

(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 t1677c (G559G) mutation 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 *embC* c2781t (R927R) mutation was present in all ETB-S and ETB-R isolates included in the present study. This polymorphism has also been reported for all isolates in the PolyTB database (http://pathogenseq.lshtm.ac.uk/polytb). This single nucleotide polymorphism (SNP) could be the result of a sequencing discrepancy in *M. tuberculosis* reference strain H37Rv deposited in GenBank (accession number AL123456.3).

DISCUSSION

Few publications have reported sequencing of the entire *embCAB* locus (16, 20, 46); 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% (46). In our study, 70/71 (98%) 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 the MTBC (Table 2), 4 of which are well described in the literature: the EmbC T270I and EmbB E378A mutations in ancestral MTBC lineages 1, 5, and 6 and *M. bovis* (PolyTB database [http://pathogenseq.lshtm.ac.uk/polytb]) (16, 44, 45); the *embA* c228t (C76C) mutation in the Beijing lineage (PolyTB database) (20); and the EmbC V981L mutation in the Haarlem lineage (16, 20). We found the EmbC V981L mutations not only in Haarlem strains but also in type X, T1, T3 variant, and Ghana isolates. In the PolyTB database, this mutation has been reported for all H1; all X; all ambiguous T2T3 and T2X1; some T1, T2, and T5; and one Manu2 (but not Manu1) strain. Taken together, these data suggest that the EmbC V981L mutation 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 Haarlem, X, Ghana, and some T strains. Five other mutations found in our study are also associated with phylogenetic lineages of the MTBC according to the PolyTB database. Several of the mutations were found in ETB-S strains, including the *embC* c1035g (R345R) and *embB* g2895a (P965P) mutations in the X lineage (PolyTB database), the *embB* c351t (V117V) mutation in all AFRI-1 isolates and *M. bovis*, and the *embC* c3108t (T1036T) and EmbB N13S mutations in *M. bovis* (PolyTB database), whereas others were detected in ETB-R isolates, including the *embA* a114g (Q38Q) mutation specific to a subgroup of the Beijing lineage and associated with the EmbB N296H mutation in three Beijing isolates in our study (PolyTB database) (20), the CAS-specific EmbC R738Q polymorphism associated with the *embCA* -c16t mutation (PolyTB database) (20), and the EmbB M423T polymorphism, which characterizes the LAM4 lineage from Portugal and was associated with the EmbB M306V and *embCA* -c16t mutations in our study (Table 2). Finally, eight SNPs that are probably not involved in ETB resistance were also identified in 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, including the *embB* g132t (T44T) and t2280c (N760N) mutations, the *embC* g363t (L121L) mutation, and the nonsynonymous EmbC A426T mutation (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, 46). In our study, 90% of ETB-R isolates had mutations in 18 EmbB codons, corresponding to 22 mutations including the above-mentioned 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 MICs and can be considered canonical EmbB mutations in ETB resistance (37, 47–50). Accordingly, in our study, only one isolate that was phenotypically susceptible to ETB harbored the M306I mutation, and a strong statistical association was found between ETB resistance and a mutation at codon 306 alone ($P < 0.001$) or EmbB mutations at codons 306, 406, and 497 ($P < 0.001$). Some authors (38, 50–52) have argued that mutations at codon 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, 50–52). 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, 46, 51, 52). 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/liter depending on the phenotypic method used for susceptibility testing (39), and the ETB MICs induced by mutations in codon 306 could be close to the breakpoint defining ETB resistance (37, 50).

Other mutations in EmbB that are likely responsible for ETB resistance were identified in our study: the D354A mutation was previously reported for ETB-R strains selected *in vitro* (37). For the F330I and N399I mutations, other mutations (F330V/S/L and N399T/H/D) were reported in the same codons in ETB-R strains ($n = 9$) in five studies (16, 17, 23, 36, 53) and in a single ETB-S strain in one study (53). Similarly, the L402V and Y334H mutations were previously described for ETB-R ($n = 3$) strains in three studies (16, 20, 35) and an ETB-S ($n = 1$) strain in one study (53), respectively. A total of three new EmbB mutations were identified in our study: S426N, in a 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 a 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 highlighting 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 first-line drug 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, 16 of which 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, 46, 53). 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 2 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 the -c11a mutation, the mutations at positions −8, −12, and −16 and the deletion of cg at positions −21 and −20, which are located within/adjacent to a predicted TATA box (15, 16, 53), were found in ETB-R strains carrying no other mutations involved in ETB resistance, confirming their involvement in ETB resistance. The role of the -c11a mutation 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*, with the MICs of ETB for strains with both *embC-embA* intergenic region mutations and *embB* mutations being much higher than those for strains with only an *embB* mutation (53).

Notably, we detected no mutation that is clearly associated with ETB resistance in *embC* and *embA*, despite the percentages 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, 46). 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, and R738Q). Among these mutations, the absence of a role of the EmbC T270I mutation in ETB resistance was experimentally confirmed by site-directed mutagenesis (54). Regarding the last

two mutations in EmbC (A426T and V987A), a similar mutation in codon 426 but not with the same substitution (A426P) was described (PolyTB database [http://pathogenseq.lshtm.ac.uk/polytb]). In our study, the A426T and V987A mutations were found in clinical isolates that were 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 the G5V mutation in EmbA.

Finally, we found no isolate with a mutation in *embR*, despite its 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 levels of M306, G406, and Q497 and (ii) the DNA segment extending from positions −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 the M306 (56%), G406 (11%), or Q497 (3%) codon of EmbB; 13% had 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 codon 306, 406, or 497). Importantly, the commercial Genotype MTBDRsl DNA strip assay, 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 of the *embB* ERDR and the *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% by testing codons 306, 406, and 497 and nucleotide positions −8, −12, and −16 and by up to 98% by testing the entire *embB* gene and the *embC-embA* intergenic region from positions −8 to −21.

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