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Transfer of *embB* Codon 306 Mutations into Clinical *Mycobacterium tuberculosis* Strains Alters Susceptibility to Ethambutol, Isoniazid, and Rifampin▽†

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Implicated as a major mechanism of ethambutol (EMB) resistance in clinical studies of *Mycobacterium tuberculosis*, mutations in codon 306 of the *embB* gene (*embB*306) have also been detected in EMB-susceptible clinical isolates. Other studies have found strong associations between *embB*306 mutations and multidrug resistance, but not EMB resistance. We performed allelic exchange studies in EMB-susceptible and EMB-resistant clinical *M. tuberculosis* isolates to identify the role of *embB*306 mutations in any type of drug resistance. Replacing wild-type *embB*306 ATG from EMB-susceptible clinical *M. tuberculosis* strain 210 with *embB*306 ATA, ATC, CTG, or GTG increased the EMB MIC from 2 μg/ml to 7, 7, 8.5, and 14 μg/ml, respectively. Replacing *embB*306 ATC or GTG from two high-level EMB-resistant clinical strains with wild-type ATG lowered EMB MICs from 20 μg/ml or 28 μg/ml, respectively, to 3 μg/ml. All parental and isogenic mutant strains had identical isoniazid (INH) and rifampin (RIF) MICs. However, *embB*306 CTG mutants had growth advantages compared to strain 210 at sub-MICs of INH or RIF in monocultures and at sub-MICs of INH in competition assays. CTG mutants were also more resistant to the additive or synergistic activities of INH, RIF, or EMB used in different combinations. These results demonstrate that *embB*306 mutations cause an increase in the EMB MIC, a variable degree of EMB resistance, and are necessary but not sufficient for high-level EMB resistance. The unusual growth property of *embB*306 mutants in other antibiotics suggests that they may be amplified during treatment in humans and that a single mutation may affect antibiotic susceptibility against multiple first-line antibiotics.

The emergence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* and extensively drug-resistant tuberculosis represents a major threat to global health (8, 42). An improved understanding of the molecular mechanisms that lead to drug resistance may help efforts to control this severe form of disease. Plasmid- or transposon-mediated drug resistance does not appear to occur naturally in *M. tuberculosis*. Instead, drug resistance develops in association with small deletions or point mutations in drug-activating genes, genes encoding drug targets, or in the promoter regions of drug targets (24, 28). A few such mutations have been conclusively proven to cause drug resistance (39, 41). Most forms of drug resistance are believed to occur in a single step as a consequence of a single mutation, although some isoniazid (INH)-resistant clinical strains contain two mutations that could each independently confer INH resistance (20). Until the current study, no single mutation had been shown to change susceptibility to more than one type of antibiotic at a time.

Ethambutol (EMB) is a first-line antituberculosis agent that is especially important when used in drug combinations to prevent the emergence of drug resistance or to treat single-drug-resistant tuberculosis. Accurate assessment of EMB susceptibility is critical when EMB is used for this purpose. A number of biochemical and genetic studies have suggested that the *embCAB* operon is involved in EMB resistance. EMB appears to inhibit arabinosyl transferases encoded by the *embCAB* operon, which are involved in polymerizing arabinose into the arabinan components of arabinogalactan and lipoarabinomannan (3, 11, 13, 35, 40). Mutations at codon 306 of the *M. tuberculosis embB* gene (*embB*306) are found in 30 to 68% of all EMB-resistant clinical isolates (1, 22, 34). This association is so strong that *embB*306 mutations have been proposed as both a cause of EMB resistance and a marker for EMB resistance in diagnostic assays (10, 27). However, *embB*306 mutations have recently been identified in clinical *M. tuberculosis* isolates that are susceptible to EMB (1, 12, 15). One large study on an international collection of isolates found that 46% of *embB*306 mutants were fully susceptible to EMB and, like other studies, reported a lack of these mutations in pan-susceptible strains. Instead, mutants in *embB*306 were found to be strongly associated with resistance to any antibiotic and to MDR strains, suggesting that *embB*306 mutations were responsible for broad antibiotic resistance rather than simply for EMB resistance (9). Other studies have supported the association between EMB resistance and INH resistance (19) or *embB*306 mutations and MDR (31, 32).

We performed allelic exchange studies to unequivocally define the causative role for *embB*306 mutations in EMB resistance and to investigate the intriguing association between these mutations and MDR tuberculosis even in EMB-suscep-

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tible isolates. Our results demonstrate that *embB*306 mutations play a complex but important role in EMB resistance. We also demonstrate that *embB*306 mutations reduce susceptibility to drugs other than EMB and potentially to drugs used in combination. This study reveals the complexity of drug resistance evolution and suggests a potentially important role for *embB*306 mutations in the development of MDR tuberculosis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Mycobacterium tuberculosis* strains (see Tables 1 and 2, below) were cultured at 37°C either in Middlebrook 7H9 broth containing 0.05% (wt/vol) Tween 80 or on Middlebrook 7H10 agar supplemented with 0.5% (vol/vol) glycerol, both enriched with 10% oleic acid-albumin-dextrose-catalase (OADC; Becton Dickinson).

DNA isolation, PCR, and DNA sequencing. Genomic DNA was extracted as described previously (38). To amplify DNA fragments for *M. tuberculosis* transformation or DNA sequencing, the PCR was performed using a mix containing 1 ng of genomic DNA, 5 pmol of each primer, 200 μM deoxynucleoside triphosphates, 1× PCR buffer, and 1 U of high-fidelity *Pfx Taq* polymerase (Invitrogen). All PCR products were purified using a gel extraction kit (Qiagen). The direct bidirectional sequencing of the *embB* gene and PCR products used for the cloning experiments was performed with a Dye Terminator kit and analyzed with an ABI 3100 genetic analyzer (Applied Biosystems).

Isogenic strain construction. A 2,211-bp fragment spanning the *embB*306 point mutations was amplified by PCR using the F1-*embB*306CL and R1-*embB*306CL primers (see Table S1 in the supplemental material). The fragment carrying either the *embB*306 ATG, *embB*306 ATC, *embB*306 CTG, or *embB*306 GTG codon was amplified using genomic DNA extracted from the *M. tuberculosis* strain 210 (see Table 1, below) or the *M. tuberculosis* strains 30167, 5041, or 5310, respectively (see Table 2, below). The fragments were purified and digested with HindIII and Acc65I (both from Promega). The 2,211-bp fragment harboring the *embB*306 ATA codon was obtained by PCR-based site-directed mutagenesis (26). The first DNA fragment was amplified using F2-*embB*306CL and R1-*embB*306CL primers (see Table S1 in the supplemental material), purified, and digested with BamHI and Acc65I (both from Promega). To introduce the single-point mutation *embB*306 ATA, the second DNA fragment was amplified by two sequential PCRs. First, a DNA fragment was amplified from wild-type strain 210 genomic DNA using the F1-*embB*306CL primer and the mutagenic primer R1-*embB*306ATA (see Table S1 in the supplemental material). The product of this first PCR was purified and used as a template for the second PCR using F1-*embB*306CL and the second mutagenic primer, R2-*embB*306ATA (see Table S1 in the supplemental material). The final PCR product was purified and digested with BamHI and HindIII. Except for the *embB*306 ATA point mutation, where the two DNA fragments were ligated simultaneously, the 2,211-bp fragments were cloned into the p2NIL vector, followed by the insertion of a PacI cassette containing the *sacB* and *lacZ* genes (see Table S1 in the supplemental material) (18). The sequences of all cloned 2-kb fragments were confirmed by direct DNA sequencing. The recombinant plasmids were transformed in *M. tuberculosis* strains, as described previously (29). Blue colonies containing single-crossover events were plated on medium containing 2% (wt/vol) sucrose and 50 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and incubated for 4 weeks. The mutant white colonies obtained by double-crossover events were screened using a standardized *embB*306 molecular beacon assay (see Table S1 in the supplemental material) (21) and were confirmed by sequencing. These screening methods were also used to determine the homogeneity of the constructed clone.

In vitro selection of EMB-resistant clones. *M. tuberculosis* strain 210 was grown in 7H9 medium to an optical density at 600 nm of 1.5. The bacterial cells were washed with fresh 7H9 medium and concentrated by centrifugation. Series of plates of 7H10 medium containing 4, 8, 16, and 32 μg/ml EMB were inoculated with 10^9 cells and incubated for 4 weeks. Colonies in each plate were screened for *embB*306 mutations by the molecular beacon assay, and the MICs of the *embB*306 mutants were determined as described below.

MIC determination and agar susceptibility testing. The MICs of all strains were determined using the standard radiometric Bactec 460TB method (Becton Dickinson and Company, Sparks, MD), as recommended by the manufacturer. Briefly, each strain was grown in 7H9 medium to approximately 2 McFarland standard turbidity units. The cultures were then diluted to approximately 2×10^5 CFU per starting inoculum, and vials of 12B medium containing different concentrations of antibiotics were inoculated. Antibiotic-free 12B media inoculated with the primary inoculum or a 1:100 dilution were used as controls. The 12B vials were incubated at 37°C for 10 to 11 days. The method was standardized so that the 1:100 control reached a growth index of 30 after 6 to 8 days of incubation. Each strain was initially tested against serial twofold increases in antibiotic concentration. Once the approximate MIC was identified, more precise MIC determinations were then achieved by repeat testing in small concentration increments. The first antibiotic concentration that inhibited growth compared to growth of the 1:100 dilution defined the MIC (33).

To confirm the Bactec results, the same inoculum that was used for the Bactec vial inoculation was also inoculated onto plates of 7H10 medium containing serial concentrations of antibiotics (17). Antibiotic-free 7H10 plates were used to confirm inoculum CFU. The plates were incubated at 37°C for 3 to 4 weeks, and MICs were determined by the antibiotic concentration that inhibited growth to <1% of growth on antibiotic-free 7H10 medium (30).

Competition assays. *M. tuberculosis* strains 210 and NJT210CTG were cultured under identical conditions to reach an optical density at 600 nm of 0.6 on the same day. The cultures were diluted 250 times, and 100-μl aliquots of the dilutions (approximately 2×10^5 CFU) were mixed and inoculated into 7H9 medium containing 0.0075% Tween 80 (this low concentration of Tween was used to diminish interference with antibiotic activity while retaining an anti-clumping effect) and 10% OADC, supplemented with sub-MICs of INH at 0.04 or 0.02 μg/ml. The inocula were also individually plated on 7H10 medium to confirm that equal cell ratios of strains 210 and NJT210CTG had been mixed. The cultures were incubated at 37°C under gentle shaking until visible growth was observed. The optical density was measured at 600 nm, and serial dilutions were plated on 7H10 medium. Fifty or more colonies were picked from each plate and tested for the presence of either wild-type *embB*306 ATG or mutant *embB*306 CTG by standardized molecular beacon assays. Colonies positive for each molecular beacon were recorded, and the ratio of strain 210 to NJ210CTG was determined. Mixed colonies were discarded.

Measuring activities of antibiotic combinations. Strain 210 or NJT210CTG was inoculated into vials of 12B medium in a checkerboard concentration pattern of either INH plus EMB, INH plus rifampin (RIF), or RIF plus EMB as described elsewhere (4, 7). The growth index of each culture was compared to the 1:100 control to identify the MICs for each antibiotic combination for each strain. A fractional inhibitory concentration (FIC) index for each combination was then derived using the following formula: FIC = [(concentration of drug number 1 at the drug combination MIC)/(MIC of drug 1)] + [(concentration of drug 2 at the drug combination MIC)/(MIC of drug 2)], as described previously (4, 14). FICs of ≤0.5 defined synergistic effects; FICs of >0.5 to 0.75 defined partial effects; FICs of >0.75 to 1 defined additive effects; FICs of >1 to 4 defined indifferent effects, as described elsewhere (14).

Statistical analysis. All data were analyzed using a nonrepeated measures analysis of variance or Student's *t* test, as appropriate. A P value of <0.05 was considered significant. GraphPad Prism software version 4 was used to perform the analysis of variance, and Microsoft Excel was used to perform Student's *t* test.

RESULTS

Transfer of mutant *embB*306 alleles into wild-type drug-susceptible *M. tuberculosis*. We performed a series of allelic exchange experiments to unequivocally determine the relationship between prevalent *embB*306 alleles and EMB resistance. Although not previously used for this purpose, the *sacB*-based counterselection method described by Parish et al. (18) enabled us to cleanly introduce point mutations into *embB* without an accompanying selectable marker. Mutations were introduced into the clinical *M. tuberculosis* 210 strain, a drug-susceptible member of the W-Beijing strain family whose genome has been partially sequenced (5). The sequence of the 210 strain *embB* gene is identical to that of the laboratory strain H37Rv (http://www.tigr.org/and http://www.ncbi.nlm.nih.gov/GenBank/index.html). Exchanging the wild-type 210 strain *embB*306 ATG (Met) codon for either *embB*306 ATA (Ile), *embB*306 ATC (Ile), *embB*306 CTG (Leu), or *embB*306 GTG (Val) codons, we created the isogenic mutants NJT210ATA, NJT210ATC, NJT210CTG, and NJT210GTG, respectively. The introduction of each point mutation was confirmed by DNA sequencing. The

TABLE 1. MICs and the *embB* genotypes of *M. tuberculosis* strain 210 and its isogenic mutants

M. tuberculosis strain	MIC (μg/ml)			*embB*306 genotype[a]
	EMB	INH	RIF	
210	2	0.05	0.05	ATG
NJT210ATA	7	0.05	0.05	ATA
NJT210ATC	7	0.05	0.05	ATC
NJT210CTG	8.5	0.05	0.05	CTG
NJT210GTG	14	0.05	0.05	GTG
NJT210ATC-ATG	2	0.05	0.05	ATG
NJT210CTG-ATG	2	0.05	0.05	ATG
NJT210EMB-R-C1[b]	14	0.05	0.05	GTG
NJT210EMB-R-C2[b]	14	0.05	0.05	GTG
NJT210EMB-R-C3[b]	8	0.05	0.05	ATA

[a] The remaining sequence of the *embB* gene is identical in all strains.
[b] EMB-resistant strain obtained by in vitro selection.

TABLE 2. MICs and *embB* genotypes of clinical *M. tuberculosis* isolates and isogenic strains

M. tuberculosis strain	MIC (μg/ml)			*embB*306 genotype[a]
	EMB	INH	RIF	
5310	28	2.5	>240	GTG
NJT5310ATG	3	2.5	>240	ATG
NJT5310ATG-ATA	16	2.5	>240	ATA
NJT5310ATG-ATC	16	2.5	>240	ATC
NJT5310ATG-CTG	20	2.5	>240	CTG
NJT5310ATG-GTG	28	2.5	>240	GTG
30167	20	0.07	>240	ATC
NJT30167ATG	3	0.07	>240	ATG
5041	16[b]	ND[c]	ND	CTG
A2558	5	ND	ND	ATC

[a] The remaining sequence of the *embB* gene is identical in all strains.
[b] The MIC is between 8 and 16 μg/ml.
[c] ND, not determined.

entire *embB* gene of each strain was also sequenced to confirm that no other mutation had been indivertibly transferred.

We measured EMB MICs for the wild type and each isogenic mutant using the Bactec 460TB method (Table 1). This method is considered one of the most accurate ways to measure EMB MICs (17, 30, 36). Small (0.5- to 1.0-μg/ml) increments of increasing EMB concentration were used to precisely measure the MIC of each strain. Our results demonstrated that transfer of *embB*306 mutant alleles produced moderate increases in the EMB MIC. While the wild-type 210 strain had an EMB MIC of 2 μg/ml, both NJT210ATA and NJT210ATC had MICs of 7 μg/ml, NJT210CTG had an MIC of 8.5 μg/ml, and NJT210GTG had an MIC of 14 μg/ml (Table 1). Each MIC was confirmed by the agar proportion method of susceptibility testing (data not shown). To confirm the relationship of codon usage to MIC, we repeated EMB MIC measurements of other strain 210 isogenic clones that also contained GTG, ATC, and ATA mutations. We found that each EMB MIC remained consistent within each mutant allele type. It was possible that EMB resistance was accidentally introduced during the allelic exchange at another location within the *M. tuberculosis* genome. We reintroduced the wild-type *embB*306 ATG codon into both NJT210CTG and NJT210ATC, creating NJT210CTG-ATG and NJT210ATC-ATG, to confirm that this had not occurred. The reintroduction of the wild-type *embB*306 sequence into both NJT210 mutants caused the EMB MIC to revert to 2 μg/ml in both cases (Table 1). DNA sequencing of the new strains confirmed that the entire *embB* gene had been reconverted to wild type. These results conclusively show that *embB*306 mutations cause an increase in the EMB MIC. However, the MICs of NJT210ATA, -ATC, and -CTG were all sufficiently close to standard breakpoints for EMB resistance, which vary from 5 to 7.5 μg/ml (17), that they might easily be identified as EMB susceptible by clinical laboratories.

Restoring the wild-type sequence to highly EMB-resistant clinical isolates. Previous studies found *embB*306 mutations in clinical *M. tuberculosis* isolates that had high-level EMB resistance (MICs, 16 to 40 μg/ml) (25). This degree of EMB resistance is well above the resistance engendered by our introduction of *embB*306 mutations into wild-type strain 210. We transferred wild-type *embB*306 ATG codons into two *embB*306 mutant and highly EMB-resistant clinical *M. tuberculosis* strains to study the role of *embB*306 mutations in high-level EMB resistance. Two parental strains were used in these experiments: the RIF- and EMB-resistant strain 30167, which had an *embB*306 ATG-to-ATC mutation and an EMB MIC of 20 μg/ml, and the INH-, RIF-, and EMB-resistant strain 5310, which had an *embB*306 ATG-to-GTG mutation and an EMB MIC of 28 μg/ml (Table 2). DNA sequencing of the whole *embCAB* operon, which included the regulatory region of *embAB* (2), showed wild-type *embAB* sequences in both strains 30167 and 5310 except at *embB*306 (Table 2). The promoter region of *embAB* in these strains was also identical to the published sequence of H37Rv and the partially sequenced 210 genome. Both strains were noted to have a C-to-T polymorphism compared to H37Rv at position 2781 of *embC*. However, this *embC* polymorphism was also present in the fully EMB-susceptible *M. tuberculosis* strain CDC1551.

Converting the mutant *embB*306 alleles in 30167 and 5310 to the wild-type ATG codon created strains NJT30167ATG and NJT5310ATG, respectively. Interestingly, we found that the EMB MICs of both NJT30167ATG and NJT5310ATG were reduced to the level of 3 μg/ml. To further support those observations, we restored the *embB*306 GTG codon into NJT5310ATG, creating NJT5310ATG-GTG. The reintroduction of this mutant allele also restored the EMB MIC back to 28 μg/ml. These results suggest that the *embB*306 ATG-to-ATC and ATG-to-GTG mutations are necessary for high-level EMB resistance, even if these mutations are not sufficient to cause high-level resistance on their own.

To confirm the relationship between parental strain type, *embB*306 allele, and EMB MIC, we transferred other *embB*306 mutant codons back into NJT5310ATG (Table 2). By introducing either *embB*306 CTG, ATC, or ATA alleles, we created the new strains NJT5310ATG-CTG, NJT5310ATG-ATC, and NJT5310ATG-ATA, respectively. While NJT5310ATG-GTG had an EMB MIC of 28 μg/ml, NJT5310ATG-CTG had an MIC of 20 μg/ml, and both NJT5310ATG-ATC and NJT5310ATG-ATA had MICs of 16 μg/ml. These results parallel at a higher level the increased MICs conferred by each mutant allele in strain 210 (i.e., EMB MIC of *embB*306 GTG > CTG > ATC and ATA). Together, these results show a strong association between specific *embB* alleles and specific MICs,

but with a variability that depends on the background of the clinical strain.

In vitro selection of *embB*306 mutants. Previous studies had failed to identify *embB*306 mutants after in vitro selection for EMB resistance (9). Repeating these studies, we cultured approximately 10^9 CFU of wild-type strain 210 on agar medium containing a variety of EMB concentrations. Mutants at *embB*306 were detected in 0 of 155 colonies selected at 4 μg/ml, 1 of 7 colonies selected at 8 μg/ml, 2 of 11 colonies selected at 16 μg/ml, and 0 of 5 colonies selected at 32 μg/ml. The one mutant identified at 8 μg/ml was found to have an *embB*306 ATG-to-ATA mutation and an EMB MIC of 8 μg/ml. The two mutants identified at 16 μg/ml were both found to have *embB*306 ATG-to-GTG mutations and EMB MICs of 14 μg/ml (Table 1). The low frequency with which the *embB*306 mutants were recovered and the fact that both of the GTG mutants could only be cultured at EMB concentrations below the 16 μg/ml of EMB used for their isolation may explain why *embB*306 mutants have not previously been identified following in vitro selection.

Enhanced in vitro growth of *embB*306 mutants in the presence of INH and RIF. The previously identified association between clinical *embB*306 mutants and resistance to drugs other than EMB raised the intriguing possibility that *embB*306 mutations might confer a form of resistance to these other antibiotics. All of the isogenic NJT210 *embB*306 mutants had MICs for INH and RIF that were identical to the parental strain 210 (0.05 μg/ml for INH and 0.05 μg/ml for RIF) (Table 1). However, the *embB*306 mutants showed a growth advantage compared to the control strains when cultured with several sub-MICs of INH or RIF. Figure 1 compares the growth rate of strain 210 and isogenic mutant NJT210CTG by using daily growth index measurements in Bactec cultures. Under these conditions, NJT210CTG grew well in 0.03 μg/ml and 0.04 μg/ml of INH and 0.04 μg/ml of RIF ($P < 0.001$) (Fig. 1). However, the parental control was mostly inhibited at these concentrations. Growth was also detectible in the CTG mutant at 0.05 μg/ml of RIF, a concentration that completely inhibited the parental control (these results did not change our decision to call the MIC for NJT210CTG at 0.05 μg/ml of RIF because of the requirement for >1% growth). The growth advantage appeared to be directly attributable to the *embB* CTG allele, because conversion back to the ATG allele (in strain NJT210CTG-ATG) eliminated any growth difference compared to the control (data not shown). To confirm this observation, we repeated the INH sub-MIC growth studies, this time comparing the EMB- and RIF-resistant 30167 clinical strain to the isogenic EMB-susceptible and RIF-resistant NJT30167ATG strain (created by transfer of the "wild-type" *embB*306 ATG codon into 30167). Figure 2 demonstrates that transfer of the ATG codon resulted in substantially poorer growth in sub-MICs of INH ($P < 0.001$) (Fig. 2). Thus, in both strain 210 and strain 30167, the sequence of the *embB*306 allele directly correlated with growth rates at sub-MICs of an antibiotic other than EMB.

We performed competition assays to determine whether *embB*306 mutants would be positively selected in mixed cultures to further investigate the growth differences at sub-MICs of INH. NJT210CTG was mixed at equal cell ratios with strain 210, cultured in liquid medium containing sub-MICs of INH,

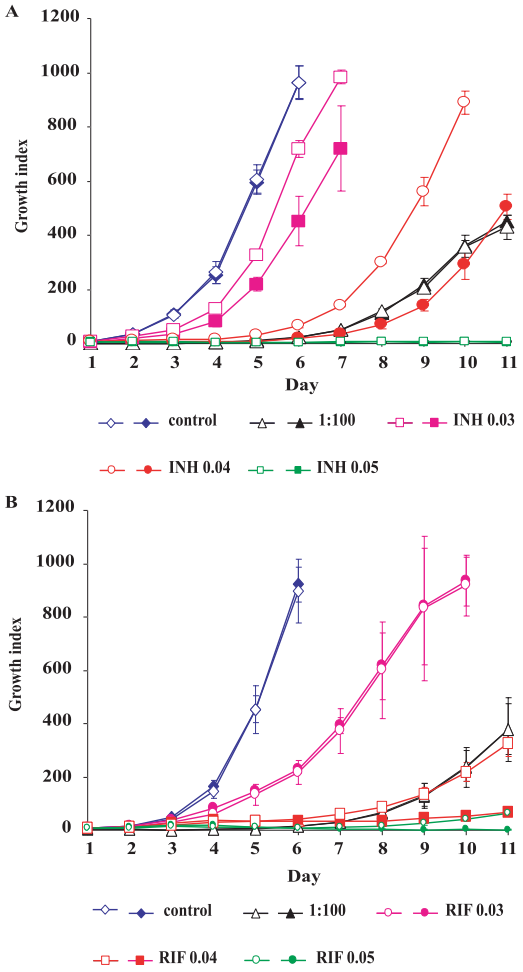

FIG. 1. Differential growth rates of the isogenic mutant NJT210CTG and *M. tuberculosis* strain 210. Strains 210 (closed symbols) and NJT210CTG (open symbols) were cultured in different sub-MICs of INH (A) and RIF (B) (concentrations are shown below the graphs and are in micrograms per milliliter). The daily growth index for each culture is shown. The 1:100 data represent results for no-antibiotic control cultures inoculated after a 1:100 dilution in the medium. The means and standard deviations of three independent experiments are shown.

and then plated on solid medium lacking antibiotics. Figure 3 shows that the CTG mutant outcompeted the wild-type 210 strain when cultured in the presence of two sub-MICs of INH ($P < 0.02$) (Fig. 3). Positive selection for NJT210CTG failed to occur in the absence of INH, confirming that NJT210CTG lacked an intrinsic growth advantage. These results strongly suggest that *embB*306 mutations provide a slight, though significant, decrease in INH and RIF susceptibility that is not detectible using standard measurements of MICs. This sub-MIC resistance may contribute to the association between *embB*306 mutants and MDR tuberculosis noted in clinical studies.

Limited activities of drug combinations. We studied the relative activity of drug combinations to further explore the relationship between *embB*306 mutations and the emergence of MDR tuberculosis (Table 3). The strain 210 control or NJT210CTG was cultured with various combinations of INH, RIF, and EMB, and the MIC of each drug combination was

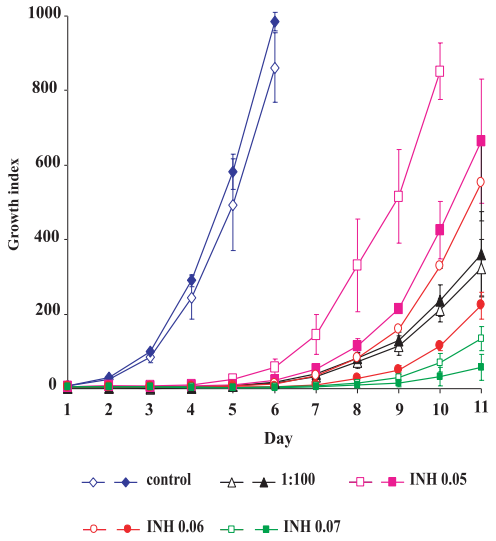

FIG. 2. Differential growth rates of *M. tuberculosis* strains 30167 and NJT30167ATG. Strains 30167 (open symbols) and NJT30167ATG (closed symbols) were cultured in different sub-MICs of INH (concentrations are shown below the graph and are in micrograms per milliliter). The daily growth index for each culture is shown. The 1:100 data represent results for no-antibiotic control cultures inoculated after a 1:100 dilution in the medium. In both Fig. 1 and 2, the strains with the *embB*306 ATG allele show repressed growth at sub-MICs. The means and standard deviations of three independent experiments are shown.

determined. An FIC was calculated using both the MICs for the individual antibiotics and the antibiotic combinations, as described previously (14). In the 210 strain, INH combined with either EMB or RIF had additive effects, as the lowest FIC was 0.85 and 0.9, respectively (with synergy, additive effects and indifference were defined as FICs of <0.75, 0.75 to 1.0, and 1 to 4, respectively). RIF combined with EMB had a synergistic effect, as the lowest FIC was 0.45. INH plus RIF plus EMB also had a synergistic effect in strain 210, as the lowest FIC of the three-drug combination was 0.425. Surprisingly, the same concentrations of RIF, INH, and/or EMB did not inhibit the growth of NJT210CTG (Table 3). We noted that the additive or synergistic effects of these two-drug combinations could only be observed in NJT210CTG when substantially higher concentrations of EMB were used. Furthermore, the lowest FIC of the RIF plus EMB combination was increased from 0.45 in strain 210 to 0.65 in NJT210CTG. Higher EMB concentrations were also required in the three-drug combinations, and the observed synergy was again diminished (the lowest three-drug FIC for strain 210 was 0.425, while the lowest three-drug FIC for NJT210CTG was 0.55). Interestingly, the FIC for INH plus RIF was increased from 0.9 in strain 210 to 1.1 in NJT210CTG, even though this drug combination did not include EMB. Taken together, these results provide additional evidence for an effect on antibiotic susceptibility in *embB*306 mutants that extends beyond simple resistance to EMB. The diminished effectiveness of antibiotic combinations may be particularly relevant to clinical scenarios where multidrug treatment is the rule.

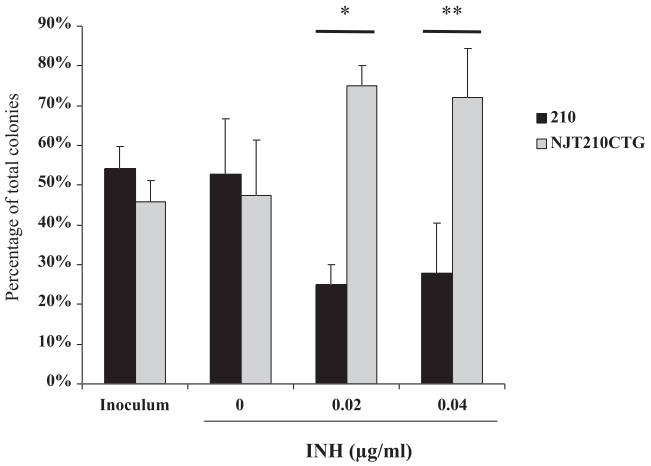

FIG. 3. Competition assays at sub-MICs of INH. Equal numbers of strains 210 and NJT210 CTG were cultured with no antibiotics or in different sub-MICs of INH, as shown. Significant differences in growth are indicated as follows: *, $P < 0.01$; **, $P = 0.01$. The means and standard deviations of three independent experiments are shown.

TABLE 3. Effects of combined antibiotic treatment on growth of strains 210 and NJT210CTG

Combination	Concn of each (μg/ml)	Results for strain 210		Results for NJT210CTG	
		Growth	FIC index	Growth	FIC index
INH/EMB	0.02/1	−	0.9	+	NA[a]
	0.03/0.5	−	0.85	+	NA
	0.0375/0.25	−	0.87	+	NA
	0.02/4.25	−	NA	−	0.9
	0.03/2.125	−	NA	−	0.85
	0.0375/1.0625	−	1.28	−	0.87
RIF/EMB	0.02/0.25	−	0.52	+	NA
	0.01/0.5	−	0.45	+	NA
	0.01/1	−	0.7	+	NA
	0.03/1.0625	−	1.13	−	0.72
	0.02/2.215	−	NA	−	0.65
	0.01/4.25	−	NA	−	0.7
INH/RIF	0.025/0.02	−	0.9	+	NA
	0.0125/0.04	−	1.05	+	NA
	0.025/0.03	−	1.1	−	1.1
	0.0125/0.05	−	1.25	−	1.25
RIF/EMB/INH	0.0025/0.25/0.02	−	0.575	+	NA
	0.005/0.25/0.01	−	0.425	+	NA
	0.0025/0.5/0.02	−	0.7	+	NA
	0.005/0.5/0.01	−	0.55	+	NA
	0.0025/1.0625/0.03	−	1.13	−	0.775
	0.005/1.0625/0.02	−	1.03	−	0.625
	0.0025/2.125/0.02	−	NA	−	0.7
	0.005/2.125/0.01	−	NA	−	0.55

[a] NA, not applicable.

DISCUSSION

This study reveals the potential complexity of drug resistance evolution in *M. tuberculosis*. All of the *embB*306 mutations that we selected in vitro or introduced into wild-type strain 210 caused an increase in the EMB MIC. Depending on the breakpoints used, the ATA and ATC mutants would likely appear to be either EMB susceptible or EMB resistant in clinical laboratories, while the CTG and GTG mutants would likely be more consistently identified as low-level EMB resis-

tant. Mutations at *embB*306 also appeared to be necessary, but not sufficient, to produce high-level EMB resistance in the clinical strains that we tested. Thus, *embB*306 mutations must contribute indirectly to high-level EMB resistance, possibly by interacting with mutations in other genes. The requirement for more than one mutation to produce high-level EMB resistance suggests that this type of resistance evolves through multiple steps. Mutations in *embB*306 are likely to occur early in this process, as they confer only a small increase in the MIC. The effects of *embB*306 mutations also appeared to extend beyond simple EMB resistance. We found that *embB*306 mutations provided a growth advantage to *M. tuberculosis* cultured in sub-MICs of INH and RIF. Furthermore, these mutations decreased susceptibility to additive and synergistic effects of multidrug combinations, even when these combinations did not contain EMB. The discovery that a single mutation can affect susceptibility to multiple antibiotics is a completely novel finding in *M. tuberculosis*. Taken together, these results suggest a new importance to *embB*306 mutations in drug resistance evolution.

This investigation resolves a conflict between prior clinical studies concerning the association between *embB*306 mutations and EMB resistance. Most clinical laboratories use breakpoints of 5.0 or 7.5 μg/ml to define EMB resistance. Therefore, it is easy to see how *embB*306 mutants could appear to be EMB susceptible, since transfer of ATC, ATA, and CTG *embB*306 codons into strain 210 only increased EMB MICs to 7 or 8.5 μg/ml. Furthermore, the effects of *embB*306 mutations may vary with the intrinsic EMB susceptibility of the wild-type parental strain (16). Thus, the clinical *M. tuberculosis* strain A2558 harbors an *embB*306ATC mutation despite having an EMB MIC as low as 5 μg/ml (Table 2). Our results suggest that we should revise conclusions made by some of us previously, based on studies of clinical *M. tuberculosis* isolates, that *embB*306 mutations do not cause EMB resistance (9). Instead, we suggest that *embB*306 mutations increase EMB MICs in clinical *M. tuberculosis* isolates and cause overt EMB resistance in a subset of these isolates. An important implication of these conclusions is that *embB*306 mutants are likely to have EMB MICs close to the breakpoints that define EMB resistance, even if they appear to be EMB susceptible according to standard susceptibility tests. It follows that strong consideration should be given to treating all patients infected with *embB*306 mutants as if their disease were fully EMB resistant. This point is most relevant in patients who are already infected with *M. tuberculosis* strains that are resistant to other antituberculosis drugs. An alternative possibility is that EMB breakpoints might be lowered to ensure that all *embB*306 mutants are categorized into the resistant group. However, both of these suggestions require clinical confirmation.

Clinical studies have also revealed strong associations between high-level EMB resistance and *embB*306 mutations. Our somewhat surprising discovery that two highly EMB-resistant clinical isolates became EMB susceptible after receiving a transfer of the wild-type *embB*306 codon provides one explanation for this clinical association. It is apparent that the *embB*306 mutations cannot be solely responsible for the high-level EMB-resistant phenotype in these clinical strains. However, the requirement for *embB*306 mutations for full expression of high-level EMB resistance would tightly link *embB*306 mutations to high-level EMB resistance in clinical association studies.

Several clinical studies have found a strong association between *embB*306 mutations and resistance to INH or RIF or MDR (9, 31, 32), and others have described similar findings (6). Our results suggest a biological explanation for this clinical association. Mutations in *embB*306 appear to have a small effect on the activity of INH and RIF. These results were confirmed for INH in competition studies. The competition studies may also shed light on bacterial growth and selection in human disease. They suggest that *embB*306 mutants may have a selective advantage when humans are treated with antibiotics. EMB mono-resistance is rare in clinical tuberculosis, and *embB*306 mutations have not been detected in pan-susceptible *M. tuberculosis*. Thus, it is unlikely that *embB*306 mutations play a major role in the evolution of INH resistance in the clinical context. However, INH-resistant isolates that are also *embB*306 mutants (whether phenotypically EMB resistant or EMB susceptible) do occur with relatively high frequency (9). These mono-resistant mutants may be predisposed to develop RIF resistance or to acquire resistance to second-line drugs. Therefore, our observations may explain the strong association observed between *embB*306 mutations and RIF resistance and MDR in clinical studies (6, 9, 15, 23, 31, 32, 37).

Mutations in *embB*306 also appear to inhibit the additive or synergistic activities of antituberculosis drugs used in combination. Although the effect of *embB*306 mutations on susceptibility to a single drug other than EMB may be small, this effect may be compounded in the treatment of a disease such as tuberculosis, where therapy with multiple drugs is required to prevent the emergence of drug resistance. Many repeated treatments over many months also serve to amplify a small effect. We can only speculate on the mechanism underlying these observations, such as the possibility that *embB*306 mutations cause subtle changes in cell wall permeability.

Our study is also significant in that it is the first to use unmarked transfer of point mutations into clinical *M. tuberculosis* strains to definitively identify a drug resistance target. The only other study of this type in *M. tuberculosis* used a phage-based method of allelic exchange that also required insertion of a hygromycin resistance cassette into the *M. tuberculosis* genome (39). While this prior work was a landmark in definitively identifying the drug target for INH, the introduction of a new antibiotic resistance cassette may have unpredictable effects on the more subtle forms of drug resistance examined in our results. Our study demonstrates the ability of genetic tools to precisely define the link between specific mutations and drug resistance in *M. tuberculosis*. It is well known that a mechanism other than *embB*306 mutations, such as mutations elsewhere in the *embCAB* operon or in other genes, may be involved in EMB resistance (25). We suggest that allelic exchange techniques such as the ones described here should be used to study these associations.

In conclusion, our results suggest that *embB*306 mutants have substantial clinical relevance. These mutations appear to identify *M. tuberculosis* that is predisposed to developing high-level EMB resistance. The clinical implication is that all *embB*306 mutants should be treated as strains with altered EMB susceptibility, even if they appear to be EMB susceptible on conventional testing. Our results also suggest that *embB*306

mutations may provide *M. tuberculosis* an advantage with a propensity to develop INH resistance or RIF resistance and a diminished susceptibility to potentially potent effects of antibiotic combinations. These properties suggest that human tuberculosis caused by *embB*306 mutants may be more likely to evolve into MDR and extensively drug-resistant strains during treatment. Our results suggest that all clinical *M. tuberculosis* isolates with any form of drug resistance should be tested for *embB*306 mutations. Patients infected with *embB*306 mutants may not receive the expected benefits of EMB treatment and should be carefully monitored for treatment failure and the possible emergence of MDR.

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