

Phenotypic and Molecular Characterization of *Mycobacterium tuberculosis* Isolates Resistant to both Isoniazid and Ethambutol

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In performing radiometric susceptibility testing on over 2,000 patient isolates of *Mycobacterium tuberculosis* during the past 6 years, we found that resistance to 7.5 µg/ml ethambutol (EMB) occurred only in isolates that are also resistant to 0.4 µg/ml isoniazid (INH). Using 157 selected isolates in the present study, we performed radiometric and agar proportion susceptibility tests and DNA sequencing of genetic regions associated with resistance to these two drugs. The goal was to study the occurrence of the common mutations associated with resistance to each drug and also to determine whether any particular INH-resistance-associated mutation occurred more often in combination with any particular EMB-resistance-associated mutation. In an analysis of 128 isolates resistant to 0.4 µg/ml INH, we found that a mutation at *katG* Ser315 was more common in isolates also resistant to 7.5 µg/ml EMB (61 of 67 = 91.0%) than in isolates either susceptible to EMB or resistant to 2.5 µg/ml EMB (39 of 60 = 65.0%). These observations suggest that INH-resistant strains with a mutation at *katG* Ser315 are more likely to acquire resistance to 7.5 µg/ml EMB than are isolates with INH-resistance-associated mutations at other sites. In addition, we found that 64 of 67 (95.5%) isolates resistant to 7.5 µg/ml EMB contained a mutation in either codon 306 or codon 406 of *embB*. Met306Val was the most common *embB* mutation, present in 52 (77.6%) of the 67 isolates. Most occurrences of this mutation (49 of 52 = 94.2%) were found in isolates that also contained the *katG* Ser315Thr mutation. Finally, sequencing this region of *embB* appears to be sufficiently sensitive for use as a rapid screening tool for detection of high-level resistance to EMB.

At present, the two greatest threats to global tuberculosis (TB) control are the association of this disease with the HIV epidemic and the increase in resistance to the most effective anti-TB drugs. Of greatest concern is multidrug resistance (MDR), defined as resistance to isoniazid (INH) and rifampin (RIF), the most effective anti-TB drugs. The most successful strategy to limit the spread of MDR-TB is rapid detection of drug resistance, followed by prompt, effective, and complete therapy. Routine laboratory diagnosis of drug resistance in TB requires a viable, pure culture of *Mycobacterium tuberculosis*. Use of liquid media has decreased the turnaround time for susceptibility test results; however, because of the slow growth of *M. tuberculosis* and other members of the TB complex, these assays can still take 10 to 14 days. Alternatively, an increased understanding of the molecular basis of resistance to the anti-TB drugs may (i) greatly contribute to further decreasing the turnaround time, (ii) lead to more appropriate and timely

treatment regimens, and (iii) ultimately prevent the transmission of drug-resistant TB.

During the past 10 years, significant progress has been made in identifying the biochemical targets for each of the most effective anti-TB drugs: INH, RIF, ethambutol (EMB), pyrazinamide (PZA), and streptomycin (STR) (reviewed in references 34, 40, and 43). In *M. tuberculosis*, drug resistance develops when random chromosomal mutations occur in a gene encoding a drug target or a drug-activating enzyme, and MDR-TB results from step-wise accumulation of individual mutations. Although much progress has been made, the variable intragenic locations of the mutations have presented challenges to the detection of multidrug resistance by molecular methods. Furthermore, resistance to certain drugs can involve a mutation in one of several possible genes or gene complexes. These problems can be significant in any effort to develop and validate rapid and accurate methods for detection of resistance to multiple drugs.

In the Clinical Mycobacteriology Laboratory of the Wadsworth Center, New York state's public health laboratory, resistance to the most effective anti-TB drugs as determined by the radiometric susceptibility assay is routinely confirmed by the agar proportion method and by DNA sequencing to detect the mutations most commonly associated with resistance to RIF, INH, EMB, and PZA in the population served by this

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laboratory. During the past several years, in isolates with resistance to more than one drug, we have found associations among resistances to the various drugs. The most striking observation is that in the radiometric susceptibility assay, resistance to the high concentration of EMB, 7.5 µg/ml (Hi-EMB), occurs only in isolates that are also resistant to the high concentration of INH, 0.4 µg/ml (Hi-INH). Furthermore, monoresistance to Hi-INH is often seen, but we have never identified a patient isolate that is resistant to only Hi-EMB. In addition, and in agreement with results reviewed by others (28, 43), we have found that certain mutations occur more often than others in drug-resistant isolates.

In the present study, we performed radiometric and agar proportion susceptibility testing and we analyzed by DNA sequencing the genetic regions associated with resistance to INH and EMB in 157 selected isolates of *M. tuberculosis*. The goal of the study was to determine (i) how often the common mutations occurred in the targeted genes in isolates displaying resistance in the phenotypic assays, and (ii) whether any particular INH-resistance-associated mutation occurred more often than others in combination with any particular EMB-resistance-associated mutation. To further characterize all isolates, we also sequenced the region associated with resistance to RIF, and we genotyped all isolates by spoligotype analysis.

MATERIALS AND METHODS

Selection of bacterial isolates for testing. From approximately 2,000 isolates of *Mycobacterium tuberculosis* obtained from patient specimens in the Clinical Mycobacteriology Laboratory, Wadsworth Center, Albany, New York, between 1998 and 2004, only 132 isolates were found in the radiometric susceptibility assay to be resistant to both isoniazid (INH) and ethambutol (EMB). These isolates had been frozen and stored at -80°C since shortly after isolation. Only four isolates characterized over the past 6 years with this resistance profile could not be recovered and thus could not be included in this study. Of the 128 available isolates, 67 were resistant to 0.4 µg/ml INH (Hi-INH) and 7.5 µg/ml EMB (Hi-EMB), 50 were resistant to Hi-INH and 2.5 µg/ml EMB (Lo-EMB), and 11 were resistant to 0.1 µg/ml INH (Lo-INH) and Lo-EMB. During this time period, no isolates were found in our laboratory to be monoresistant to 7.5 µg/ml EMB or to be resistant to this high level of EMB unless the isolate was also resistant to Hi-INH. In addition to the 128 isolates described above, 29 randomly selected isolates with commonly found resistance profiles (susceptible to both concentrations of EMB but resistant to either Hi-INH or Lo-INH or susceptible to INH) were included as controls.

The 157 isolates (specimens from patients in New York City [55%], upstate New York [40%], and out of state [5%]) were identified as members of the *M. tuberculosis* complex by the direct amplification assay (MTD; GenProbe, San Diego, CA) or by DNA probe (AccuProbe; GenProbe, San Diego, CA) and were further speciated to the final identification of *M. tuberculosis* using deletion analysis (23). Several patients had more than one isolate tested in our laboratory; however, only the first isolate from each patient is included in this study.

Drug susceptibility testing. Drug resistance assays using standard radiometric BACTEC procedures (22, 33) included tests of susceptibility or resistance to 0.1 and 0.4 µg/ml INH (Lo-INH and Hi-INH) and 2.5 and 7.5 µg/ml EMB (Lo-EMB and Hi-EMB). The isolates were also tested in the radiometric assay with 2.0 µg/ml rifampin (RIF), 100 µg/ml pyrazinamide (PZA), and 2.0 and 6.0 µg/ml streptomycin (STR).

The agar proportion method was performed, according to a standard protocol with drug solutions incorporated in Middlebrook 7H10 agar (22), for isolates resistant to at least one of the anti-TB drugs tested as described above in the radiometric BACTEC procedure. The following final drug concentrations were used in the agar proportion assay: 0.2 and 1.0 µg/ml for INH, 5.0 and 10 µg/ml for EMB, 1.0 µg/ml for RIF, and 2.0 and 10 µg/ml for STR (17). The control strain, H37Ra, was run weekly in both the radiometric and agar proportion methods.

DNA sequencing of genes associated with drug resistance. A suspension of bacterial cells from each isolate was heat-killed at 80°C for 1 h, and particular gene targets were amplified by PCR. DNA sequencing focused on the most

common gene target regions associated with resistance to each drug. For INH resistance, the region encoding amino acid Ser315 of *katG* (catalase-peroxidase, the enzyme that activates INH) was sequenced following amplification using primers Tb86 (5' GAA-ACA-GCG-GCG-CTG-ATC-GT 3') and Tb87 (5' GTT-GTC-CCA-TTT-CGT-CGG-GG 3') (35) (product size, 209 bp) and PCR conditions of 5 min denaturation at 95°C and then 35 amplification cycles (each cycle was 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) and a final extension cycle of 7 min at 72°C. For EMB resistance, the region encoding amino acids Met306 and Gly406 of *embB* (an arabinosyl transferase involved in cell wall biosynthesis) was sequenced following amplification using primers embB-F (5' TGA-TAT-TCG-GCT-TCC-TGC-TC 3') and embB-R (5' ACC-GCT-CGA-TCA-GCA-CAT-AG 3') (this study) (product size, 417 bp) and PCR conditions of 5 min denaturation at 95°C and then 40 amplification cycles (each cycle was of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min) and a final extension cycle of 10 min at 72°C. For RIF resistance, an 81-bp region encoding 27 amino acids of *rpoB* (beta-subunit of RNA polymerase, the enzyme necessary for prokaryotic transcription) was sequenced following amplification using primers rpo95 (5' CCA-CCC-AGG-ACG-TGG-AGG-CGA-TCA-CAC 3') and rpo397 (5' CGT-TTC-GAT-GAA-CCC-GAA-CGG-GTT-GAC 3') (14) (product size, 320 bp) and PCR conditions of 5 min denaturation at 95°C and then 35 amplification cycles (each cycle was 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min) and a final extension cycle of 7 min at 72°C. After the gene targets had been sequenced, mutations were identified by comparing the sequence to that of wild-type drug-susceptible strains. Because this study was focused on mutations associated with resistance to INH and EMB, the *katG* and *embB* regions were sequenced in all 157 isolates; however, the *rpoB* region was sequenced only in RIF-resistant isolates.

Spoligotype analysis of isolates. Spoligotyping, a PCR-based method detecting 43 known spacer sequences that are interspersed with the direct repeats in the genomic direct repeat region of *M. tuberculosis* complex strains, was performed as previously described (16, 39). In brief, PCR amplifications were performed on extracted DNA or cell suspensions, which had been heat-killed for 1 h at 80°C. Spoligotype patterns were analyzed with BioImage Whole Band Analysis v3.4 software (Genomic Solutions, Ann Arbor, MI) on a Sun Ultra10 workstation (Sun Microsystems Inc., Santa Clara, CA). Spoligotype patterns were given a 15-digit octal code, a descriptive nomenclature according to a standard method (8), and a unique arbitrary numeric designation.

RESULTS

Radiometric susceptibility testing. The 157 isolates of *M. tuberculosis* were divided into six groups based on resistance or susceptibility to INH and EMB in the radiometric susceptibility assay. These groupings were established because two observations suggested an association between resistances to these two important anti-TB drugs. First, as stated above, resistance to 7.5 µg/ml EMB (Hi-EMB) has not been seen in isolates that were not also resistant to 0.4 µg/ml INH (Hi-INH). Second, certain mutations are found more often than others in drug-resistant isolates, and the groupings of isolates were used in this study to test the hypothesis that a relationship exists between the particular mutations that are present when resistances to high concentrations of INH and EMB occur in the same isolate. Any observed relationships could be due to one or more of the following reasons: the relative fitness of the organism containing a particular combination of mutations, the effectiveness of particular mutations in conferring resistance to each drug, and the presence of a predominant genotype in the population served by this laboratory.

The groups as described above were designated groups I to VI and consisted of the following isolates: group I, 67 isolates resistant to Hi-INH and Hi-EMB; group II, 50 isolates resistant to Hi-INH and Lo-EMB and susceptible to Hi-EMB; group III, 11 isolates resistant to Lo-INH and Lo-EMB and susceptible to Hi-INH and Hi-EMB; group IV, 10 isolates resistant to Hi-INH and susceptible to both concentrations of EMB; group V, 9 isolates resistant to Lo-INH and susceptible

to Hi-INH and both concentrations of EMB; and group VI, 10 isolates susceptible to both concentrations of both INH and EMB.

Group I isolates (Hi-INH^r and Hi-EMB^r). As stated above, group I included 67 isolates from individual patients that were found to be resistant to high concentrations of both INH and EMB. Of these 67 isolates, 65 (97.0%) were MDR strains, since they were also resistant to RIF. In addition, 47 (70.1%) were resistant to STR and 23 (34.3%) were resistant to PZA. One isolate (1.5%) was only resistant to Hi-INH and Hi-EMB, 8 (11.9%) were also resistant to one other drug, 47 (70.1%) were also resistant to two other drugs, and 11 (16.4%) were also resistant to the other three highly effective anti-TB drugs.

Table 1 summarizes the incidence of the most common mutations in *katG* and *embB* associated with resistance to INH and EMB in the group I isolates. A total of 61 of the 67 isolates (91.0%) contained a mutation at Ser315 in *katG*. Sixty of the 61 replaced the Ser with Thr, and 30 (50%) of those contained the relatively rare dinucleotide change of AGC to ACA. Of those 61 isolates with Ser315 mutations, 54 (54 of 61 = 93.1%) had a mutation at Met306 in *embB*, 3 (3 of 58 = 5.2%) had a mutation at Gly406, and 3 (3 of 61 = 4.9%) did not contain a mutation at the common *embB* codons. The 6 group I isolates that did not contain a mutation at Ser315 in *katG* all had a mutation at either Met306 (5 of 6 = 83.3%) or Gly406 (1 of 6 = 16.7%) in *embB*, indicating that the common mutations in *embB* can be found in strains without a Ser315 mutation. Overall, mutations were found in *embB* Met306 or Gly406 in 64 (95.5%) of the 67 isolates resistant to Hi-EMB. The most common mutation in *embB*, Met306Val, was found in 52 (77.6%) of the 67 group I isolates (including 6 that contained a mixed population of mutant and wild-type alleles, a phenomenon called heteroresistance) (31), and this substitution was most often found in combination with the *katG* Ser315Thr mutation (49 of 52 = 94.2%).

In order to determine whether the 67 isolates in group I are closely related genetically, we performed spoligotyping, and we identified 17 distinct types. The majority of the isolates in this group (40 isolates = 59.7%) belong to S00034 (octal code 00000000003771), the spoligotype that includes the W-Beijing family strains often associated with major outbreaks of MDR TB. In addition, five isolates belong to spoligotype S00009, three each to spoligotypes S00002 and S00086, two each to 3 other spoligotypes, and one each to 10 other spoligotypes.

To determine whether the relationship between *katG* Ser315Thr and *embB* Met306Val was not simply due to a predominance of a certain strain in the population, we eliminated duplicate isolates that had the same *katG*, *embB*, and *rpoB* profile and spoligotype. Thirty-four isolates with distinctly different profiles remained. Of the 34, the *katG* Ser315Thr mutation was present in 28, and the *embB* Met306Val mutation was present along with *katG* Ser315Thr in 18 of these 28 (64.3%).

Group II isolates (Hi-INH^r, Lo-EMB^r). Included in group II were 50 isolates that had been found to be resistant to Hi-INH and Lo-EMB. In this group, fewer isolates, compared to the proportion in group I, were resistant to one or more of the other most effective anti-TB drugs (33 = 66.0%). Of these 50 isolates, 27 (54.0%) were also resistant to RIF (MDR strains), 15 (30.0%) were resistant to STR, and 12 (24.0%) were resis-

tant to PZA. In total, 17 (34.0%) isolates were resistant only to Hi-INH and Lo-EMB, 18 (36.0%) isolates were also resistant to one other drug, 9 (18.0%) isolates were also resistant to two other drugs, and 6 (12.0%) isolates were also resistant to the other three drugs.

Table 1 lists the incidence of the most common mutations associated with resistance to INH and EMB in the 50 isolates in group II. In contrast to 91.0% (61 of 67) of the group I isolates with a *katG* Ser315 mutation, only 33 (66.0%) of the 50 isolates in group II contained a mutation at this site, including 1 with this region deleted (no PCR product obtained) and 1 containing a mixed population of mutant and wild-type Ser315 alleles (heteroresistant). None of the 26 isolates in group II with the *katG* Ser315Thr mutation contained the relatively rare dinucleotide change of AGC to ACA found in 50% of the group I isolates with this amino acid substitution. Of the 33 isolates with Ser315 mutations, only 3 (3 of 33 = 9.1%) contained a *embB* mutation at Met306, while 30 (90.9%) had wild-type sequence at both Met306 and Gly406. Seventeen isolates (34.0%) did not contain a mutation at Ser315 in *katG*; 12 (12 of 17 = 70.6%) of those did not contain a mutation at either Met306 or Gly406, while the remaining 5 (5 of 17 = 29.4%) contained the Met306Ile mutation. Four of those five containing the Met306Ile mutation could have been related, since all were also resistant to RIF (all containing the same *rpoB* mutation), STR, and PZA and were spoligotype S00235. Overall, mutations were found in *embB* Met306 in only 8 (16.0%) of the 50 isolates resistant to Lo-EMB but susceptible to Hi-EMB. Only one (1 of 50 = 2%) of these was a Met306Val mutation, while seven (7 of 50 = 14%) were Met306Ile. In contrast to the results obtained for group I, only one of the 26 isolates with the *katG* Ser315Thr mutation (1 of 26 = 3.8%) also had the *embB* Met306Val mutation.

Spoligotyping of the isolates in group II demonstrated that the isolates were more genetically diverse than were the group I isolates. Of the 50 group II isolates, 27 different spoligotypes were identified. Only seven isolates (14.0%) in group II belonged to the W-Beijing family spoligotype S00034 (versus 59.7% in group I). In addition, six isolates belonged to spoligotype S00002, four each to spoligotypes S00009 and S00235, two each to 6 other spoligotypes, and one each to 17 other spoligotypes.

Group III isolates (Lo-INH^r, Lo-EMB^r). Included in group III were 11 isolates that had been found to be resistant to Lo-INH and Lo-EMB. In this group, six isolates (54.5%) were resistant only to Lo-INH and Lo-EMB, and five (45.5%) were also resistant to one or more of the other primary drugs (none of the isolates in group III was resistant to more than one drug besides Lo-INH and Lo-EMB). Of these 11 isolates, 4 (36.4%) were also resistant to RIF (MDR strains), and 1 (9.1%) was resistant to STR. No resistance to PZA was seen in this group.

Table 1 lists the incidence of the most common mutations associated with resistance to INH and EMB in the 11 isolates in group III. Only one isolate (9.1%) contained a mutation at *katG* Ser315, and the amino-acid substitution was Gly rather than the commonly seen Thr substitution. Ten isolates (90.9%) did not contain a mutation at Ser315 in *katG*, and none of the 11 isolates in group III contained an *embB* mutation at either Met306 or Gly406.

Spoligotyping of the 11 isolates in group III demonstrated

TABLE 1. Mutations in *katG* and *embB* present in the 157 isolates in groups I to VI^a

Group (no. of isolates)	<i>katG</i>	<i>embB</i>
I (67 isolates resistant to Hi-INH and Hi-EMB)	Ser315Thr (60) Ser315Asn (1)	Met306Val (43) Met306Ile (5) Met306Phe (1) Heteroresistant (Met306Val or Ile or no mutation at these codons) ^b (6) Gly406Asp or Ser (3) No mutation at Met306 or Gly406 (3)
	No mutation at Ser315 (6)	Met306Val (3) Met306Ile (2) Gly406Asp (1)
II (50 isolates resistant to Hi-INH and Lo-EMB)	Ser315Thr (26) Ser315Asn (4) Ser315Ile (1) Ser315Arg/Leu/Ile ^b (1) Region deleted (1)	No mutation at Met306 or Gly406 (30) Met306Val or Ile (3)
	No mutation at Ser315 (17)	No mutation at Met306 or Gly406 (12) Met306Ile (5)
III (11 isolates resistant to Lo-INH and Lo-EMB)	Ser315Gly (1)	No mutation at Met306 or Gly406 (1)
	No mutation at Ser315 (10)	No mutation at Met306 or Gly406 (10)
IV (10 isolates resistant to Hi-INH and susceptible to EMB)	Ser315Thr (6)	No mutation at Met306 or Gly406 (6)
	No mutation at Ser315 (4)	No mutation at Met306 or Gly406 (4)
V (9 isolates resistant to Lo-INH and susceptible to EMB)	No mutation at Ser315 (9)	No mutation at Met306 or Gly406 (9)
VI (10 isolates susceptible to both INH and EMB)	No mutation at Ser315 (10)	No mutation at Met306 or Gly406 (10)

^a A complete listing of the 157 isolates and their genotypes is available from the corresponding author.

^b A heteroresistant population consists of both drug-susceptible and drug-resistant members, identified by the presence of multiple peaks on the DNA sequencing chromatographs at the nucleotides encoding amino acid 306 in *embB*.

that these isolates, in similarity to those in group II, were genetically diverse, with nine different spoligotypes represented (2 isolates each belonged to two spoligotypes, and 1 isolate each belonged to seven spoligotypes). There were no isolates belonging to the W-Beijing spoligotype S00034 in group III.

Groups IV (Hi-INH^r, EMB^s), V (Lo-INH^r, EMB^s), and VI (INH^s, EMB^s). The isolates in these three control groups were all susceptible to both concentrations of EMB, and they differed only in susceptibility or resistance to INH. Group IV consisted of 10 isolates resistant to Hi-INH and susceptible to EMB; group V consisted of 9 isolates resistant to Lo-INH and susceptible to EMB; and group VI consisted of 10 isolates susceptible to both concentrations of both INH and EMB.

Of the 10 Hi-INH-resistant, EMB-susceptible isolates in group IV, 6 (60.0%) contained the common Ser315Thr mutation in *katG* (AGC to ACC), and none had the common *embB* mutations (Table 1). Three of the 10 were also resistant to other drugs, 2 to RIF (MDR strains), and 1 to PZA. No mutations were found at the common sites in *katG* or *embB* in any of the isolates in groups V and VI. In group V, one isolate was also resistant to RIF, and two other isolates were also resistant to STR. The isolates in group VI were pansusceptible.

These groups were the most genetically diverse, since there was no spoligotype shared by any of the isolates in groups IV, V, and VI. One of the isolates in group VI (pansusceptible group) belonged to spoligotype S00034, demonstrating that the MDR phenotype is not always associated with this spoligotype.

Level of resistance seen in isolates having common mutations in *katG* and *embB*. Tables 2 and 3 present the results of the agar proportion method (considered to be the "gold standard" for susceptibility testing) for INH and EMB, respectively, in relation to the presence of the common mutations in *katG* and *embB*. Not all isolates could be tested, due to lack of growth on 7H10 agar. As shown in Table 2, 123 of the 127 Hi-INH-resistant isolates from groups I, II, and IV were tested in the agar proportion assay. Of those, the 96 with the *katG* Ser315 mutation had a higher proportion of resistant colonies (87.5% and 78.1% had between 80 and 100% resistant colonies at 0.2 and 1.0 µg/ml INH, respectively), while only 66.7% and 29.6% of the 27 isolates with no Ser315 mutation had between 80 and 100% resistant colonies at these two concentrations of INH.

In addition, of the 20 isolates resistant to Lo-INH from groups III and V that were tested in the agar proportion assay, only 1 had a Ser315 mutation in *katG*; the other 19 did not. The mutation

TABLE 2. INH agar proportion susceptibility results relative to a mutation in *katG* Ser315 in 143 isolates resistant to either 0.4 µg/ml INH (Hi-INH) or 0.1 µg/ml INH (Lo-INH)

% Resistance	No. (%) of isolates exhibiting resistance at the indicated concn (µg/ml)			
	<i>katG</i> Ser 315 mutation		No mutation at <i>katG</i> Ser 315	
	0.2	1.0	0.2	1.0
Hi-INH (<i>n</i> = 123) ^a				
80–100	84 (87.5)	75 (78.1)	18 (66.7)	8 (29.6)
30–75	12 (12.5)	21 (21.9)	8 (29.6)	16 (59.3)
0–25	0	0	1 (3.7)	3 (11.1)
Lo-INH (<i>n</i> = 20) ^b				
80–100	0	0	7 (36.8)	0
30–75	1 (100)	0	6 (31.6)	0
0–25	0	1 (100)	6 (31.6)	19 (100)

^a Number of isolates with *katG* Ser 315 mutation, 96; number of isolates with no mutation at *katG* Ser 315, 27.

^b Number of isolates with *katG* Ser 315 mutation, 1; number of isolates with no mutation at *katG* Ser 315, 19.

found in the single isolate was Ser315Gly, an amino-acid change not found in our experience in isolates resistant to Hi-INH. In the agar proportion method, this isolate showed fairly low-level resistance (30 to 75% and 0% resistant at 0.2 and 1.0 µg/ml, respectively). For the other 19 isolates resistant to Lo-INH, the percentages of resistant colonies differed greatly at 0.2 µg/ml, and there were no colonies resistant to 1.0 µg/ml.

The agar proportion results were more variable when 64 of the 67 group I isolates resistant to Hi-EMB were tested, as shown in Table 3. Although the agar proportion assay has not been easy to standardize for EMB (18), these variable results could suggest the presence of a more heterogeneous population relative to EMB resistance, further supported by our finding of multiple nucleotide peaks at the Met306 codon in 6 of the 60 Hi-EMB resistant isolates with mutations at this site (Table 1). In addition, we have experienced difficulties in verifying high-level resistance to EMB in the radiometric susceptibility assay, after isolates have grown for several generations in the laboratory in the absence of antibiotic selection (data not shown). A comparison of the agar proportion results between Hi-EMB resistant isolates with and without the common mutations is difficult, since only three isolates were without a mutation in Met306 or Gly406. Finally, the agar proportion results for the 61 Lo-EMB-resistant isolates from groups II and III demonstrated very low-level resistance at either 5 or 10 µg/ml.

RpoB mutations in RIF-resistant isolates. A total of 99 (63.1%) of the 157 isolates tested were resistant to RIF. All 99 contained a mutation in the 81-bp region of *rpoB*, with the most common mutations at Ser531 (50 of 99 = 50.5%; 49 were Ser531Leu, and 1 was Ser531Trp) and His526 (45 of 99 = 45.5%; 40 were His526Tyr, 2 were His526Arg, 2 were His526Asp, and 1 had deleted the His526 codon). Less-common mutations were found at Asp516 (three were Asp516Val) and Gln513 (one was Gln513Lys).

Although 76% (76 of 100) of the Hi-INH-resistant isolates with a mutation at *katG* Ser315 were also RIF resistant, there was not a clear correlation of RIF resistance with this muta-

TABLE 3. EMB agar proportion susceptibility results relative to a mutation in either *embB* Met306 or in Gly406 in 125 isolates resistant to either 7.5 µg/ml EMB (Hi-EMB) or 2.5 µg/ml EMB (Lo-EMB)

% Resistance	No. (%) of isolates exhibiting resistance at the indicated concn (µg/ml)			
	Mutation in <i>embB</i> Met 306 or Gly 406		No mutation in <i>embB</i> Met 306 or Gly 406	
	5	10 ^c	5	10 ^c
Hi-EMB (<i>n</i> = 64) ^a				
80–100	16 (26.2)	2 (4.8)	0	
30–75	26 (42.6)	7 (16.7)	3 (100)	
0–25	19 (31.1)	33 (78.6)	0	
Lo-EMB (<i>n</i> = 61) ^b				
80–100	0	0	1 (1.9)	0
30–75	4 (50.0)	2 (33.3)	4 (7.5)	0
0–25	4 (50.0)	4 (66.7)	48 (90.6)	41 (100)

^a Number of isolates with a mutation in *embB* Met 306 or Gly 406, 61 at 5 µg/ml and 42 at 10 µg/ml; number of isolates with no mutation in *embB* Met 306 or Gly 406, 3 at 5 µg/ml and 0 at 10 µg/ml.

^b Number of isolates with a mutation in *embB* Met 306 or Gly 406, 8 at 5 µg/ml and 6 at 10 µg/ml; number of isolates with no mutation in *embB* Met 306 or Gly 406, 53 at 5 µg/ml and 41 at 10 µg/ml.

^c Results for 10 µg/mL EMB were not entered into the database prior to 2002.

tion, since 66.7% (18 of 27) of the isolates with no Ser315 mutation were also resistant to RIF. However, there did appear to be an association between the most common *rpoB* mutations and the *katG* Ser315 mutation; 73.3% (33 of 45) of the isolates with *rpoB* Ser531Leu and 97.5% (39 of 40) of those with *rpoB* His526Tyr also had the *katG* Ser315 mutation.

DISCUSSION

In performing susceptibility testing on over 2,000 isolates of *M. tuberculosis* during the past 6 years, we have found, when using the radiometric susceptibility assay, that resistance to the high concentration of EMB, 7.5 µg/ml (Hi-EMB), occurs only in isolates that are also resistant to the high concentration of INH, 0.4 µg/ml (Hi-INH). Furthermore, monoresistance to Hi-INH is often seen, but we have never found a patient isolate that is resistant to only Hi-EMB. This observation is in agreement with the nonoccurrence or very rare occurrence of EMB monoresistance in isolates of *M. tuberculosis* as reported by others (19, 37). Therefore, in the present study, we analyzed resistance to INH and EMB detected in 128 isolates, using both phenotypic and molecular methods.

Mutations at *katG* Ser315 in isolates of *M. tuberculosis* mediate high-level resistance to INH, one of the most effective anti-TB drugs (13). Furthermore, a significant correlation has been established between the common *katG* Ser315 mutations in INH-resistant strains and the presence of resistances to other drugs (20, 25, 38). These observations were confirmed in the present study, when a mutation at *katG* Ser315 was found to be more common in Hi-INH-resistant isolates also resistant to Hi-EMB (61 of 67 = 91.0%) than in Hi-INH-resistant isolates either susceptible to EMB or resistant to only 2.5 µg/ml (Lo-EMB) (39 of 60 = 65.0%) (Fisher's exact test; *P* = 0.00002). These observations suggest that INH-resistant strains with mutations at Ser315 of *katG* are more likely to acquire

resistance to Hi-EMB. Resistance to RIF (as well as the presence of RIF-resistance-associated mutations) was also found in most of the isolates resistant to Hi-INH; however, the correlation with the *katG* Ser315 mutation was not as clear as with Hi-EMB. While 76% of the isolates with the *katG* Ser315 mutation were also RIF resistant, 66.7% of the isolates with no Ser315 mutation were additionally resistant to RIF.

M. tuberculosis katG encodes a catalase peroxidase (an enzyme important in protecting the organism from oxidative stress) that converts the prodrug INH to its bioactive form, whose primary activity involves interference with biosynthesis of cell-wall components (13, 15, 42). The *katG* Ser315Thr alteration results in an enzyme that cannot activate INH but that still maintains significant catalase-peroxidase activity, thus permitting the INH-resistant organism to survive in the presence of oxidative stress (26). Therefore, these strains have overcome the fitness deficit usually imposed by resistance-associated mutations. Most often, fitness deficits resulting from drug-resistance-associated mutations can be reduced, through compensatory mutations arising at other sites (4, 7). When such mutations compensate for a fitness deficit, there is little likelihood of spontaneous reversion to susceptibility. Thus, in addition to the organism's having gained resistance to INH, this genetic background may favor the emergence of multi-drug-resistant strains that can survive under stressful conditions and maintain transmissibility.

An important family of MDR-TB, the W-Beijing family (spoligotype S00034), includes the New York strain W, the causative agent of a large outbreak of MDR TB in New York City in the early 1990s (2, 3). The New York strain W was resistant to INH, RIF, EMB, and STR and contained a distinguishing marker not shared by other W strains: a rare dinucleotide change (AGC to ACA) in *katG* resulting in a Ser315Thr mutation (3, 21). Of the 60 group I isolates in this study with Ser315Thr mutations, 30 (50%) contain this dinucleotide change, are spoligotype S00034, and thus are likely in the New York strain W lineage. The other 30 with the Ser315Thr mutation have the more common single-nucleotide change (AGC to ACC), as do the 32 isolates in groups II and IV with the Ser315Thr mutation. In addition, the 30 potential New York strain W isolates from group I all contained a Met306Val mutation in *embB* and a His526Tyr mutation in *rpoB* (previously reported to occur in the New York strain W) (3, 21) while the other 30 isolates with the AGC-to-ACC nucleotide change were more genetically diverse, containing 17 different combinations of mutations in *embB* (six heteroresistant) and in *rpoB* and belonging to 13 different spoligotypes. Also confirming the persistence of New York strain W from 8 to 12 years after the original outbreak, all 30 of the recently obtained isolates were resistant to STR in addition to INH, EMB, and RIF (3, 21), and 21 (70%) were obtained from patients in New York City.

According to previous studies, the acquisition of resistance to EMB is a multistep process that may involve several genes (1, 36). The first step, likely resulting in low to moderate resistance, involves overexpression of the EmbCAB proteins, arabinosyl transferases involved in biosynthesis of important components of the mycobacterial cell wall (9). The second step involves a mutation in the region of *embB* that is associated with both natural and acquired high-level resistance to EMB (1). Most mutations in isolates with higher EMB MICs occur at

the codon for amino acid 306, with only a few substitutions (Val, Ile, Leu) tolerated to replace the wild-type Met. In addition, a strong correlation between MIC and the particular amino-acid substitution has been reported. Strains with the Met306Ile replacement were found to have MICs that were generally lower (20 µg/ml) than those for strains with either Met306Val or Met306Leu (40 µg/ml) (29). Less-common resistance-associated mutations have been found in *embB* amino acid 406, replacing the wild-type Gly with Ala, Asp, Cys, or Ser (29). Overall, these observations suggest that Met306 and Gly406 form a critical part(s) of the glycosyl transferase active site to which EMB binds (1, 36).

In the present study, we found that 64 of 67 (95.5%) isolates resistant to Hi-EMB contained a mutation in either codon 306 or 406 of *embB*. These results suggest that the sequencing of this region is sufficiently sensitive to use as a rapid screening tool for detection of high-level resistance to EMB. Met306Val was the most common mutation, present in 52 (77.6%) of the 67 group I isolates. The other 12 isolates contained either Met306Ile or Phe (9 isolates) or Gly406Asp or Ser (4 isolates). Only three isolates did not have an *embB* mutation at one of these two common sites. Of the 52 Hi-EMB-resistant isolates with the *embB* Met306Val mutation, most (49 of 52 = 94.2%) also contained the *katG* Ser315Thr mutation. These observations suggest that strains with this combination of mutations remain relatively fit and can persist in the population for years.

Isolates resistant to Lo-EMB and susceptible to Hi-EMB in the radiometric susceptibility assay have been more problematic to characterize. Questions have arisen about whether resistance at this level is indicative of the potential for treatment failure in the patient (19). In the present study, we found that 53 (86.9%) of the 61 isolates resistant to Lo-EMB did not have one of the common *embB* mutations, suggesting that this resistance could have resulted from a "first-step" mutation. Only eight isolates contained one of the common *embB* mutations, seven with a Met306Ile alteration (previously reported to have a lower MIC than do Met306 substitutions with Val or Leu) (29) and one with Met306Val. Three of the isolates having the mutations (one with Met306Val and two with Met306Ile) also had the *katG* Ser315Thr mutation. The results of the agar proportion assay (Table 3) on the isolates with the *embB* Met306 mutation that were only resistant to Lo-EMB in the radiometric susceptibility assay demonstrate that low proportions of resistant colonies were present. Thus, the low proportion of resistant organisms in the population may not have been sufficient to demonstrate resistance in the presence of Hi-EMB in the broth-based radiometric assay.

A complicating factor in phenotypic analysis of resistance to EMB is that susceptibility testing to this drug is not well standardized (18). Standardization has been hampered by discrepancies encountered when comparing results obtained in liquid versus solid media, suggesting that the conditions used in the current assays may greatly influence the interaction of EMB with *M. tuberculosis*. In earlier studies on the effects of exposure time, drug concentration, and temperature on the activity of EMB against *M. tuberculosis*, it was found that the bactericidal action of EMB is dependent upon all of these criteria (10). Thus, the problems encountered in attempts to standardize the assays in any medium may be due to the general issue of poor stability of EMB caused by storage, incubation tem-

perature, or interaction with divalent cations, as suggested by recent work (27). Lack of reproducibility has resulted in problems in determining the MICs of susceptible strains and—most problematic for patient care—in determining the critical concentrations of EMB to be used in routine susceptibility testing (5, 12, 24, 41).

The basis of the agar proportion assay is the phenomenon called heteroresistance, which is the simultaneous occurrence of resistant and susceptible organisms in the same sample and is a valid phenomenon in clinical tuberculosis (5, 6, 31). Most often heteroresistance is due to development of a resistant subpopulation of TB in the patient during the course of treatment. Acquisition of resistance results in an at least temporary decrease in the growth rate of the cells with the mutation (11) and provides the opportunity for any faster-growing cells to overwhelm those that grow more slowly. For example, the slower-growing resistant population would be selected for in the presence of drug in the patient, but during *in vitro* culturing and subculturing in the absence of drug, the faster-growing susceptible cells could become the predominant population. Differences in growth rates of susceptible and resistant cells within the population can have significant effects, particularly on the results of broth-based susceptibility assays, since these assays are interpreted based on growth of the total population. In particular, it is unknown whether certain procedures such as decontamination of the sputum specimen will affect the selection of particular populations.

Heteroresistance has been demonstrated in isolates of *M. tuberculosis* resistant to EMB, as well as those resistant to INH and RIF (30, 32). Furthermore, heteroresistance may contribute to the difficulties encountered in standardizing phenotypic EMB resistance assays (18, 19, 24). In the present study, three observations suggest that heteroresistance could be a factor in our EMB susceptibility results. The first was our inability to confirm high-level resistance to EMB (originally demonstrated in the radiometric assay and confirmed by the agar proportion method) in several isolates following subculturing, freezing, reviving, and retesting of the isolates (data not shown). This result suggests the existence of a heteroresistant population, with the susceptible organisms overgrowing the resistant population that had been predominant when the isolate was tested immediately after isolation from the patient. Second, in DNA sequencing of regions of *embB*, we found multiple peaks at codon 306 in six of the isolates resistant to Hi-EMB (Table 1). This result suggests the presence of both mutant (resistant) and wild-type (susceptible) cells in the population. Third, among the isolates that were found to be resistant to Hi-EMB in the radiometric assay and that contained the common resistance-associated mutations in *embB*, there were fewer EMB-resistant colonies than would have been expected at 5 µg/ml in the agar proportion assay (Table 3). However, this last discrepancy could have been due to lack of equivalence or comparability in the drug concentrations in the two media.

In summary, we found that 64 of 67 (95.5%) isolates resistant to Hi-EMB contained a mutation in codon 306 or 406 in *embB*, but only 8 of 50 (16.0%) of isolates resistant to Lo-EMB contained such a mutation. This result suggests that the sequencing of this region of *embB* is sufficiently sensitive for use as a rapid screening tool for detection of high-level resistance to EMB, especially in the population served by our laboratory.

This finding is of increased significance due to the difficulties inherent in phenotypic EMB susceptibility testing and again brings up the question of the appropriate concentration of EMB used in phenotypic susceptibility assays that will best correlate with drug resistance in the patient.

In contrast to the *embB* 306 and 406 results, the molecular assay that detected a mutation at *katG* Ser315 was not sensitive enough to use as a screening tool for INH resistance (for either high- or low-level resistance). Only 78.7% (100 of 127) of the isolates with resistance to 0.4 µg/ml INH had a mutation at Ser315. Of those with resistance to 0.1 µg/ml INH, even fewer, 20% (1 of 20), had the mutation. Thus, to increase the sensitivity of molecular detection of INH resistance, we must, in addition to investigating *katG* Ser315, look for mutations in other regions of *katG* or in other resistance-associated genes. Furthermore, until automated assays are available to simply and rapidly test multiple drug targets, we and others believe that phenotypic susceptibility testing remains the most sensitive assay for detection of resistance to INH (37).

Finally, an association between the occurrence of the common mutations in isolates resistant to INH and EMB was found only in the group I isolates. Of the 67 isolates resistant to both Hi-INH and Hi-EMB, 61 contained a mutation at *katG* Ser315, with 93.1% (54 of 61) of those also containing an *embB* mutation at Met306; more specifically, 80.3% (49 of 61) contained the Met306Val mutation. This result suggests not only that these mutations are effective in providing resistance to the respective drugs but also that the organisms with such mutations are fit enough to survive in the individual patient. Most notable in this respect, our findings provide strong evidence for stability and transmission of the New York W strain within the patient population served by our laboratory.

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