Role of *embB* Codon 306 Mutations in *Mycobacterium tuberculosis* Revisited: a Novel Association with Broad Drug Resistance and IS*6110* Clustering Rather than Ethambutol Resistance

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Mutations at position 306 of *embB* (*embB*306) have been proposed as a marker for ethambutol resistance in *Mycobacterium tuberculosis*; however, recent reports of *embB*306 mutations in ethambutol-susceptible isolates caused us to question the biological role of this mutation. We tested 1,020 clinical *M. tuberculosis* isolates with different drug susceptibility patterns and of different geographical origins for associations between *embB*306 mutations, drug resistance patterns, and major genetic group. One hundred isolates (10%) contained a mutation in *embB*306; however, only 55 of these mutants were ethambutol resistant. Mutations in *embB*306 could not be uniquely associated with any particular type of drug resistance and were found in all three major genetic groups. A striking association was observed between these mutations and resistance to any drug ($P <$ 0.001), and the association between *embB*306 mutations and resistance to increasing numbers of drugs was highly significant ($P <$ 0.001 for trend). We examined the association between *embB*306 mutations and IS*6110* clustering (as a proxy for transmission) among all drug-resistant isolates. Mutations in *embB*306 were significantly associated with clustering by univariate analysis (odds ratio, 2.44; $P =$ 0.004). In a multivariate model that also included mutations in *katG*315, *katG*463, *gyrA*95, and *kasA*269, only mutations in *embB*306 (odds ratio, 2.14; $P =$ 0.008) and *katG*315 (odds ratio, 1.99; $P =$ 0.015) were found to be independently associated with clustering. In conclusion, *embB*306 mutations do not cause classical ethambutol resistance but may predispose *M. tuberculosis* isolates to the development of resistance to increasing numbers of antibiotics and may increase the ability of drug-resistant isolates to be transmitted between subjects.

The antibiotic ethambutol (EMB) appears to inhibit the growth of both *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* by blocking the synthesis of arabinogalactan. Arabinogalactan biosynthesis is dependent on the activity of the *embABC* gene cluster, which encodes the arabinotransferases

that mediate the polymerization of arabinose into arabinan. Several lines of evidence suggest that EMB exerts its toxic effect on mycobacteria by inhibiting the *embABC*-encoded proteins (26, 27, 34, 35, 46), and mutations in *embABC* also appear to play a key role in the development of EMB resistance in both *M. tuberculosis* and *M. smegmatis* (34). Associations between EMB resistance and mutations in *embA*, *embB*, and *embC* have been reported in clinical strains of *M. tuberculosis* (41), and mutations in codon 306 of the *embB* gene (*embB*306) in *M. tuberculosis* are seen in approximately 50% of all EMB-

TABLE 1. Clinical *M. tuberculosis* isolates included in the study

Geographic origin	No. (%) of isolates			No. (%) of isolates resistant to the following no. of antibiotics:				No. (%) of isolates with the following membership[b]:		No. (%) of *embB*306 mutants
	Total	Pansusceptible	EMB[ra]	One	Two	Three	Four	Clustered	Unique	
Australia	56	26 (46)	4 (7)	21 (38)	6 (11)	3 (5)	0	0	56 (100)	1 (2)
Colombia	304	163 (54)	42 (14)	7 (2)	31 (10)	72 (24)	31 (10)	214 (70)	90 (30)	48 (15)
India	48	10 (21)	10 (21)	13 (27)	14 (29)	8 (17)	3 (6)	12 (25)	36 (75)	4 (8)
Mexico	197	73 (37)	17 (9)	46 (23)	51 (26)	15 (8)	9 (5)	56 (28)	141 (72)	24 (13)
New York City	149	121 (82)	11 (7)	7 (5)	8 (5)	2 (1)	10 (7)	66 (44)	83 (56)	12 (8)
Spain	109	98 (90)	3 (3)	1 (1)	6 (5)	4 (4)	0	69 (64)	39 (36)	0
Texas	157	91 (58)	21 (13)	30 (19)	11 (7)	8 (5)	17 (11)	58 (37)	99 (63)	11 (7)
Total	1,020	582 (57)	108 (10)	122 (12)	130 (13)	112 (11)	69 (7)	372 (44)	480 (56)	100 (10)

[a] EMB[r], resistant to EMB. The EMB resistance breakpoints used were 2 µg/ml in Spain, 2.2 µg/ml in Colombia, 2.5 µg/ml in Australia and New York City, 5 µg/ml in India and Texas, and 7.5 µg/ml in Mexico.

[b] Isolates clustered by IS*6110*-based RFLP analysis. One pansusceptible isolate from Spain was not typed by RFLP analysis.

resistant clinical isolates. In the six *embB*306 nucleotide polymorphisms that have been described, the wild-type methionine is changed to either isoleucine, leucine, valine, or threonine (12, 31, 33, 34, 36, 41, 45, 52). Each mutation has been associated with an 8- to 16-fold increase in the MIC of EMB (1, 33, 41, 45).

The association between *embB*306 mutations and EMB resistance in clinical *M. tuberculosis* isolates is so strong that it has been proposed as a marker for EMB resistance in diagnostic tests (13, 33, 36, 43, 52, 54). Thus, it was a surprise when Mokrousov et al. (37) first described 48 clinical *M. tuberculosis* isolates from Russia that were susceptible to EMB yet that had mutations in *embB*306. All of these EMB-susceptible *embB*306 mutants were resistant to at least one antibiotic. Several other small studies confirmed these findings (31, 36, 39, 48). We hypothesized that EMB might have two function: one that inhibits the growth of *M. tuberculosis* at concentrations above its MIC and another that prevents the development of resistance to antibiotics other than EMB at concentrations below its MIC. We postulated that the second function might be abrogated by *embB*306 mutations, even in the absence of overt EMB resistance. This hypothesis was consistent with data suggesting that the *embABC* gene cluster is responsible for the biosynthesis of several different cell wall components (10, 13, 35, 57) and prior work showing that sub-MICs of EMB are synergistic with other antibiotics (13, 20). Confirmation of this possibility might have an important impact on tuberculosis treatment strategies (6).

In this study, 1,020 clinical *M. tuberculosis* isolates with different drug susceptibility patterns and of different geographical origins were tested for detection of associations between *embB*306 (and other) mutations, drug resistance patterns, and membership in a cluster defined by restriction fragment length polymorphism (RFLP) analysis. We found that *embB*306 mutations were strongly associated with resistance to increasing numbers of drugs and were independently associated with membership in a cluster. These results suggest that one function of EMB is prevention of the emergence of resistance to other antibiotics and that this function is distinct from its ability to inhibit the growth of *M. tuberculosis*. The development of *embB*306 mutations may predispose *M. tuberculosis* to the development of resistance to multiple antibiotics and may

increase the ability of these multiple-drug-resistant isolates to be transmitted between subjects.

MATERIALS AND METHODS

***M. tuberculosis* clinical isolates.** A total of 1,020 clinical *M. tuberculosis* isolates were obtained from Australia, Colombia, India, Mexico, New York City, Spain, and Texas. The 56 samples from Australia consisted of all of the isolates resistant at least to isoniazid (INH) collected in the state of Victoria at the Victorian Mycobacterium Reference Laboratory in Melbourne during 2001 and 2002 and 26 pansusceptible isolates. The samples from Colombia consisted of 142 isolates resistant to at least rifampin (RIF) and 163 pansusceptible isolates selected from the strain bank of the Mycobacterial Group, Instituto Nacional de Salud, composed of clinical *M. tuberculosis* isolates collected from all regions of Colombia between 1992 and 2000. The samples from India consisted of all 38 drug-resistant isolates of any type identified in the Vallabhbhai Patel Chest Institute in New Delhi between January 2001 and January 2002 plus 10 randomly selected susceptible control isolates recovered during the same period. The samples from Mexico consisted of 197 clinical isolates selected from a sample collection of the Laboratory of Clinical Microbiology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, collected in Mexico City, Huauchinango, Puebla, and Orizaba between 1991 and 1996 (7, 16, 24). The samples from Spain consisted of all isolates collected at the General Penitentiary Hospital Microbiology Laboratory from a longitudinal study of tuberculosis performed between January 1993 and June 1994 (11). The samples from Texas consisted of all resistant isolates collected by the Texas Department of Health and the University of Texas Health Center at Tyler between May 1992 and August 1994 and 91 sensitive isolates collected in a population-based study of Tarrant County between May 1992 and December 1996 (55, 56) that were submitted to the mycobacteriology research laboratory at Central Arkansas Veterans Healthcare System for genotype analysis. Samples from New York City consisted of a random selection of all isolates collected at the Montefiore Medical Center, Bronx, between 1989 and 1996 (2).

Antibiotic susceptibility testing and cluster analysis. All isolates included in this study were subjected to susceptibility testing and DNA fingerprinting as described below. Each center or laboratory tested the isolates for their susceptibilities to at least INH, RIF, streptomycin (STR), and EMB by the agar proportion method (25) (Colombia, India, New York, and Spain), with the BACTEC MGIT 960 system (21) (Australia), or by the radiometric BACTEC 460 method (32) (Mexico and Texas); extracted chromosomal DNA; and performed IS*6110*-based RFLP analysis (2, 5). MICs for selected isolates were determined with the BACTEC system (19) for isolates from Mexico and Australia or by the colorimetric assay (14) for isolates from Colombia. Isolates were considered to be clustered if they had an RFLP pattern identical to that of another isolate collected from the same study site over the study period. Isolates with low band numbers were confirmed to be identical by secondary methods (variable numbers of tandem repeats and spoligotyping) (15, 22). A description of the isolates included in the study is shown in Table 1.

Mutation detection. Mutations in *katG*315, *katG*463, *gyrA*95, and *kasA*269 were detected by hairpin primer (HP) assays, as described previously (17), with the primers listed in Table 2. Mutations in *katG*315 were confirmed by a previ-

TABLE 2. Molecular beacons, sequencing primers, and HPs used in this study

Molecular beacon or primer name	Sequence[a]	Remarks	Amplicon length (bp)
B*embB*306	FAM[b]-cgcgacatcctgggcatggccgtcgcg-D[c]	Wild-type (ATG)[d] molecular beacon	
B*embB*M306L-c	TET[e]-cgcgacatcctgggcCtggccgtcgcg-D	Mutant (CTG)[f] molecular beacon	
B*embB*M306I-c	FAM-cgcgacatcctgggcatCgccgtcgcg-D	Mutant (ATC)[g] molecular beacon	
B*embB*M306I-a	FAM-cgcgacatcctgggcatAgccgtcgcg-D	Mutant (ATA)[g] molecular beacon	
FB2*embB*306	cgaattcgtcggacgacg	PCR primers for beacon assay	65
RB2*embB*306	catgtagccggcgtggtc		
FSeq*embB*306	accgacgccgtggtgata	Sequencing primers	129
RB*embB*306	gcggaaatagttggacatgtagc		
RHP*gyrA*95	gcctgggccatCcgcaccaggc	Constant primer *gyrA*F3, cgagaccatgggcaactaccacc	69
RHP*gyrA*95mut	ccctgggccatCcgcaccaggG		
FHP*katG*463	cggatctagcctttagagccagatccg	Constant primer R*katG*463, gagacagtcaatcccgatgc	41
FHP*katG*R463L	aggatctgagcctttagagccagatccT		
FHP-A-*katG*315	tggtgatcggtaaggacgAgatcacca	Constant primer R*katG*321, aaactgttgtcccatttcgtcgg	70
FHP-A-*katG*S315R	gggtgatcggtaaggacgAgatcaccC		
FHP-*katG*315[h]	ctggtgatcggtaaggacgAgatcaccag	Constant primer R*katG*321, aaactgttgtcccatttcgtcgg	71
FHP-*katG*S315L[h]	agggtgatcggtaaggacgAgatcaccCT		
RHP*katG*315	gcggccatacgTcctcgatgccgc	Constant primer F*katG*315, ccggtaaggacgcgatcac	40
RHP*katG*S315I	tcggccatacgTcctcgatgccgA		
RHP*katG*S315N	acggccatacgTcctcgatgccgT		
RHP*katG*S315T	ccggccatacgTcctcgatgccgG		
RHP*katG*S315Tdouble	caggcacatacgTcctcgatgccTG		
FHP*kasA*269	cggcacgattCctgggtgccg	Constant primer R*kasA*269, aaaggcgtccgaggtgatac	36
RHP*KasA*G269S	tggcacgattCctgggtgccA		

[a] Underlined sequences indicate the stem of the molecular beacon or the tail of the HP; residues shown in capital letters correspond to either the mutated residue, a secondary mutation inserted into the oligonucleotide, or the intended SNP.
[b] FAM, 6-carboxyfluorescein.
[c] D, [4-(4'-dimethylaminophenylazo)benzoic acid]succimidyl ester (DABCYL).
[d] Encodes a methionine.
[e] TET, tetrachlorofluorescein.
[f] Encodes a leucine.
[g] Encodes an isoleucine.
[h] Assays for other *katG* alleles are described previously (17).

ously described molecular beacon assay (38). Mutations in *embB*306 were screened by a set of four molecular beacons in which the molecular beacon B*embB*306 fluoresced brightly in real-time PCRs containing wild-type *embB*306 sequences while molecular beacons B*embB*M306L-c, B*embB*M306I-c, and B*embB*M306I-a detected common mutant *embB*306 alleles (Table 2). Samples that were negative by all molecular beacon assays were directly sequenced to identify the mutation present. A subset of samples that were positive for each mutant molecular beacon was also sequenced to confirm the accuracy of each assay. All molecular beacon assays were performed at 60°C, following the recommendations described previously (49, 50).

DNA sequencing. The *embB*306 position was amplified by PCR and sequenced with primers FSeq*embB*306 and RB*embB*306 (Table 2). DNA sequencing was performed with a Dye Terminator kit (Applied Biosystems), and the reactions were analyzed with an ABI 3100 genetic analyzer.

Statistical analysis. Chi-square tests for trend with and without adjustment for country of origin were performed to test whether the proportion of mutant *embB*306 isolates increased with the number of drugs to which an isolate was resistant. Pearson chi-square tests were used to test for an association between the *embB*306 genotype and clustering. We employed an overall multifactor logistic regression model to evaluate whether independent associations existed between clustering and the chosen set of single-nucleotide polymorphisms (SNPs). All mutations were initially entered into the overall model, and individual SNPs found to be insignificant were sequentially removed from the model. The best-fitting model was obtained for resistant isolates obtained from studies

that took place over 2 years and resistant cases in general. SAS version 8.02 was used for all statistical analyses.

RESULTS

Associations between *embB*306 mutations and drug resistance. A total of 1,020 clinical isolates of *M. tuberculosis* were evaluated by the *embB*306 molecular beacon assays and DNA sequencing. One hundred isolates (10%) contained a mutation in *embB*306. Forty-six of the *embB*306 mutants were susceptible to EMB, while 54 were EMB resistant (Table 3). Mutants were present in samples from all countries except Spain at frequencies between 2% and 15%; samples from Spain did not have any *embB*306 mutants (Table 1). The susceptibilities to EMB of 104 isolates from Australia, Colombia, India, Mexico, Spain, and Texas were retested. Twenty-eight of these isolates were *embB*306 mutants; however, the investigators responsible for retesting EMB susceptibility were blinded to the earlier results. Only five isolates (three with *embB*306 mutations) were found to have discrepant susceptibilities, with resistant isolates

TABLE 3. Distribution of *embB*306 mutations in clinical *M. tuberculosis* isolates used in this study

| Drug resistance[a] | No. of isolates with the following genotype at *embB*306: | | | | | | | No. of *embB*306 mutants/no. of wild-type isolates (%) |
| | Wild-type ATG[b] | Mutant | | | | | | |
		CTG[c]	TTG[c,d]	GTG[e]	ATC[f]	ATA[f]	ATT[f]	
Pansusceptible	582							0/582 (0)
INH	93	1			1	2		4/97 (4)
RIF	9							0/9 (0)
STR	18							0/18 (0)
EMB	1							0/1 (0)
INH + RIF	76			2	5	14		21/97 (22)
INH + STR	23					2		2/25 (8)
INH + EMB	1					1		1/2 (50)
RIF + STR	1							0/1 (0)
RIF + EMB	0				1			1/1 (100)
STR + EMB	4							0/4 (0)
INH + RIF + STR	64			11	1	6	1	19/83 (23)
INH + RIF + EMB	15	2		2	2	2	1	9/24 (37)
RIF + STR + EMB	2							0/2 (0)
INH + STR + EMB	1		1			1		2/3 (67)
INH + RIF + STR + EMB	29	3		20	2	13	3	41/70 (59)
Resistant to one or more antibiotics	338	6	1	35	12	41	5	100/438 (23)
Total	920	6	1	12	35	41	5	100/1,020 (10)

[a] The antibiotics tested were INH, RIF, STR and EMB.
[b] Encodes a methionine.
[c] Encodes a leucine.
[d] Novel mutation.
[e] Encodes a valine.
[f] Encodes an isoleucine.

falsely classified as susceptible. The results for all samples with discrepant results were reconfirmed at least one more time prior to the final analysis. We also determined the EMB MICs for 55 isolates from Australia, Colombia, and Mexico, including 12 *embB*306 mutants. The EMB MICs ranged from <1 to >32 μg/ml, and no association was observed between the *embB*306 genotype and the MIC (Table 4). Notably, the isolate with the lowest EMB MIC was found to have an *embB*306 mutation.

Mutations in *embB*306 can result in several different amino acid substitutions (12, 31, 33, 34, 36, 41, 45, 52). We examined each amino acid change separately to determine if any particular substitution had a unique association with EMB resistance. However, no such associations were observed (Tables 3 and 4). A striking association was observed between *embB*306 mutations and drug resistance in general. Thus, none of the 582 pansusceptible isolates contained an *embB*306 mutation, while 100 of 438 (23%) of the isolates that were resistant to at least one antibiotic contained a mutation at this locus ($P <$ 0.001 for association with broad drug resistance). This association may explain why the Spanish samples did not include any *embB*306 mutants, as only 11 of 109 (10%) of the Spanish isolates were resistant to any drug.

The overall drug resistance patterns of the *embB*306 mutants were examined in order to determine whether *embB*306 mutations could be responsible for resistance to any antibiotic other than EMB. Nine of the 100 *embB*306 mutants were susceptible to RIF, 36 were susceptible to STR, and 1 was susceptible to INH (Table 3). We considered the possibility that a mistake had been made in the analysis of the single INH-susceptible *embB*306 mutant; it was retested twice to con-

TABLE 4. MICs of EMB for selected wild-type and mutant isolates with mutations at position *embB*306

Isolate	EMB susceptibility	EMB MIC (μg/ml)[a]	Genotype at *embB*306
Mexico 111	Susceptible	<1	Mutant (ATA)
Colombia 717	Susceptible	1	Wild type
Colombia 125	Susceptible	2	Wild type
Australia 2558	Susceptible	2.5	Mutant (ATC)
H37*Rv*	Susceptible	2.5	Wild type
Colombia 1311	Susceptible	4	Wild type
Colombia 1314	Susceptible	4	Wild type
Mexico 5306	Susceptible	6	Mutant (ATA)
Mexico 5523	Susceptible	6	Mutant (ATA)
Mexico 5041	Moderately resistant	8[b]	Mutant (CTG)
Mexico 12563	Moderately resistant	8	Mutant (ATA)
Colombia 1194	Moderately resistant	8[b]	Wild type
Colombia 1299	Moderately resistant	8[b]	Wild type
Mexico 5036	Resistant	10	Wild type
Mexico 30787	Resistant	12	Wild type
Colombia 003	Resistant	16	Wild type
Colombia 444	Resistant	16	Wild type
Colombia 959	Resistant	16	Wild type
Colombia 828	Resistant	16	Mutant (ATC)
Mexico 5309	Resistant	>16	Mutant (CTG)
Mexico 30167	Resistant	>16	Mutant (ATC)
Mexico 16703	Resistant	>16	Wild type
Colombia 227	Resistant	>32	Mutant (GTG)
Colombia 1125	Resistant	>32	Mutant (GTG)
Colombia 1231	Resistant	>32	Mutant (ATT)

[a] The breakpoint value is 7.5 μg/ml.
[b] The EMB susceptibilities of these isolates were reconfirmed twice by the agar proportion method, but the MIC classifies them as moderately resistant.

TABLE 5. INH resistance-associated mutations detected in the INH monoresistant *embB*306 mutants

Isolate	Mutation at *embB*306	INH resistance-associated mutations	Major genetic group
Australia 2558	M306I (ATG→ATC)	*inhA* promoter (−17 G→T) *inhA*I194T (ATC→ACC) *ahpC* promoter (−46 G→A)	I
Mexico 111	M306I (ATG→ATA)	*katG*S315T (AGC→ACC)	II
Mexico 5041	M306L (ATG→CTC)	*inhA* promoter (−15 C→T) *inhA*S94A (TCG→GCG)	II
Mexico 5306	M306I (ATG→ATA)	*katG*S315T (AGC→ACC)	II

firm its antibiotic susceptibility, and the presence of the *embB*306 mutation was also reconfirmed by direct DNA sequencing. We also determined that each of the four INH-monoresistant *embB*306 mutants contained other mutations that could account for their resistance to INH using assays described previously (38) (Table 5), and their patterns of susceptibility to all antibiotics were reconfirmed. These results strongly indicate that *embB*306 mutations are not one of the causes of INH resistance. Thus, mutations in *embB*306 could not be uniquely associated with any particular type of drug resistance.

Genotype at position *embB*306 after in vitro selection for EMB resistance. Approximately 50% of EMB-resistant clinical isolates have *embB*306 mutations (12, 31, 33, 36, 37, 43, 52). One would expect to find a similar proportion of *embB*306 mutants during in vitro selection for EMB resistance, if this mutation truly conferred resistance to EMB. Thirty-seven EMB-resistant colonies were isolated after approximately 4 × 10^7 CFU of strain H37Rv was plated onto Middlebrook 7H10 medium containing 20 μg/ml of EMB. All 37 colonies were found to have the wild-type sequence at the *embB*306 codon. We also tested whether lower concentrations of EMB could select for *embB*306 mutations in isolates that were grown at the MIC of EMB. H37Rv was plated on medium containing 2.5 μg/ml of EMB. All of the 55 colonies isolated under these conditions were EMB susceptible; none contained an *embB*306 mutation. We also considered the possibility that *embB*306 mutations did indeed confer EMB resistance but that the resistant phenotype was being suppressed by another mutation in the *embB* gene. The complete *embB* gene of two EMB-susceptible clinical strains (Australia 2558 and Mexico 5041) with *embB*306 mutations was sequenced to test this hypothesis. Both EMB-susceptible strains had wild-type *embB* sequences except at *embB*306.

Phylogenetic studies. We considered the possibility that the association between the *embB*306 mutations and drug resistance was not due to a change in the biological activity of the *embB* gene. It was possible that *embB*306 mutations were simply phylogenetic markers for a related family of isolates that were predisposed to the development of drug resistance through a mechanism unrelated to the *embB* gene activity. This hypothesis would have been consistent with reports that the Beijing family of *M. tuberculosis* is predisposed to the development of multidrug resistance (4, 28, 47). The *embB*306 mutants were examined for membership in the three major ge-

netic groups of *M. tuberculosis* (44). Mutants were found in all three major genetic groups: 22 (22%) in group I, 66 (66%) in group II, and 12 (12%) in group III. These findings suggest a biological rather than a phylogenetic role for *embB*306 mutations (3).

Association between *embB*306 mutants and resistance to increasing numbers of antibiotics. Based on the results described above, we questioned whether *embB*306 mutations could confer a general predisposition to the development of resistance to any antibiotic. We reasoned that factors with the ability to nonspecifically potentiate drug resistance should be observed at increasing frequencies in isolates with resistance to an increasing number of drugs. Analysis of all 438 drug-resistant isolates revealed a strong association between the presence of the *embB*306 mutations and resistance to increasing number of antibiotics ($P < 0.001$ for trend) (Fig. 1). This association remained significant even after geographic region was controlled for as a confounder ($P < 0.001$). Furthermore, this trend was essentially unchanged when the effect of the IS*6110*-defined cluster size was removed by examining each drug resistance pattern separately and counting each cluster as a single isolate (for this analysis, a cluster that contained isolates with both wild-type and *embB*306 mutant sequences was
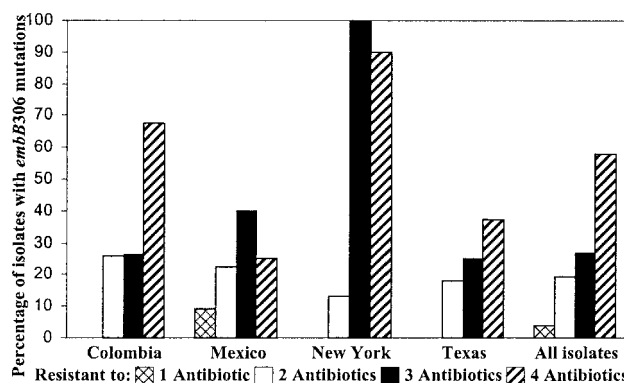

FIG. 1. Proportion of drug-resistant *M. tuberculosis* isolates with *embB*306 mutations. The proportion of *embB*306 mutants is shown by geographical origin and the number of antibiotics to which each isolate was resistant. Only geographical sites in which at least 10 *embB*306 mutant isolates were identified are shown. The "all isolates" category corresponds to all isolates included in the study.

TABLE 6. Univariate analysis showing association between *embB*306 mutations and clustering

No. of antibiotics to which the isolate was resistant	*embB*306 genotype	All studies				Studies occurring over >2 yr			
		No. (%) of isolates in a cluster	No. (%) of unique isolates	P (χ^2)	Odds ratio	No. (%) of isolates in a cluster	No. (%) of unique isolates	P (χ^2)	Odds ratio
All isolates									
Two or more	Mutant	65 (68)	31 (32)	0.017	1.84	64 (70)	28 (30)	0.092	1.58
	WT[b]	115 (53)	101 (47)			104 (59)	72 (41)		
One or more	Mutant	67 (67)	33 (33)	<0.001	2.44	66 (69)	29 (31)	0.004	2.06
	WT	153 (45)	184 (55)			136 (53)	123 (47)		
One isolate per cluster[a]									
Two or more	Mutant	22 (42)	31 (58)	0.034	2.05	21 (43)	28 (57)	0.085	1.86
	WT	35 (26)	101 (74)			29 (29)	72 (71)		
One or more	Mutant	22 (40)	33 (60)	0.008	2.27	21 (42)	29 (58)	0.046	1.94
	WT	54 (23)	184 (77)			46 (27)	123 (73)		

[a] Each cluster was counted as a single isolate. In cases where a cluster contained isolates with both wild-type and *embB*306 mutant sequences, the cluster was counted twice but was assigned to both the mutant and the wild-type categories.
[b] WT, wild type.

counted twice but was assigned to both the mutant and the wild-type categories) (data not shown).

Association between *embB*306 mutants and clustering as a marker for transmissibility. Several recent studies have suggested that multidrug-resistant *M. tuberculosis* strains may be transmitted less frequently than pansusceptible strains (9, 16). The novel association between *embB*306 mutations and resistance to increasing number of drugs led us to question whether this mutation could identify a subgroup of drug-resistant isolates with a heightened propensity for transmission. The clusters identified by IS*6110*-based RFLP analysis have become accepted as a proxy for measurements of transmission and accelerated disease progression (3, 16, 51). We found an association between *embB*306 mutations and presence in a cluster using a sample that consisted of all drug-resistant study isolates. This association was significant for all types of drug resistance as well as for isolates that were resistant to at least two antibiotics (Table 6). Similar results were obtained when this analysis was repeated after all isolates in each cluster were counted as a single isolate to eliminate the effect of cluster size (Table 6). This analysis was also repeated after removal of the Colombian and Australian "outlier" populations, which had the highest and lowest proportions of clustered isolates, respectively. Again, the same associations were found (data not shown). Some of the populations in this study were based on isolates collected over a relatively short period of time. Time is a known confounder in cluster analysis; therefore, the analysis was repeated a fourth time by limiting the samples to those from studies that had collected isolates over more than 2 years (which included only isolates from Colombia, Mexico, New York City, and Texas). The association between *embB*306 mutations and clustering remained significant in this final analysis (Table 6).

Multivariate analysis. The association between *embB*306 mutations and clustering that we observed could have been biased by our failure to include other mutations potentially associated with clustering in our analysis. Hidden biases in the study population or biases due to sampling could also have

influenced our results. We tested all drug-resistant study isolates for mutations in *katG*315, *katG*463, *gyrA*95, and *kasA*269 to control for these possibilities. Previous studies have suggested that *katG*315 mutations are associated with clustering and drug-resistant tuberculosis (40, 53). This mutation was chosen as the best approximation of a positive control. As negative controls we used mutations at *katG*463, *gyrA*95, and *kasA*269, which are known to be neutral phylogenetic markers (3, 38, 42, 44). Like *embB*306 mutations, these four mutations occur in genes that are involved in antibiotic resistance. Univariate analysis initially suggested that each of these mutations was associated with clustering (data not shown). However, when these SNPs were placed in a multifactor logistic regression model, only mutations at *embB*306 and *katG*315 showed an independent significant association with clustering (Table 7). In the case of *katG*463, we observed a significant association with unique rather than clustered isolates when all isolates were included in the analysis, but this significance was lost when we limited our analysis to study sites that provided isolates collected for more than 2 years (Table 7).

TABLE 7. Multivariate regression model of association between various mutations and clustering

Study	Position	Odds ratio	95% CI[a]	P value
All studies	*embB*306	2.41	1.43–4.07	0.001[b]
	*katG*315	1.74	1.07–2.83	0.027[b]
	*katG*463	0.50	0.29–0.86	0.013
	*kasA*269	2.28	0.77–6.77	0.138
	*gyrA*95	1.15	0.56–2.38	0.696
Studies occurring over >2 yr	*embB*306	2.14	1.22–3.76	0.008[b]
	*katG*315	1.99	1.15–3.44	0.015[b]
	*katG*463	1.01	0.50–2.02	0.981
	*kasA*269	2.16	0.72–6.47	0.167
	*gyrA*95	1.22	0.58–2.57	0.595

[a] CI, confidence interval.
[b] Significant association with clustering.

DISCUSSION

Our results strongly suggest that *embB*306 mutations do not cause EMB resistance in *M. tuberculosis*. Rather, we found that *embB*306 mutations may predispose *M. tuberculosis* to become resistant to any antibiotic and to become multidrug resistant. A careful search for alternate explanations and hidden biases is necessary. Some of the results of this study could be due to errors in EMB susceptibility testing, which can occasionally be unreliable (17–19, 29, 30). However, the EMB-susceptible *embB*306 mutants were detected in six of the seven laboratories in this study, and 46 of the *embB*306 mutants were EMB susceptible. An error in EMB susceptibility testing of this magnitude is highly unlikely, given that our discrepancy rate is similar to that described for other reference laboratories (29, 30). We observed *embB*306 mutations in 50% (54 of 108) of the EMB-resistant isolates, a percentage similar to those described in previous reports (12, 31, 33, 36, 37, 43, 52). Finally, measurements of EMB MICs demonstrated that *embB*306 mutations could be present in highly EMB-susceptible isolates. It should also be noted that potential errors in EMB susceptibility testing would not have affected our discovery of an association between *embB*306 mutations and clustering, as this analysis was performed only with isolates known to be antibiotic resistant.

One might question why mutations in *embB*306 have been associated with EMB resistance in many prior studies. We suggest that those studies did not include the proper EMB-susceptible control isolates in their analyses. The controls in the prior studies predominantly consisted of pansusceptible *M. tuberculosis* isolates, while our study found *embB*306 mutations only among the EMB-susceptible isolates that were resistant to other antibiotics. Thus, it is easy to see how previous investigations could have erroneously concluded that *embB*306 mutations were associated with EMB resistance. Our results are supported by those of other studies that used controls similar to ours (31, 37, 39, 48). The biological evidence supporting a role for *embB*306 mutations in EMB resistance is also weak. Overexpression of *embB* or replacement of wild-type *embB* with an I289M mutation (which corresponds to *embB*303 in *M. tuberculosis*) has been shown to cause EMB resistance in *M. smegmatis* (34). However, to our knowledge, EMB resistance has never been documented in *M. tuberculosis* either by overexpressing *embB* or by transferring a single copy of the *embB* gene containing any of the mutated *embB*306 alleles. In the current study, we also show that *M. tuberculosis* isolates selected for EMB resistance in vitro do not appear to acquire mutations in *embB*306.

We observed that *embB*306 mutations were present only in isolates that were resistant to one or more drugs. One possible explanation for this observation is that *embB*306 mutations increase the likelihood that any type of drug resistance will develop but do not otherwise increase bacterial fitness. This hypothesis predicts that *embB*306 mutations will rarely, if ever, be detected in pansusceptible isolates. However, once a rare *embB*306 mutant acquires a drug resistance mutation, it would then have a strong evolutionary advantage over wild-type cells. It has been shown that sub-MICs of EMB can increase *M. tuberculosis* susceptibility to hydrophobic antibiotics by increasing cell wall permeability (13, 20). It is possible that *embB*306

mutations render *M. tuberculosis* resistant to this effect of EMB while the isolate remains fully EMB "susceptible," as measured by MIC or antibiotic breakpoint testing. Verification of this hypothesis could have important implications for the treatment of tuberculosis.

Clustering has been widely accepted as a marker for recently transmitted *M. tuberculosis* with rapid progression to disease (3, 8, 16, 42, 51). Thus, our results suggest that *embB*306 mutations could also be associated with disease transmission. This investigation was open to a number of biases, given its retrospective design and the combined use of several studies with different enrollment and selection criteria. An effort was made to reduce potential biases by limiting analysis to drug-resistant isolates and by including other mutations in a multivariate analysis with *embB*306. We found that both *embB*306 and *katG*315 mutations were independently associated with clustering, while the other control mutations did not have such an association. Mutations in *katG*315 have been associated with clustering among drug-resistant isolates in another large study (53). Our ability to confirm these results for *katG*315 in the current investigation further validates our study populations and suggests that the association which we found between *embB*306 and clustering is valid. Further confirmation of this hypothesis must await a large population-based study that includes all tuberculosis cases over an extended period.

Mutations in *katG*315 are known to cause INH resistance in *M. tuberculosis* (23), and it is postulated that the association between *katG*315 mutations and clustering can be explained by the fact that this mutation is less likely to attenuate bacterial virulence than other mutations that cause INH resistance (40). In contrast, we have shown that *embB*306 mutations are not associated with resistance to any particular antibiotic; thus, there is no obvious biological explanation for the observed association between *embB*306 mutations and clustering. However, the *embABC* genes are intimately involved in the biosynthesis of the *M. tuberculosis* cell wall. Mutations in *embB*306 could change the *M. tuberculosis* cell wall in unknown ways, in the presence or absence of EMB, altering virulence properties and increasing the transmission rates or disease progression.

In conclusion, we have demonstrated that *embB*306 mutations in *M. tuberculosis* are associated with broad drug resistance and clustering of drug-resistant isolates. Our results suggest as well that *embB*306 and/or *katG*315 could serve as a marker for tuberculosis cases that are at increased risk for the development and spread of drug-resistant disease. Additional studies are required to confirm these results and to determine the biological mechanism underlying these observations. These observations could lead to new treatment approaches that would ultimately make a significant contribution to tuberculosis control.

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