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Allelic Exchange and Mutant Selection Demonstrate that Common Clinical *embCAB* Gene Mutations Only Modestly Increase Resistance to Ethambutol in *Mycobacterium tuberculosis*▽

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Mutations within codon 306 of the *Mycobacterium tuberculosis embB* gene modestly increase ethambutol (EMB) MICs. To identify other causes of EMB resistance and to identify causes of high-level resistance, we generated EMB-resistant *M. tuberculosis* isolates in vitro and performed allelic exchange studies of *embB* codon 406 (*embB*406) and *embB*497 mutations. In vitro selection produced mutations already identified clinically in *embB*306, *embB*397, *embB*497, *embB*1024, and *embC*13, which result in EMB MICs of 8 or 14 μg/ml, 5 μg/ml, 12 μg/ml, 3 μg/ml, and 4 μg/ml, respectively, and mutations at *embB*320, *embB*324, and *embB*445, which have not been identified in clinical *M. tuberculosis* isolates and which result in EMB MICs of 8 μg/ml, 8 μg/ml, and 2 to 8 μg/ml, respectively. To definitively identify the effect of the common clinical *embB*497 and *embB*406 mutations on EMB susceptibility, we created a series of isogenic mutants, exchanging the wild-type *embB*497 CAG codon in EMB-susceptible *M. tuberculosis* strain 210 for the *embB*497 CGG codon and the wild-type *embB*406 GGC codon for either the *embB*406 GCC, *embB*406 TGC, *embB*406 TCC, or *embB*406 GAC codon. These new mutants showed 6-fold and 3- to 3.5-fold increases in the EMB MICs, respectively. In contrast to the *embB*306 mutants, the isogenic *embB*497 and *embB*406 mutants did not have preferential growth in the presence of isoniazid or rifampin (rifampicin) at their MICs. These results demonstrate that individual *embCAB* mutations confer low to moderate increases in EMB MICs. Discrepancies between the EMB MICs of laboratory mutants and clinical *M. tuberculosis* strains with identical mutations suggest that clinical EMB resistance is multigenic and that high-level EMB resistance requires mutations in currently unknown loci.

Ethambutol (EMB) is a first-line antituberculosis drug that is often used in combination with other drugs to treat tuberculosis and to prevent the emergence of drug resistance. EMB also has a place in the treatment of drug-resistant and multidrug-resistant tuberculosis (2). The recent global increase in the incidence of drug-resistant tuberculosis has produced many strains that are resistant to EMB. Therefore, it is prudent to test isolates from all tuberculosis patients for their EMB susceptibility, especially when EMB is used to treat multidrug-resistant tuberculosis. Unfortunately, conventional culture-based EMB susceptibility test methods have poor intertest and interlaboratory reproducibilities (8, 21). This has made it difficult to firmly rule out the presence of EMB resistance by the use of conventional assays. Culture-based *Mycobacterium tuberculosis* drug susceptibility tests are also quite slow (12, 20).

Genetic tests for EMB resistance are potentially more rapid and more accurate than conventional culture-based resistance testing. Genetic assays identify resistance by detecting mutations that encode EMB resistance on the *M. tuberculosis* chromosome, principally within the *embB* gene (5, 17, 25). The results of genetic assays can be available within hours; they have high interassay reproducibilities and have the potential to have high sensitivities (5, 25). However, genetic testing for EMB resistance has been hindered by a persistent uncertainty concerning the role of specific mutations in EMB resistance. Initially, the role of mutations within codon 306 of the *embB* gene (*embB*306) was questioned. Although *embB*306 mutations were present in 30 to 68% of EMB-resistant clinical isolates (1, 13, 22), some studies had noted a widespread presence of *embB*306 mutations in EMB-susceptible isolates (1, 7, 9). The role of *embB*306 mutations was firmly established to be a cause of low- and moderate-level (two to seven times the MIC for the wild type) EMB resistance in a recent allelic exchange study (19). However, that study also demonstrated that *embB*306 mutations do not in themselves cause high-level (MICs $>$ 20 μg/ml) EMB resistance. Furthermore, the cause of EMB resistance in the 32 to 70% of clinical EMB-resistant *M. tuberculosis* isolates that did not have *embB*306 mutations remained an open question.

Several clinical studies have suggested that other mutations in the *embCAB* operon are responsible for at least some of the remaining EMB-resistant tuberculosis cases. The most commonly occurring *embCAB* mutations other than *embB*306 have been found in *embB*406 and *embB*497. Importantly, these two mutations have been detected in clinical isolates with high-

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TABLE 1. Plasmids, primers, and molecular beacons used in this study

Plasmid, primer, or molecular beacon	Description or sequence[a]
Plasmids	
p2NIL	Suicide vector containing kanamycin resistance gene and OriE
pGOAL17	Vector containing ampicillin resistance gene, *sacB-lacZ* cassette, and OriE
p2NIL::embB497CAG-PacCassette	p2NIL vector with 3,500-bp fragment spanning embB497 CAG and *sacB-lacZ* cassette inserted
p2NIL::embB497CGG-PacCassette	p2NIL vector with 3,500-bp fragment spanning embB497 CGG and *sacB-lacZ* cassette inserted
p2NIL::embB406GCC-PacCassette	p2NIL vector with 2,211-bp fragment spanning embB406 GCC and *sacB-lacZ* cassette inserted
p2NIL::embB406GAC-PacCassette	p2NIL vector with 2,211-bp fragment spanning embB406 GAC and *sacB-lacZ* cassette inserted
p2NIL::embB406TGC-PacCassette	p2NIL vector with 2,211-bp fragment spanning embB406 TGC and *sacB-lacZ* cassette inserted
p2NIL::embB406TCC-PacCassette	p2NIL vector with 2,211-bp fragment spanning embB406 TCC and *sacB-lacZ* cassette inserted
Primers and molecular beacons	
F-embB406Bcn	CGTGGATGCCGTTCAACAA
R-embB406Bcn	GCTCGATCAGCACATAGGTG
Bcn-embB406	FAM-CGCCAGGAGGGCATCATCGCGCTGGCG-D
F-embB497Bcn	TCATCCTGACCGTGGTGT
R-embB497Bcn	AACCCTGGTGGCTTCCAA
Bcn-embB497	FAM-CGCCAGGACCAGACCCTGTCAACGCTGGCG-D
F2-embBCL	TTTGGTACCGGTCCGTTCCTGTTCACC
R1-embBOEx	AACACCACGGTCAGGATGACG
F2-embBOEx	CGCATTCACACTGGGTGTGCA
R2-embBOEx	TTCCAAGCTTCGCTATGGACCAATTCGGATC
F2-embB406Ala	CGGAGGCCATCATCGCGCTCGGCTCGC
F3-embB406Ala	GGCCATGGTCTTGCTGACCGCGTGGATGCCGTTCAACAACGGCCTGCGGCCGGAG GCCATCATCGCGCTCGGCTCGC
F2-embB406Asp	CGGAGGACATCATCGCGCTCGGCTCGC
F3-embB406Asp	GGCCATGGTCTTGCTGACCGCGTGGATGCCGTTCAACAACGGCCTGCGGCCGGAG GACATCATCGCGCTCGGCTCGC
F2-embB406Cys	CGGAGTGCATCATCGCGCTCGGCTCGC
F3-embB406Cys	GGCCATGGTCTTGCTGACCGCGTGGATGCCGTTCAACAACGGCCTGCGGCCGGAG TGCATCATCGCGCTCGGCTCGC
F2-embB406Ser	CGGAGTCCATCATCGCGCTCGGCTCGC
F3-embB406Ser	GGCCATGGTCTTGCTGACCGCGTGGATGCCGTTCAACAACGGCCTGCGGCCGGAG TCCATCATCGCGCTCGGCTCGC
F4-embB406	TGAGGTGCTGCCCCGCCTCGGGCCGGCGGTGGAGGCCAGCAAACCCGCCTACTGG GCGGCGGCCATGGTCTTGCTGACCGCGTGGATGCC
F5-embB406	TCTGTGGATGCGCCTGCCAGACCTGGCCGCCGGGCTAGTGTGCTGGCTGCTGCTGT CGCGTGAGGTGCTGCCCCGCCTCGGGCCGGCGGT
F6-embB406	CGGAGGATCCCTTCGGCTGGTATTACAACCTGCTGGCGCTGATGACCCATGTCAGC GACGCCAGTCTGTGGATGCGCCTGCCAGACCTGGCCGC
R-embB306CL	TTCCGGTACCTGTGCTGACTGTGATCCC
F-embB306CL	TTCCAAGCTTGGTCCGTTCCTGTTCACC
R1-embB406CL	GCCAGCAGGTTGTAATACC

[a] Underlining indicates the codons that were changed. FAM, 6-carboxyfluorescein; D, 4-(4-dimethylaminophenylazo)benzoic acid succinimidyl ester.

level EMB resistance (11, 14). However, other studies identified *embB*406 mutations in EMB-susceptible clinical isolates (7, 15, 23). Other mutations in the *embB* and *embC* genes have also been identified in EMB-resistant clinical *M. tuberculosis* isolates (6, 22, 23), but at low frequencies, making it difficult to firmly establish associations with EMB resistance. Thus, the actual role of non-*embB*306 mutations in EMB resistance has not been proven.

In the study described here, we examined the role of *embB* mutations outside of the *embB*306 codon in EMB resistance. Using in vitro-selected mutants and allelic exchange techniques, our results demonstrate that non-*embB*306 mutations in the *embCAB* operon play an important role in EMB resistance, but like mutations in *embB*306, these mutations confer only a low to a moderate increase in EMB MICs. Our study strongly suggests that unrecognized mycobacterial gene targets for EMB resistance and high-level resistance remain to be discovered.

MATERIALS AND METHODS

Bacterial culture and MIC testing. *M. tuberculosis* strains were cultured as described previously (18). The MICs for EMB, isoniazid (INH), and rifampin (RIF; rifampicin) were determined by the standard radiometric Bactec 460TB method (Becton Dickinson and Company, Sparks, MD) and by the 7H10 agar proportion method, as described previously (19). The Top10 *Escherichia coli* strain (Invitrogen) was used as the host for all plasmid constructions. Top10 was grown in Luria-Bertani broth or agar medium (Sigma), which was supplemented with 50 μg/ml kanamycin where appropriate.

In vitro mutant selection and mutant testing. Cultures of *M. tuberculosis* strain 210 were grown in liquid culture to an optical density at 600 nm of approximately 1.5, spun down, and resuspended in 2 ml of 7H9 medium. Approximately 10^9 *M. tuberculosis* cells were then plated on 7H10 medium containing either no EMB or EMB (Sigma) at a final concentration of 8, 16, or 32 μg/ml. The cells were then incubated at 37°C for 4 weeks. The resulting colonies were cultured in 7H9 medium without EMB prior to MIC testing. Genomic DNA was extracted from each colony after subculture in the absence of EMB. Mutations in *embB*406 and *embB*497 were detected by a real-time PCR assay consisting of the primers and molecular beacons listed in Table 1, following the recommendations described previously (4). The entire *embB* gene of all EMB-resistant colonies that were found not to contain mutations in *embB*406 or *embB*497 were sequenced. Sub-

TABLE 2. Detection of mutations in the *embB* gene by standardized molecular beacon assays

EMB concn (μg/ml)	No. of colonies	No. of mutants/mutation		
		*embB*306 ATG[a]	*embB*406 GGC	*embB*497 CAG
8	7	1/ATA	0	0
16	11	2/both GTG	0	2/both CGG
32	5	0	0	2/both CGG

[a] From previously published data (19).

sequently, the entire *embCAB* operon of colonies that were not found to have mutations in the *embB* gene was sequenced.

***embCAB* operon sequencing.** The PCR products of the *embCAB* locus were generated from genomic DNAs by using a TaKaRa LA PCR kit (version 2; Takara Bio) and standard PCR conditions. Five overlapping PCR products (amplicon 1, primers 1 [5′-CGCACATAACAGCTACACCC-3′] and 2 [5′-CG AAGGTCTGATCACGAAAG-3′]; amplicon 2, primers 3 [5′-CTTTCGTGAT CAGACCTTCG-3′]) and 4 [5′-ACCAGCCAGTCCAGGAACAC-3′]; amplicon 3 primers 5 [5′-GTGTTCCTGGACTGGCTGGT-3′] and 6 [5′-GATCGACCG TTCCACCAACA-3′]; amplicon 4, primers 7 [5′-TGTTGGTGGAACGGTCG ATC-3′] and 8 [5′-CCACCGACAACACAAAGCCA-3′]; amplicon 5, primers 9 [5′-GGTCCGTTCCTGTTCACC-3′] and 10 [5′-CGCTATGGACCAATTCGG ATC-3′] were amplified from genomic DNAs to span the *embCAB* locus. Overlapping *embCAB* amplicons were purified with a QIAquick PCR cleanup column (Qiagen) and were used as templates for Sanger sequencing (Applied Biosystems). Sequence reads were assembled by using TIGR_Assembler (J. Craig Venter Institute) software. In order to identify sequence polymorphisms, the consensus sequence from each contig for each strain was compared to the sequences of *M. tuberculosis* strains 210 and NJT210GTG by using NUCMER sequence analysis software (3). Electropherograms were inspected by using Cloe sequence analysis software (http://cloe.sourceforge.net) for all polymorphisms identified to assess the quality of the read. Areas of low coverage or poor quality were resequenced to increase sequence coverage and quality. The genome coordinates of confirmed polymorphisms were determined by BLAST analysis by using *M. tuberculosis* strain H37Rv as a reference.

Isogenic strain construction. DNA isolation and PCRs were performed as described previously (18). To create the *embB*497 isogenic strains, a 3,500-bp DNA fragment spanning the *embB*497 codon was created in two separate PCR steps by using genomic DNA extracted either from *M. tuberculosis* strain 16703, which contained a single CAG-to-CGG point mutation at *embB*497, or from wild-type strain 210. The left and right fragments were amplified with primer pair F2-embBCL and R1-embBOEx and primers pair F2-embBOEx and R2-embBOEx (Table 1), respectively. The resulting left fragment was digested with Acc65I and BamHI, and the right fragment was digested with BamHI and HindIII (Promega). To create the *embB*406 isogenic strains, a 2,211-bp fragment carrying either the *embB*406 GCC (Ala), *embB*406 GAC (Asp), *embB*406 TGC (Cys), or *embB*406 TCC (Ser) codon was obtained in two separate PCR steps by use of a site-directed PCR mutagenesis method (16, 19), as follows. A first DNA fragment was amplified from strain 210 genomic DNA by using primers F-embB306CL and R1-embB406CL (Table 1). The amplicon was then purified and digested with HindIII and BamHI. To introduce different single point mutations at *embB*406, a second DNA fragment was obtained by five sequential PCRs with primer R-embB306CL and mutagenic primers F2-embB406, F3-embB406, F4-embB406, F5-embB406, and F6-embB406 (Table 1) in succession, as described previously (19). The final PCR products were purified and digested with BamHI and Acc65I. To obtain the *embB*497 or *embB*406 point mutations, the left and right amplicons for each mutation (or the wild-type control) were ligated and simultaneously cloned into the p2NIL vector, followed by the insertion of a PacI cassette containing the *sacB* and *lacZ* genes (10) (Table 1). The recombinant plasmids were transformed into *M. tuberculosis* strain 210, and the mutant colonies obtained by double-crossover events were screened by standardized molecular beacon assays (Table 1). We confirmed that only the intended single point mutations were introduced into the *embB* gene by direct DNA sequencing of the isogenic strain DNA, as described previously (19).

TABLE 3. MICs and the *embCAB* mutations of *M. tuberculosis* strain 210 and its in vitro-selected clones

EMB exposure concn (μg/ml)	*M. tuberculosis* strain	EMB MIC (μg/ml) by the following method:		*embCAB* mutation
		7H10 agar proportion	Bactec 460TB	
NA[a]	210	2	2	Wild type
8	8C1	6	2	Wild type
	8C2	8	4	*embB* wild type
	8C3	ND[b]	4	ND
	8C5	8	4	*embC*13 CGG-CTG (Arg-Leu)[c]
	8C6	4	2	Wild type
	8C7	4	2	*embB*445 CAG-CGG (Gln-Arg)[c]
16	16C6	8	8	*embB*320 TTC-TTG (Phe-Leu)[c]
	16C7	ND	8	*embB*445 CAG-CGG (Gln-Arg)[c]
	16C9	ND	12	*embB*497 CAG-CGG (Gln-Arg)[d]
	16C10	6	2	Wild type
	16C11	8	5	*embB*397 CCG-TCG (Pro-Ser)[2]
	16C12	8	3	Wild type
	16C14	16	8	*embB*445 CAG-CGG (Gln-Arg)[c]
	16C15	ND	12	*embB*497 CAG-CGG (Gln-Arg)[d]
	16C16	8	3	*embB*1024 GAC-AAC (Asp-Asn)[c]
32	32C1	6	2	*embB*445 CAG-CGG (Gln-Arg)[c]
	32C2	ND	12	*embB*497 CAG-CGG (Gln-Arg)[d]
	32C3	ND	12	*embB*497 CAG-CGG (Gln-Arg)[d]
	32C4	ND	4	*embB*445 CAG-CGG (Gln-Arg)[c]
	32C5	8	8	*embB*324 GGC-CGC (Gly-Arg)[c]

[a] NA, not applicable.
[b] ND, not done.
[c] Mutations detected by *embB* gene sequencing and/or *embCAB* operon sequencing.
[d] Mutations detected by molecular beacon assays.

RESULTS

In vitro selection for *embB*406 and *embB*497 mutants. Mutations at *embB*406 and *embB*497 have frequently been described in clinical studies of EMB-resistant tuberculosis. We searched for *embB*406 and *embB*497 mutations in *M. tuberculosis* colonies that were picked after they were cultured on plates with different EMB concentrations (Table 2). Three of these 23 colonies had previously been identified as *embB*306 mutants (19). No *embB*406 mutants were detected in the 23 colonies screened; however, 4/23 colonies had *embB*497 mutations. Two of the 5 colonies selected with 32 μg/ml of EMB and 2 of 11 colonies selected with 16 μg/ml of EMB contained *embB*497 CAG-to-CGG (Gln-to-Arg) mutations (Table 2). All of these *embB*497 mutants had EMB MICs of 12 μg/ml (Table 3). This is in contrast to the findings of clinical studies, which have

reported on *embB*497 mutants with much higher EMB MICs (14).

Mutations within the entire *embCAB* operon. We performed additional sequencing of the in vitro-selected EMB-resistant colonies to search for mutations other than *embB*306, *embB*406, and *embB*497. We first sequenced the entire *embB* gene of each colony, and then we sequenced the *embCA* genes of all colonies that had wild-type sequence at *embB*. We detected mutations previously described in EMB-resistant clinical isolates at *embB*397, *embB*1024, and *embC*13. These mutants had very low levels of EMB resistance, with EMB MICs being between 3 and 5 μg/ml with the Bactec 460TB system and 8 μg/ml by the agar proportion method (Table 3). Three mutations that resulted in slightly higher levels of EMB resistance were identified at *embB*320, *embB*324, and *embB*445. The *embB*320 and *embB*324 mutants had EMB MICs of 8 μg/ml by the use of both the Bactec 460TB and the agar proportion methods (Table 3). Previous molecular studies also detected spontaneous mutants at *emB*320 or *embB*324 by in vitro selection methods (24); however, the *embB*445 mutation has not been described previously. The five *embB*445 mutants had identical *embB*445 CAG-to-CGG (Gln-to-Arg) changes; yet, surprisingly these colonies had a range of MICs by use of the Bactec 460TB system that varied from 2 to 8 μg/ml. These results were confirmed by repeat MIC testing two times. Complete sequencing of the entire *embCAB* operon confirmed the absence of any other mutation besides *embB*445 in each case, suggesting that additional mutations outside of the *embCAB* operon were responsible for the variability in the MICs. Interestingly, three EMB-resistant colonies (MICs, 4 to 6 μg/ml by the agar proportion method) had wild-type *embCAB* gene sequences. These colonies did not appear to be resistant by use of the Bactec 460TB method. It is possible that these clones contained mutations which were situated outside of the *embCAB* operon. These as-yet-unknown mutations may be at least partially responsible for the discrepancies in the results obtained between the liquid and solid EMB resistance testing methods sometimes observed in clinical studies (8, 12, 21). An additional three colonies had an intermediate EMB resistance phenotype, with the EMB MICs being 8 μg/ml by the agar proportion method and 3 to 4 μg/ml by use of the Bactec 460TB system. The significance of these MIC increases is unclear, even though they were reproducible.

Allelic exchange studies of *embB*406 point mutations. Our inability to identify *embB*406 mutations in any of the in vitro-selected EMB-resistant colonies caused us to question the significance of this mutation, despite previously observed associations between *embB*406 mutations and EMB resistance in clinical studies. We performed allelic exchange experiments to definitively explore this issue (Table 4). We exchanged the wild-type clinical *M. tuberculosis* 210 strain *embB*406 GGC (Gly) codon for either the *embB*406 GCC (Ala), *embB*406 GAC (Asp), *embB*406 TGC (Cys), or *embB*406 TCC (Ser) codon, creating isogenic mutants NJT210GCC, NJT210GAC, NJT210TGC, and NJT210TCC, respectively. These mutations were selected because each of them has been reported in clinical studies of EMB-resistant tuberculosis (7, 11, 14). The unique transfer of the intended point mutation into *embB* was confirmed by sequencing the entire *embB* gene. Our results show that *embB*406 mutations are responsible for small increases in EMB MICs. Strains NJT210GCC, NJT210GAC, and NJT210TGC each showed an EMB MIC of 7 μg/ml and strain NJT210TCC had an MIC of 6 μg/ml (Table 3), whereas the EMB MIC was 2 μg/ml for parental strain 210. The small increase in the EMB MICs that we observed may explain our inability to detect *embB*406 mutants by in vitro selection.

TABLE 4. MICs and *embB* genotypes of *M. tuberculosis* strain 210 and its isogenic mutants constructed by allelic exchange methods

M. tuberculosis strain	MIC (μg/ml)			*embB* genotype[a]
	EMB	INH	RIF	
210	2	0.05	0.05	Wild type
NJT210CGG	12	0.05	0.05	*embB*497 CAG-CGG (Gln-Arg)
NJT210CGG-CAG	2	0.05	0.05	Wild type
NJT210GCC	7	0.05	0.05	*embB*406 GGC-GCC (Gly-Ala)
NJT210GAC	7	0.05	0.05	*embB*406 GGC-GAC (Gly-Asp)
NJT210TGC	7	0.05	0.05	*embB*406 GGC-TGC (Gly-Cys)
NJT210TCC	6	0.05	0.05	*embB*406 GGC-TCC (Gly-Ser)

[a] The remaining sequence of the *embB* gene was identical in all strains.

Allelic exchange studies of *embB*497 point mutations. Mutations at *embB*497 have been reported to be associated with high-level EMB resistance in clinical strains. We performed allelic exchange studies at *embB*497 to study the impact of mutation of this allele on the EMB MIC. We replaced the wild-type *embB*497 CAG (Gln) codon in strain 210 with the *embB*497 CGG (Arg) codon, creating isogenic mutant strain NJT210CGG (Table 4). This mutant showed a moderately increased EMB MIC (12 μg/ml). This increase is comparable to that seen when the more commonly occurring mutant *embB*306 GTG allele is moved into wild-type strain 210. However, neither mutation produces high-level EMB resistance. We also reintroduced the wild-type *embB*497 CAG sequence back into NJT210CGG, creating strain NJT210CGG-CAG. This was done to confirm that the increased EMB MIC that we observed was not caused by an accidental introduction of mutations at other locations within the *M. tuberculosis* genome. As expected, the reintroduction of the wild-type *embB*497 sequence into NJT210CGG-CAG caused its EMB MIC to revert to the wild-type level.

Differential growth in the presence of INH and RIF. We had previously shown that *embB*306 mutants show less growth inhibition in the presence of subinhibitory concentrations of INH or RIF. An *embB*306 CTG isogenic mutant was also able to outcompete its wild-type parental strain during coculture in subinhibitory concentrations of INH (19). We performed similar growth studies with INH and RIF using the mutants created in this study. Upon exposure to either drug, none of the *embB*406 or *embB*497 mutants showed a difference in growth compared to the growth of the parental control.

DISCUSSION

This study demonstrates that mutations at *embB*497 and *embB*406 are definitive causes of EMB resistance in *M. tuberculosis*. Other than mutations at *embB*306, these mutations are

the ones that are the most frequently detected in clinical EMB-resistant strains. This study demonstrates that the clinically observed mutations at *embB*397, *embB*1024, and *embC*13 also increase the EMB MICs, although their effects are much smaller (resulting in 1.5- to 2-fold increases in the MICs). We also confirm that mutations in *embB*320 and *embB*324 result in EMB resistance, supporting the findings of a previous in vitro selection study (24); and we identified a new mutation at *embB*445 to be a cause of EMB resistance. The level of proof supplied by our in vitro selection studies is somewhat lower than that supplied by the allelic exchange experiments performed to investigate *embB*406 and *embB*497 mutations. We cannot rule out a contribution of mutations outside of the *embCAB* operon that could have been coselected during growth in the presence of EMB. In fact, our observation that the five separate clones harboring the same *embB*445 Gln-to-Arg mutation had EMB MICs ranging from 2 to 8 μg/ml strongly suggests the coselection of additional mutations, along with the mutation of the *embB*445 allele.

Our study provides further support for the hypothesis that several mutations, one within the *embCAB* operon and one or more outside of the *embCAB* operon, may be required for high-level EMB resistance. Safi et al. previously demonstrated that *embB*306 mutations were necessary but not sufficient for high-level EMB resistance by performing allelic exchange studies with several clinical *M. tuberculosis* strains (19). In the current study, we show that *embB*497 and *embB*406 mutations also confer a relatively low level of EMB resistance when they are introduced as single mutations. This finding is in contrast to those of studies that have observed the same mutations in clinical strains with high-level EMB resistance (11, 14). Similarly, the *embB*397, *embB*1024, and *embC*13 mutations which were selected in vitro had very small elevations in EMB MICs, even though the same mutations are found in clinical *M. tuberculosis* isolates that have much higher EMB MICs. Together, these results demonstrate that EMB resistance is much more complex than was previously thought.

Our results suggest that phenotypic tests for EMB resistance may have trouble consistently detecting some *embB* mutations, despite their clear role in altering EMB susceptibility. The transfer of GCC, GAC, TGC, and TCC *embB*406 codons into wild-type *M. tuberculosis* increased the EMB MICs only from 2 to 6 or 7 μg/ml. Most clinical laboratories use breakpoints of 5.0 or 7.5 μg/ml to define EMB resistance. Thus, it is not surprising that many *embB*406 mutants have been identified as EMB susceptible in clinical studies. It is not possible for us to determine whether these small increases in the EMB MIC can adversely affect treatment outcomes by themselves. However, it is reasonable to assume, at the least, that these mutations will decrease the genetic barrier against high-level EMB resistance and, at the worst, that these mutations could render EMB ineffective at standard doses.

It has previously been shown that *embB*306 mutations confer on strains the ability to grow more vigorously than wild-type strains in the presence of sub-MICs of INH and RIF. Antibiotic synergy is also adversely affected. We did not observe the same phenomenon in the *embB*497 or *embB*406 mutants. The reasons for this difference are unclear. It is possible that *embB*306 codon changes affect cell wall permeability by altering the arabinogalactan or the lipoarabinomannan content or structure within the *M. tuberculosis* cell wall. This may not occur to the same extent in *embB*406 and *embB*497 mutants.

This study may have important implications for the design of genetic drug resistance tests and for the unraveling of the molecular mechanism of EMB resistance. More effective therapeutic and diagnostic measures for tuberculosis are desperately needed. An improved understanding of the basic mechanisms of *M. tuberculosis* drug resistance is central to the development of new molecular diagnostic assays and new drugs. Our study reveals the complexity of EMB resistance in *M. tuberculosis*. These results strongly suggest that *embB* mutations confer a low to moderate level of EMB resistance and that other mutations elsewhere in the chromosome are needed for full resistance. More clinical studies are needed to fully identify the range of EMB resistance-associated mutations. This should be complemented by whole-genome sequencing investigations to discover the unrecognized target genes for EMB resistance. In addition to providing improved diagnostic assays, this work is likely to also reveal new EMB drug targets, and it may identify new ways in which *M. tuberculosis* can be inhibited or killed by using novel classes of drugs.

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