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Mutations at *embB* Codon 306 Are an Important Molecular Indicator of Ethambutol Resistance in *Mycobacterium tuberculosis*▽

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Ethambutol resistance in clinical *Mycobacterium tuberculosis* isolates is associated primarily with missense mutations in the *embB* gene. However, recent reports have described the presence of *embB* mutations, especially those at *embB* codon 306, in isolates susceptible to ethambutol. To clarify the role of *embB* mutations in ethambutol resistance, we sequenced the ethambutol resistance-determining region in spontaneous ethambutol-resistant mutants. In our study, 66% of spontaneous mutants contained a single point mutation in *embB*, with 55% of these occurring at *embB* 306. The MIC of ethambutol for spontaneous mutants was increased two- to eightfold relative to the pansusceptible *M. tuberculosis* strains from which the mutants were generated. To further characterize the role of *embB* 306 mutations, we directly introduced mutant alleles, *embB*(*M306V*) or *embB*(*M306I*), into pansusceptible *M. tuberculosis* strains and conversely reverted mutant alleles in spontaneous ethambutol-resistant mutants back to those of the wild type via allelic exchange using specialized linkage transduction. We determined that the MIC of ethambutol was reduced fourfold for three of the four spontaneous ethambutol-resistant *embB* 306 mutants when the mutant allele was replaced with the wild-type *embB* allele. The MIC for one of the spontaneous mutants genetically reverted to wild-type *embB* was reduced by only twofold. When the wild-type *embB* allele was converted to the mutant allele *embB*(*M306V*), the ethambutol MIC was increased fourfold, and when the allele was changed to M306I, the ethambutol MIC increased twofold. Our data indicate that *embB* 306 mutations are sufficient to confer ethambutol resistance, and detection of these mutations should be considered in the development of rapid molecular tests.

Tuberculosis is a global public health problem that results in approximately 2 million deaths each year (7). Efforts to treat patients and control the spread of tuberculosis have been hindered by the emergence of *Mycobacterium tuberculosis* bacilli resistant to both first- and second-line antituberculosis drugs (28, 29). To address this growing crisis, there is an urgent need to study the genetic basis for antimicrobial resistance in *M. tuberculosis*. Understanding the molecular basis of resistance is essential for developing rapid molecular diagnostic tests which can facilitate treatment decisions as well as possibly reveal approaches to alternative treatment strategies (13).

Ethambutol (EMB) is an important first-line drug for the treatment of tuberculosis. EMB is thought to target the arabinosyl transferases (*embCAB*), thereby inhibiting the biosynthesis of the cell wall components arabinogalactan and lipoarabinomannan (5, 33, 34). Sequence analysis of EMB-resistant (EMB^r) clinical isolates has shown that EMB resistance is associated primarily with missense mutations within the EMB resistance determining region (ERDR) (3, 34) of the gene *embB*. The most commonly found mutations occur at *embB* codon 306 in 50 to 70% of clinical isolates (23–25, 32, 34). Due to this strong correlation, mutations at *embB* 306 have been proposed as a marker for the rapid detection of EMB resistance (2, 11, 18, 26, 36).

The precise role of *embB* 306 mutations in resistance to EMB remains controversial. Reports have included the detection of *embB* 306 mutations in EMB-susceptible clinical isolates (1, 14, 19, 35) and that the presence of these mutations may be a marker for increasing resistance to other first-line antituberculosis drugs (10, 30, 31). Conflicting reports on the association of *embB* 306 mutations with EMB^r led us to investigate the contribution of these mutations to resistance. In the present study, we examined the frequency and distribution of *embB* mutations in spontaneous EMB^r mutants generated from pansusceptible strains of *M. tuberculosis*. In addition, we directly examined the role of *embB* 306 mutations in EMB resistance by allelic replacement of mutant and wild-type *embB* alleles in the bacterial chromosome of spontaneous EMB^r and pansusceptible *M. tuberculosis* strains. Our data demonstrate that mutations at *embB* 306 can cause EMB^r and are an important molecular indicator of EMB resistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Plasmids and phages used in this study are described in Table 1. *Escherichia coli* Top10 and HB101 (Invitrogen) were grown either in Luria-Bertani broth (LB) or on LB agar plates at 37°C. Liquid cultures of *Mycobacterium smegmatis* LR222 (17) and *M. tuberculosis* strains were grown at 37°C in Middlebrook 7H9 broth supplemented with 10% (vol/vol) albumin-dextrose-catalase (ADC) enrichment (Becton, Dickinson and Company) and 0.05% (vol/vol) Tween 80. Solid medium for growth of *M. tuberculosis* was Middlebrook 7H10 agar supplemented with 10% (vol/vol) oleic acid-ADC (OADC) enrichment (Becton, Dickinson and Company). When required, hygromycin B (HYG; Invitrogen) was added to the medium at a final concentration of 200 μg/ml for *E. coli* and 50 μg/ml for *M. tuberculosis*. EMB (Sigma) was used at 5 μg/ml for isolation of spontaneously arising resistant mutants.

Isolation of spontaneous EMB-resistant mutants. Two pansusceptible strains of *M. tuberculosis*, H37Rv-type strain and Beijing F2, a well-characterized clinical

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TABLE 1. Plasmids, cosmids, and shuttle phages used in this study

Plasmids, cosmids, and phages	Characteristics	Source/reference
Plasmids		
pCR-BluntII-TOPO	Vector for cloning blunt-ended PCR products, Kan^r	Invitrogen
pAS230	2.9-kb *embB* fragment in pCR-BluntII-TOPO	This study
pAS231	0.7-kb Rv3796 fragment in pCR-BluntII-TOPO	This study
pAS235	2.9-kb *embB*(*M306V*) fragment in pCR-BluntII-TOPO	This study
pAS236	2.9-kb *embB*(*M306I*) fragment in pCR-BluntII-TOPO	This study
Cosmids		
pYUB854	Used for cloning allelic exchange substrates; contains λ phage *cos* site, Hyg^r	4
pAS232	*embB* fragment upstream of Hyg^r cassette in pYUB854	This study
pAS233	*embB* and Rv3796 fragments flanking Hyg^r cassette in pYUB854	This study
pAS237	*embB*(*M306V*) fragment upstream of Hyg^r cassette in pYUB854	This study
pAS238	*embB*(*M306I*) fragment upstream of Hyg^r cassette in pYUB854	This study
pAS239	*embB*(*M306V*) and Rv3796 fragments flanking Hyg^r cassette in pYUB854	This study
pAS240	*embB*(*M306I*) and Rv3796 fragments flanking Hyg^r cassette in pYUB854	This study
Phages		
phAE159	Temperature-sensitive shuttle phasmid derived from the mycobacteriophage TM4	4
phAS2006	pAS233 cloned into unique PacI site of phAE159	This study
phAS2007	pAS239 cloned into unique PacI site of phAE159	This study
phAS2008	pAS240 cloned into unique PacI site of phAE159	This study

isolate, were grown to an optical density at 600 nm of approximately 1.0 (corresponding to 1×10^8 CFU/ml). The cultures were serially diluted in 7H9 broth to a concentration of approximately 1×10^4 CFU/ml. For each diluted culture, 0.5 ml was added to 50 tubes containing 5 ml of 7H9 broth without antibiotic, yielding a final concentration of 1×10^3 CFU/ml. The cultures were incubated at 37°C without shaking for 23 days to an average optical density at 600 nm of 0.6. A total of 1.5 ml from each of the 5-ml cultures were transferred into a sterile microcentrifuge tube and pelleted by centrifugation. The pellet was suspended in 100 μl of medium and plated onto 7H10 plates containing the critical concentration of 5 μg/ml EMB (20) to select for spontaneous EMB^r mutants. Plates were incubated at 37°C for 3 weeks. One colony from each plate with growth, representing an independent spontaneous mutant, was subcultured into 5 ml of 7H9 containing 5 μg/ml EMB. Samples of each culture were frozen and used for isolation of genomic DNA in the analysis of mutations in *embB*.

DNA extraction, PCR amplification, and sequencing of *embB* from spontaneous EMB-resistant mutants. Genomic DNA was extracted by rapid mechanical disruption as previously described (22). A 525-bp fragment (encoding amino acids 180 to 354) including the ERDR of the *embB* gene (3, 34) was amplified by PCR using the primers embB-F2 (5′-AACCTGCGCCCGCAGATTGTC-3′) and embB-R2 (5′-GGTCTGGCAGGCGCATCC-3′). A 1.1-kb fragment downstream of the ERDR in *embB* (encoding amino acids 706 to 1069) that included a region previously reported to be associated with EMB resistance (25) was also amplified from mutants that had no mutation in the ERDR with the primers AG-1 (5′-CCGGTACCGATCGTGGCCGG-3′) and AG-2 (5′-GGTGTCGAA CTTGCGCAGGGAACC-3′). The same primer sets were used in subsequent sequencing reactions along with primer AG-3 (5′-GGTCGTCGGGCACCATC CGCC-3′). Sequences were determined using the Beckman CEQ 8000 genetic analysis system. DNA sequences of the ERDR and downstream region from each EMB^r mutant were compared to the sequences of the parental strains H37Rv and Beijing F2 using the BioEdit sequence alignment editor (9).

Determination of MICs. The MIC of EMB for each strain was determined using a modification of the conventional agar proportion method as previously described (20). Strains were plated on 7H10 agar plates with OADC enrichment containing 1.25, 2.5, 5, 10, 20, 40, or 80 μg/ml EMB, and plates were incubated at 37°C for 3 weeks. Additionally, MICs were measured at 0.0312, 0.0625, 0.125, 0.25, 0.5, and 1.0 μg/ml for rifampin (RIF) and 0.0125, 0.025, 0.05, 0.1, and 0.2 μg/ml for isoniazid (INH) by the modified agar proportion method. The MIC was defined as the lowest concentration of drug that prevented growth of >99% of the initial inoculum.

Construction of recombinant cosmids containing allelic exchange substrates. Recombinant cosmids containing allelic exchange substrates were constructed for insertion into shuttle phasmids for the introduction of point mutations at *embB* codon 306. A 2.9-kb fragment of *embB* was PCR amplified from the genomic DNA of H37Rv using Phusion high-fidelity DNA polymerase (Finnzymes) and the primers A115F (5′-ATACAGGCCTGTGATCTTGTCCG TGCCG-3′) and A115R (5′-ATAC**TCTAGA**CTATGGACCAATTCGGATCTT G-3′). The StuI site is underlined and the XbaI site is bold in the primer sequences. The resulting amplicon was cloned into the vector pCR-BluntII-TOPO (Invitrogen), resulting in the plasmid pAS230 (Table 1). To construct pAS231, a 0.7-kb fragment of Rv3796 was amplified from the genomic DNA of H37Rv using the primers A116F (5′-TATCAGATCTCGTCAGGCTCCGCAG T-3′) and A116R (5′-TATC**ACTAGT**GTGGTGTCACCCGAGAAGGT-3′). The BglII site is underlined and the SpeI site is bold in the primer sequences. The resulting Rv3796 fragment was cloned into pCR-BluntII-TOPO, resulting in the plasmid pAS231. To construct the recombinant cosmid, the 2.9-kb SpeI/XbaI fragment (wild-type *embB*) from pAS230 was subcloned into the corresponding restriction sites in pYUB854 (4) upstream of the res-hygromycin-res cassette, resulting in the cosmid pAS232 (Table 1). Subsequently, the 0.7-kb Rv3796 fragment from pAS231 was inserted into the SpeI/XbaI restriction sites of pAS232 downstream of the HYG cassette, resulting in the cosmid pAS233. The recombinant cosmids pAS237 and pAS238 (Table 1) were constructed in a similar manner, with the exception of the genomic DNA template used for amplification of the *embB* fragment. Genomic DNA from Rv8, a spontaneous *embB*(*M306V*) EMB-resistant mutant with the mutation ATG → GTG, and Rv44, an *embB*(*M306I*) mutant with an ATG → ATA substitution, was used for amplification of 2.9-kb *embB* fragments for pAS237 and pAS238, respectively.

Construction of specialized transducing phages. The specialized transducing phages used in this study were constructed as previously described (4, 6, 37). Briefly, cosmids pAS233, pAS239, and pAS240 carrying the allelic exchange substrates were linearized with PacI and ligated with phAE159 shuttle phasmid DNA digested with PacI. The recombinant phasmids were packaged using a λ in vitro packaging kit (Gigapack III XL; Stratagene) and transduced into *E. coli* HB101. The transductants were selected on LB plates containing 200 μg/ml HYG. Phasmids phAS2006, phAS2007, and phAS2008 were extracted from *E. coli* HB101 and used to transform *M. smegmatis* LR222. The resulting transducing phages were plaque purified and analyzed to ensure temperature sensitivity. The presence of the allelic exchange substrates was confirmed by PCR.

Transduction of *M. tuberculosis* for introduction of allelic exchange substrates. Spontaneous EMB-resistant *M. tuberculosis* strains Rv8, Rv44, B52, and B54 were transduced using the phage phAS2006 (wild-type *embB*). Pansusceptible *M. tuberculosis* strains H37Rv and Beijing F2 were transduced separately with the phage phAS2007, which contains the *embB*(*M306V*) allele, and phAS2008, which contains the *embB*(*M306I*) allele. Transduction experiments were conducted as previously described (6, 37). Transductants were selected at 37°C on Middlebrook 7H10 plates containing 50 μg/ml HYG.

Verification of mutations at *embB* 306 in transductants. Isolated colonies from transductant plates were inoculated into 5 ml of 7H9 broth supplemented with ADC containing 50 μg/ml of HYG and grown at 37°C for 5 to 7 days. To verify the *embB* allele of each transductant, a 525-bp fragment of *embB* was amplified by PCR using the primers embB-F2 and embB-R2 with PCR MasterMix (Pro-

TABLE 2. Sequence analysis of the *embB* region encoding amino acids 180 to 354 in spontaneous EMB-resistant mutants

Amino acid change	Nucleotide change	No. of H37Rv mutants	No. of Beijing F2 mutants	Total no. of mutants (%)
No change (wild type)	None	24	6	30 (34)
D300G	GAC → GGC	8	5	13 (15)
D300E	GAC → GAA	1	0	1 (1)
M306V	ATG → GTG	6	14	20 (23)
M306I	ATG → ATA	1	5	6 (7)
M306I	ATG → ATC	0	3	3 (3)
M306L	ATG → CTG	1	0	1 (1)
M306T	ATG → ACG	0	2	2 (2)
V309F	GTC → TTC	0	1	1 (1)
F320L	TTC → TTA	0	1	1 (1)
F320L	TTC → CTC	1	1	2 (2)
R321H	CGC → CAC	0	1	1 (1)
G324R	GGC → CGC	1	1	2 (2)
D328Y	GAT → TAT	2	2	4 (5)
D354A	GAC → GCC	0	1	1 (1)

TABLE 3. EMB MICs for each type of *embB* mutant recovered from the fluctuation test

Mutant[a]	Nucleotide change	Amino acid change	EMB MIC (μg/ml)[b]
B74	GAC → GGC	D300G	10
Rv7, Rv18, B55, B59	GAC → GGC	D300G	20
Rv8, Rv17, Rv23, B52, B56, B64	ATG → GTG	M306V	20
Rv44, B54, B85, B95	ATG → ATA	M306I	20
B84, B97	ATG → ATC	M306I	20
B70	ATG → ATC	M306I	40
Rv11	ATG → CTG	M306L	20
B51	ATG → ACG	M306T	20
B68	TTC → TTA	F320L	20
Rv10, B71	TTC → CTC	F320L	20
B99	CGC → CAC	R321H	10
Rv39, B79	GGC → CGC	G324R	20
Rv21, Rv31, B53, B96	GAT → TAT	D328Y	20
B92	GAC → GCC	D354A	20

[a] Names of spontaneous EMB^r mutants derived from H37Rv and Beijing F2 begin with Rv and B, respectively.
[b] EMB MICs for H37Rv and Beijing F2 were ≤5 μg/ml.

mega) and 1 μl of cell culture as the template. Similar to the analysis of *embB* in spontaneous EMB-resistant mutants, amplicons were sequenced using the primers described above and the Beckman Coulter CEQ 8000. DNA sequences of transductants were analyzed using BLAST at the TubercuList World Wide Web server (http://genolist.pasteur.fr/TubercuList/) for the introduction of an M306V, M306I, or wild-type allele for *embB*. The presence of the linked HYG cassette was also confirmed by PCR using the primers Hyg-10F (5′-GAGGCGTACGC GGTCCTGGC-3′) and Hyg-13R (5′-AAGATGTTGGTCCCGTGCAG-3′).

RESULTS

Sequence analysis of *embB* in spontaneous EMB-resistant mutants. We generated spontaneous EMB^r mutants using a fluctuation test approach (15) to examine the correlation between *embB* mutations and EMB^r in the absence of resistance to other drugs. Of 100 individual cultures, all 50 of the H37Rv and 45 of the Beijing F2 yielded spontaneous EMB^r mutants. Most of the plates contained between 1 and 24 colonies. A single colony from each plate representing an independent mutational event was chosen for subculturing in 7H9 broth containing 5 μg/ml EMB. If the plate contained more than 24 colonies, 2 to 4 were subcultured. Upon subculturing, only 90% (45/50) of the H37Rv-derived mutants and 96% (43/45) of the Beijing F2-derived mutants were viable in the presence of EMB.

Because mutations in *embB*, including those at codon 306, have been linked to EMB resistance (23–25, 32), the ERDR of *embB* encoding amino acids 180 to 354 in each of the independent spontaneous mutants was sequenced. The sequence of this region in *embB* was identical for multiple isolates selected from the same plate, indicating a single mutational event in the culture. Of the 88 spontaneous mutants examined, 34% (30/88) did not have a mutation in this region of *embB*, and the majority of these (80%; 24/30) were spontaneous mutants generated from one of the parent strains, H37Rv. However, 66% (58/88) did have a point mutation in *embB*, resulting in an amino acid change (Table 2). Of the mutants containing an *embB* mutation, 55% (32/58) had a mutation at *embB* 306. The most common *embB* 306 mutations were ATG → GTG (M306V; 63%; 20/32) and ATG → ATA or ATC (M306I; 28%; 9/32). Interestingly, only 18% (8/45) of spontaneous mutants derived from H37Rv contained *embB* 306 mutations compared to 56% (24/43) of mutants derived from Beijing F2. Other mutations detected in *embB* included D300G, D300E, V309F, F320L, R321H, G324R, D328Y, and D354A (Table 2). The D300G mutation was the second most common mutation in the ERDR in 15% (13/88) of all mutants. An additional region of *embB* thought to be associated with EMB^r (amino acids 706 to 1069) (25) was sequenced in mutants that did not contain a mutation in the region encoding amino acids 180 to 354. All mutants examined contained the wild-type sequence in this region (706 to 1069).

EMB, RIF, and INH MICs of spontaneous EMB^r mutants. The EMB MIC for a representative subset of each type of spontaneous mutant was measured using agar containing drug at twofold intervals between 1.25 and 80 μg/ml. The EMB MIC for the parental strains H37Rv and Beijing F2 was 5 μg/ml. The EMB MICs for spontaneous mutants ranged from 10 to 40 μg/ml (Table 3), with the exception of six mutants with a MIC of ≤5 μg/ml (data not shown). Two mutants harboring *embB* mutations (D300G and V309F) and four wild-type mutants for *embB* in the region sequenced did initially grow in liquid subcultures at 5 μg/ml in 7H9 broth but did not grow on 7H10 agar plates containing EMB. Mutants containing *embB* 306 mutations had EMB MICs that ranged from 20 to 40 μg/ml (Table 3). The EMB^r mutants wild type for *embB* had MICs that ranged from 10 to ≥20 μg/ml (data not shown). None of the spontaneous mutants selected had an EMB MIC of greater than 40 μg/ml.

Prior reports have suggested that mutations in *embB* may be associated with resistance to antibiotics in addition to EMB, so we measured the RIF and INH MICs of spontaneous EMB^r mutants that contained mutations in *embB*. MICs were measured by the agar proportion method at twofold intervals between 0.0312 and 1.0 μg/ml of RIF and 0.0125 and 0.2 μg/ml of INH. All mutants examined were susceptible to the critical concentrations of 1 μg/ml RIF and 0.2 μg/ml INH (data not shown). In addition, there was no significant difference in the

RIF MICs (at concentrations below the critical concentration) of spontaneous mutants with *embB* 306 mutations and those with other *embB* mutations (Student's *t* test; $P = 0.12$).

Contribution of mutations at *embB* 306 to EMB resistance. Due to the substantial number (36%; 32/88) of spontaneous mutants that contained *embB* 306 mutations in our fluctuation test and the controversy surrounding the precise role of these mutations in EMB resistance, we examined whether *embB* 306 mutations are sufficient to confer EMB^r. We directly replaced (i) mutant alleles with the wild-type allele in the chromosome of the spontaneous EMB^r mutants Rv8 and B52, which contain *embB*(*M306V*) mutations (GTG → ATG), and Rv44 and B54, which contain *embB* M306I mutations (ATA → ATG), and (ii) the wild-type *embB* allele with the mutant alleles *embB* M306V (ATG → GTG) and *embB*(*M306I*) (ATG → ATA) in the pansusceptible *M. tuberculosis* strains H37Rv and Beijing F2. The mutations were marked with a HYG resistance cassette, introduced via a specialized transducing phage, and incorporated into the chromosome by homologous recombination. Two independent transductants from each substitution with either a wild-type or mutant *embB* 306 allele were analyzed for their susceptibility to EMB, RIF, and INH using the agar proportion method on Middlebrook 7H10 agar.

The EMB MICs for three of the four spontaneous EMB^r mutants, Rv8, B52, and B54, decreased fourfold to levels below the critical concentration (5 μg/ml) after replacement of the *embB* mutant alleles with the wild-type allele. Rv8, which contains an *embB*(*M306V*) mutation, had a MIC of 20 μg/ml for EMB (Table 4). The MIC decreased to 5 μg/ml for the Rv8 transductants Rv8100 and Rv8101 after the *embB* 306 mutation was replaced with the wild-type sequence (GTG → ATG). The EMB MIC of the strain Rv8102, an Rv8 transductant containing the HYG cassette and *embB*(*M306V*) mutation, remained at 20 μg/ml, indicating that the presence of the HYG cassette was not responsible for the decreased MIC observed in the transductants Rv8100 and Rv8101 (Table 4). Similarly, the EMB MICs for B52 and B54 containing the *embB*(*M306V*) and *embB*(*M306I*) alleles, respectively, were reduced from 20 μg/ml to 5 μg/ml after being replaced with the wild-type allele (Table 4). However, Rv44 transductants in which the wild-type allele was substituted for the *embB*(*M306I*) allele had only a twofold reduction in the EMB MIC, from 20 μg/ml to 10 μg/ml (Table 4).

We also examined the EMB MICs of transductants after the conversion of the wild-type *embB* allele to either an *embB*(*M306V*) or *embB*(*M306I*) allele in the pansusceptible *M. tuberculosis* strains H37Rv and Beijing F2. The EMB MICs of H37Rv and Beijing F2 transductants containing the *embB*(*M306V*) mutant allele (strains RvAS1, RvAS2, BAS5, and BAS6) increased fourfold, from 5 to 20 μg/ml, relative to those of the parent strain (Table 4). In contrast, transductants RvAS3, RvAS4, BAS7, and BAS8 containing the mutant allele *embB*(*M306I*) had only a twofold increase in the EMB MIC, from 5 to 10 μg/ml. The EMB MICs of RvAS9 and BAS10, transductants wild type for *embB* and containing the HYG resistance cassette, were the same as those of the parental strains H37Rv and Beijing F2, indicating that the increase in the EMB MICs in transductants with mutant alleles was due to the introduction of the mutation and not the presence of the resistance cassette linked to the mutation (Table 4). All transductants were fully susceptible to 0.2 μg/ml INH and 1.0 μg/ml RIF.

TABLE 4. MICs of EMB for parental, spontaneous EMB^r mutants and transductants containing either mutant or wild-type alleles of *embB*

Isolate	Parent strain	HYG cassette[a]	*embB* mutation	EMB MIC (μg/ml)
Rv8	H37Rv[b]	−	M306V	20
Rv8100	Rv8[c]	+	None[d]	5
Rv8101	Rv8	+	None	5
Rv8102	Rv8	+	M306V	20
Rv44	H37Rv	−	M306I	20
Rv4400	Rv44[c]	+	None	10
Rv4401	Rv44	+	None	10
Rv4402	Rv44	+	M306I	20
B52	Beijing F2[b]	−	M306V	20
B5200	B52[e]	+	None	5
B5201	B52	+	None	5
B5202	B52	+	M306V	20
B54	Beijing F2	−	M306I	20
B5400	B54[e]	+	None	5
B5401	B54	+	None	5
B5402	B54	+	M306I	20
H37Rv	NA[f]	−	None	5
RvAS1	H37Rv	+	M306V	20
RvAS2	H37Rv	+	M306V	20
RvAS3	H37Rv	+	M306I	10
RvAS4	H37Rv	+	M306I	10
RvAS9	H37Rv	+	None	5
Beijing	NA	−	None	5
BAS5	Beijing F2	+	M306V	20
BAS6	Beijing F2	+	M306V	20
BAS7	Beijing F2	+	M306I	10
BAS8	Beijing F2	+	M306I	10
BAS10	Beijing F2	+	None	5

[a] The HYG resistance cassette was linked to allelic exchange substrates containing mutant alleles *embB*(*M306V*) and *embB*(*M306I*) and the fragments reverting mutants back to wild-type *embB* that were introduced into isolates during specialized linkage transduction.
[b] H37Rv and Beijing F2 are pansusceptible strains of *M. tuberculosis.*
[c] Rv8 and B52 are spontaneous EMB-resistant mutants with *embB*(*M306V*) mutations.
[d] Methionine is present at EmbB 306.
[e] Rv44 and B54 are spontaneous EMB-resistant mutants with *embB*(*M306I*) mutations.
[f] NA, not applicable.

DISCUSSION

In this study, we found that *embB* mutations previously reported for EMB^r clinical isolates of *M. tuberculosis* were common among laboratory-generated, spontaneously resistant mutants isolated through a fluctuation test. While other investigators have reported difficulty isolating spontaneous EMB^r mutants with *embB* mutations in vitro, particularly at *embB* 306, (10, 27), 66% (58/88) of the independent EMB^r mutants we isolated contained a single point mutation in the ERDR of *embB*, with 55% (32/58) of these mutations at *embB* 306. The proportion of mutants in our study with *embB* mutations is similar to that reported after examination of this region in sets of clinical isolates resistant to EMB (25, 32). Interestingly, of the 58 mutants with ERDR mutations, the majority (64%; 37/58) were generated from the pansusceptible strain Beijing F2. Moreover, Beijing F2-derived mutants accounted for 75% of mutants with *embB* 306 mutations. An explanation

for the lower number of H37Rv-derived mutants containing *embB* mutations is unknown, but potential differences in the propensity of some strains to accumulate mutations in this region may exist. Approximately one-third of the EMB^r mutants had a wild-type sequence in the region analyzed, indicating that additional resistance-conferring mutations occurred in regions outside of the ERDR in *embB* or other genes.

Previous reports have suggested that mutations in the ERDR, especially at *embB* 306, serve as molecular markers of resistance to INH and/or RIF and not EMB (10, 30, 31). In this study, EMB^r mutants containing *embB* mutations were fully susceptible to the critical concentrations of RIF and INH as measured by the agar proportion method. Furthermore, at concentrations below the critical concentration, we found no differences in the INH or RIF MICs in EMB^r strains. Our results suggest no correlation between these mutations and additional resistances. However, a recent study by Safi et al. proposed that *M. tuberculosis* strains harboring *embB* 306 mutations may in fact have altered susceptibility to INH and RIF and therefore have a growth advantage in the presence of these antibiotics (27). In the complex host environment during treatment with multidrug therapy, strains with *embB* 306 mutations may have an increased chance of developing resistance to other first-line antimycobacterial drugs. However, the nature of the association of *embB* 306 mutations with multidrug resistance is not clearly understood and warrants further investigation.

Using allelic exchange in pansusceptible and laboratory-generated EMB^r mutants, we determined that *embB* 306 mutations are sufficient to confer EMB resistance. Recently, a publication by Safi et al. found similar results to those reported here in that *embB* 306 mutant alleles caused an increase in the EMB MIC. Specifically, the investigators determined that introduction of the *embB*(*M306I*) and *embB*(*M306V*) alleles into a wild-type strain resulted in an increase in the EMB MIC, from 2 μg/ml to 7 μg/ml and 14 μg/ml, respectively (27). Furthermore, reintroduction of the wild-type *embB* 306 allele caused the reversion of mutant strains to being fully susceptible at an EMB MIC of 2 μg/ml. A reduction in the EMB MIC was also observed for highly EMB-resistant clinical isolates when the wild-type *embB* 306 allele was introduced (27). Our results and those of Safi et al. suggest that *M. tuberculosis* isolates with *embB* 306 mutations, especially the *embB*(*M306I*) mutation, could have EMB MICs close to the critical concentration and therefore could be inadvertently defined as EMB susceptible. Because of the fourfold-higher MIC we observed after introduction of the *embB*(*M306V*) mutation, clinical isolates harboring this allele would most likely be characterized as resistant by the agar proportion method at a critical concentration of 5 μg/ml. In our study, introduction of the wild-type *embB* allele into strains with either an M306V or M306I mutation were reverted to full susceptibility to EMB, with the exception of one strain. The EMB MIC of Rv44, a spontaneous mutant with an *embB*(*M306I*) mutation, was reduced only to 10 μg/ml after introduction of the wild-type allele. It is likely that this strain had an additional mutation contributing to EMB resistance located outside of the ERDR of *embB*.

Due to inherent difficulties with conventional testing of EMB resistance, rapid molecular methods based on the detection of genetic mutations may prove more valuable for determining EMB resistance in *M. tuberculosis*. Culture-based methods can be problematic because of the bacteriostatic nature of EMB (12). The storage conditions of the drug and the type of medium used can impact the drug activity (8, 16, 21). Furthermore, the presence of microcolonies on solid medium and the proximity of EMB MICs of clinical isolates near the critical concentration can make determination of resistance difficult. A multicenter evaluation of the reproducibility of EMB susceptibility test results found that 50% of isolates called resistant by Bactec 460TB were susceptible to EMB by the agar proportion method (16). In another study, Johnson et al. investigated potential discrepancies between phenotypic and genotypic evaluation of EMB resistance (12). In that study, 91.4% of EMB resistance was not detected during the initial phenotypic screening using the agar diffusion method and was confirmed only after identification of *embB* 306 mutations and radiometric Bactec testing (12). The results from our study confirm that *embB* 306 mutations are an important molecular indicator of EMB resistance. Although the absence of an *embB* 306 mutation in *M. tuberculosis* isolates does not rule out phenotypic EMB resistance, the presence of these mutations is confirmatory and would be useful for detection of EMB resistance in 50 to 70% of clinical isolates.

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