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Role of *embB* in Natural and Acquired Resistance to Ethambutol in Mycobacteria

FERNANDO ALCAIDE,[1] GABY E. PFYFFER,[2] AND AMALIO TELENTI[1]*

Institute for Medical Microbiology, University of Berne, Berne,[1] and Swiss National Center for Mycobacteria, Department of Medical Microbiology, University of Zurich, Zurich,[2] Switzerland

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The mycobacterial *embCAB* operon encodes arabinosyl transferases, putative targets of the antimycobacterial agent ethambutol (EMB). Mutations in *embB* lead to resistance to EMB in *Mycobacterium tuberculosis*. The basis for natural, intrinsic resistance to EMB in nontuberculous mycobacteria (NTM) is not known; neither is the practical implication of resistance to EMB in the absence of *embB* mutations in *M. tuberculosis* well understood. The conserved *embB* resistance-determining region (ERDR) of a collection of 13 strains of NTM and 12 EMB-resistant strains of *M. tuberculosis* was investigated. Genotypes were correlated with drug susceptibility phenotypes. High-level natural resistance to EMB (MIC, ≥64 μg/ml) was associated with a variant amino acid motif in the ERDR of *M. abscessus*, *M. chelonae*, and *M. leprae*. Transfer of the *M. abscessus emb* allele to *M. smegmatis* resulted in a 500-fold increase in the MICs. In *M. tuberculosis*, *embB* mutations were associated with MICs of ≥20 μg/ml while resistance not associated with an ERDR mutation generally resulted in MICs of ≤10 μg/ml. These data further support the notion that the *emb* region determines intrinsic and acquired resistance to EMB and might help in the reassessment of the current recommendations for the screening and treatment of infections with EMB-resistant *M. tuberculosis* and NTM.

Ethambutol (EMB) is a frontline antituberculous drug with a broad spectrum of antimycobacterial activity. EMB targets the mycobacterial cell wall through interaction with the arabinosyl transferases involved in arabinogalactan and lipoarabinomannan biosynthesis (1, 27). The recently identified *emb* operon encodes several homologous arabinosyl transferase enzymes—EmbC, EmbA, and EmbB in *Mycobacterium tuberculosis*, *M. smegmatis*, and *M. leprae* (31) and EmbA and EmbB in *M. avium* (1). In *M. smegmatis*, attainment of high-level resistance to EMB is a multistep process. First-step resistance results from overexpression of the Emb proteins. A further decrease in susceptibility requires a mutation in a conserved region of EmbB or additional changes in expression levels (31). EmbB mutations can be identified in 47 to 69% of EMB-resistant isolates of *M. tuberculosis* (25, 31).

EMB is widely used in the treatment of *M. avium*, *M. kansasii*, *M. xenopi*, *M. marinum*, and *M. malmoense* infections (5). However, the basis of susceptibility or resistance to EMB among these and other nontuberculous mycobacteria (NTM) is not known; neither are EMB susceptibility testing and interpretation for those species well established.

The present work aimed at further characterizing the mutational "hot spot" in *embB*—the proposed EMB resistance-determining region (ERDR)—of *M. tuberculosis* and NTM. The final goals were (i) to establish a firm correlation between the *embB* genotype and susceptibility to ethambutol and (ii) to analyze the significance of EMB resistance in the absence of mutations in the ERDR of the *embB* gene.

MATERIALS AND METHODS

Strains and susceptibility testing. *M. tuberculosis* H37Rv, a representative collection of resistant isolates of *M. tuberculosis* ($n = 12$), *M. smegmatis* mc^{2}155 and its laboratory EMB-resistant mutant imm129 (31), and reference or clinical isolates (one strain each) of *M. avium*, *M. malmoense*, *M. marinum*, *M. kansasii*, *M. fortuitum*, *M. peregrinum*, *M. chelonae*, *M. abscessus*, *M. gordonae*, and *M. nonchromogenicum* were evaluated by susceptibility testing and genotypic analysis. *M. genavense* and *M. leprae* were investigated only by analysis of genotypic data.

EMB dihydrochloride was purchased from Sigma Chemical Co. (St. Louis, Mo.). Susceptibility testing of *M. tuberculosis* was performed radiometrically (BACTEC) (22) by using a concentration of EMB of 7.5 μg/ml. In addition, susceptibility testing for *M. tuberculosis*, as well as for slowly growing NTM, was performed by the modified proportion agar dilution method by using Middlebrook 7H10 medium supplemented with oleic acid, albumin, dextrose, and catalase (OADC) in accordance with the standard procedure (8). Dilutions of EMB, 0.5, 1, 2.5, 5, 10, 20, and 40 μg/ml for *M. tuberculosis* and 0.5, 1, 2, 4, 8, and 16 μg/ml for NTM, were incorporated into the agar. Inocula were adjusted to the optical density of a 1 McFarland standard. Two dilutions of this suspension were made for *M. tuberculosis* (10^{-2}- and 10^{-4}-fold, approximately 3×10^6 and 3×10^4 cells/ml, respectively) and for NTM (10^{-3}- and 10^{-5}-fold). With a capillary pipette, each quadrant of a plate was inoculated with 3 drops (approximately 30 μl) of the respective cell suspension. Plates were incubated for 3 weeks at 37°C in an atmosphere of 5% CO_2. The lowest concentration of EMB that inhibited more than 99% of the bacterial population was considered to be the MIC.

Rapid growers were evaluated by both broth microdilution test in microtiter trays containing cation-adjusted Mueller-Hinton broth and agar dilution with 7H10-OADC. For microdilution susceptibility testing, 20 μl of a 10^{-2} dilution of a 0.5 McFarland standard suspension (approximately 3×10^4 cells/ml) was used as the inoculum in wells containing serial twofold dilutions of EMB in 180 μl of medium. Final concentrations of EMB ranged from 0.5 to 256 μg/ml. Plates were evaluated after 3 and 5 days of incubation at 30 and 37°C (11). The lowest concentration of EMB inhibiting visible growth was considered to be the MIC.

PCR, sequencing, and SSCP. Bacterial lysates were prepared by suspending a loop of the inoculum in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and then subjecting it to 20 min of deactivation at 80°C and 30 min of boiling (30). For PCR, 2.5 μl of the lysate supernatant was added to a PCR mixture (50 μl) (29) containing reverse primer TE23 (5′-GGGCTGCCGAACCAGC GGAA) and degenerated forward primer TE24d (5′-AATTCGTC[G/C]GA[G/C]GA[G/C]GG[G/C]TA), targeting a 92-bp conserved *embB* sequence region (GenBank accession no. U46844, U68480, Z80343, and U66560) (31). Amplification products were cloned into pT7T3U18 (Pharmacia, Uppsala, Sweden) and sequenced in an ALF Pharmacia automated sequencer. The collection of 12 EMB-resistant *M. tuberculosis* isolates was investigated by PCR-single-strand conformational polymorphism (SSCP) by using fluorescent primers TE17f (5′-ACGCTGAAACTGCTGGCGAT) and TE14f (5′-ACAGACTGGCGTCG CTGACA) (31) to amplify a 400-bp *embB* region as previously described (28). SSCP patterns identical to that of susceptible *M. tuberculosis* H37Rv were scored as wild type, and divergent patterns were scored as resistant. This approach has been validated by sequence analysis (25, 31).

Library construction and gene transfer. The contribution of the *M. abscessus* and *M. fortuitum emb* regions to a resistance phenotype was assessed by gene

* Corresponding author. Mailing address: Division des Maladies Infectieuses, CHUV, 1011 Lausanne, Switzerland. Phone: 41 21 314 0550. Fax: 41 21 314 1008. E-mail: telenti@imm.unibe.ch.

TABLE 1. Impact of *embB* mutations on the susceptibility of *M. tuberculosis* to EMB[a]

Strain	Genotype	Phenotype		MIC (μg/ml) in 7H10
		EMB at 5 μg/ml of 7H10	EMB at 7.5 μg/ml of BACTEC 12B	
H37Rv	WT	S	S	2.5
Group I				
5693	WT	R	S	10
5706	WT	R	S	10
5722	WT	R	S	10
5729	WT	R	S	20[b]
5700	WT	R	R	10
7902	WT	R	R	10
5698	WT	R	R	40[c]
Group II				
5691	M	R	R	20
5726	M	R	R	20
7895	M	R	R	20
7932	M	R	R	40[d]
5684	M	R	R	40

[a] Group I includes resistant isolates without a recognizable *embB* mutation. Group II includes strains with mutations identified by PCR-SSCP. R, resistant; S, susceptible; WT, wild type; M, mutant.
[b] 2.5% of colonies present at an EMB concentration of 10 μg/ml.
[c] 2% of colonies present at an EMB concentration of 20 μg/ml.
[d] 1.5% of colonies present at an EMB concentration of 20 μg/ml.

transfer experiments. Intact chromosomal DNA was subjected to partial *Sau*3A digestion. Fragments (35 to 45 kb) were ligated to the shuttle cosmid vector pYUB415, in vitro packaged (Gigapack III Gold; Stratagene, La Jolla, Calif.), and transduced into *Escherichia coli* (31). Colonies selected on Luria-Bertani agar with ampicillin at 50 μg/ml were pooled, their cosmids were extracted, and the DNA (1 μg) was electroporated into electrocompetent *M. smegmatis* mc^2155 (23). Transformants were selected on 7H10-OADC agar containing hygromycin B at 50 μg/ml (Boehringer GmbH, Mannheim, Germany) and EMB at 2.5 μg/ml. The correct identity of inserts was documented by restriction fragment length polymorphism analysis of PCR products with primers TE23 and TE24d. Susceptibility testing of the transformants was performed with microtiter plates as described above.

RESULTS

Correlation between phenotype and genotype in a collection of EMB-resistant *M. tuberculosis* isolates. In the BACTEC system, the following EMB phenotypes have been defined for clinical isolates of *M. tuberculosis*: susceptible, MIC ≤ 1.9 μg/ml; moderately susceptible, MIC = 3.8 μg/ml; moderately resistant, MIC = 7.5 μg/ml; resistant, MIC ≥ 15.0 μg/ml (10). In our study, strains classified as having a moderate-resistance phenotype (resistant to 5 μg/ml in 7H10, susceptible to 7.5 μg/ml in BACTEC) exhibited a wild-type ERDR genotype, and with one exception, the MICs in 7H10 were below 10 μg/ml (Table 1). Strains scored as resistant by both the 7H10 and BACTEC methods could be differentiated on the basis of the ERDR genotype: MICs for mutant strains were consistently ≥20 μg/ml, while for two of three strains with the wild-type genotype the MIC was 10 μg/ml. Overall, only one of seven isolates (5698) displayed a definitive high-level resistance phenotype despite having a wild-type genotype (Table 1).

Correlation between phenotype and genotype in NTM. Because of our previous observation of a variant ERDR amino acid sequence in *M. leprae* (31), an organism reported to be highly resistant to EMB (13, 19, 21), we investigated this specific region of EmbB in 12 additional NTM. Primers TE23 and TE24d allowed amplification of a 92-bp fragment from all of the mycobacteria tested. Sequence analysis identified limited polymorphism within this highly conserved region of EmbB (Table 2). Correlation of these sequences with EMB MICs identified critical amino acid residues as possibly predictive of high natural resistance to EMB (Q303/M304 in *M. leprae*, *M. chelonae*, and *M. abscessus*) and confirmed the association of the I303F mutation in *M. smegmatis* and the M306I, M306L, or M306V mutation in *M. tuberculosis* with EMB resistance (3, 25,

TABLE 2. Correlation between EMB phenotype and genotype at the ERDR in EmbB[a]

Species (phenotype)	ERDR[b]	MIC (μg/ml) or phenotype	MIC (μg/ml) after gene transfer to *M. smegmatis*
	◆		
M. tuberculosis	SDDGY **ILGM** ARVADHAGYMSN	2.5	
M. gordonae	•••• •••• ••••••••••••	2.5	
M. nonchromogenicum	•••• •••• ••••••••••••	2.5	
M. marinum	•••• •••• ••••••••••••	4	
M. malmoense	•••• •••• ••••••••••••	4–8	
M. genavense	•••• •••• ••••••••••••	S?	
M. kansasii	•••• •••• •••••R••••••	8	
M. avium	•••• •••• •••••R••••••	8	
M. smegmatis	•••• **••Q•** •T•E•••••A••	0.5	10
M. peregrinum	•••• **••Q** ••••••••••••	8	
M. fortuitum	•••• **••Q** ••••••••••••	8–16	64
M. chelonae	•••• **QM••** ••T•E•••••A•	64	
M. abscessus	•••• **QM••** ••T•E•••••A•	64	254
M. leprae	•••• **QMQ•** ••T•••S•••A•	R	
M. smegmatis (R)	•••• **F•Q•** ••T•E•••••A•	100	≥254
M. tuberculosis (R)	•••• **•••I** ••••••••••••	20	
M. tuberculosis (R)	•••• **•••L** ••••••••••••	40	
M. tuberculosis (R)	•••• **•••V** ••••••••••••	40	

[a] The critical amino acids around Met306 (◆) are in boldface. Alignment homology is represented by dots. Phenotypes for *M. genavense* (S?, susceptible?) and *M. leprae* (R, resistant) were obtained from the literature (2, 13, 19, 21, 35). MICs for the *M. smegmatis* (R) and *M. tuberculosis* (R, resistant) mutants have been previously reported (25, 31). Displayed in the last column are MICs for *M. smegmatis* mc^2155 transformed with various *emb* alleles.
[b] A, alanine; R, arginine; N, asparagine; D, aspartic acid; E, glutamic acid; Q, glutamine; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; F, phenylalanine; S, serine; T, threonine; Y, tyrosine; V, valine.

31) (Table 2). The MICs for *M. fortuitum* were moderately elevated in the face of a genotype indistinguishable from that of susceptible *M. smegmatis* at this critical region (ILQM in Table 2). Among the mycobacteria with the most common motif, ILGM, *M. malmoense* (two unrelated strains) presented the least susceptibility to EMB (MIC, 4 to 8 μg/ml). Overall, the MICs corresponded to those reported in the literature (11).

Gene transfer experiments. To assess the relative contributions of various *emb* alleles to a natural resistance phenotype, the *emb* regions of *M. abscessus* (MIC, 64 μg/ml; QMGM ERDR genotype) and *M. fortuitum* (MIC, 8 to 16 μg/ml; ILQM ERDR genotype) were cloned into a multicopy vector and used to transform the exquisitely susceptible strain *M. smegmatis* mc^2155 (MIC, 0.5 μg/ml). Constructs with *M. smegmatis* wild-type (ILQM ERDR genotype) and mutant (FLQM ERDR genotype) alleles (31) were used as controls.

Transformation of *M. smegmatis* with the *M. abscessus emb* region or with the *M. smegmatis* mutant allele resulted in a 500-fold increase in the MIC (256 μg/ml) (Table 2). In contrast, multicopy vector expression of the *M. fortuitum* or *M. smegmatis* wild-type allele increased the MIC for the recombinant *M. smegmatis* 128-fold (64 μg/ml) and 20-fold (10 μg/ml), respectively.

DISCUSSION

This report presents additional genetic evidence for a key role of the Emb proteins—cell wall arabinosyl transferases (1)—in determining the intrinsic susceptibility or resistance to EMB in mycobacteria. It also extends prior data (25, 31) by conclusively identifying EmbB mutations in *M. tuberculosis* that are associated with high-level resistance (MIC, ≥20 μg/ml), while in the absence of a mutation the MICs generally remain below 10 μg/ml. These data raise two practical issues regarding (i) the determination of critical drug concentrations for screening for EMB resistance and (ii) the value of EMB in the treatment of infections with moderately resistant *M. tuberculosis* strains.

The choice of critical drug concentrations for testing of EMB susceptibility is subject to controversy, and testing of susceptibility to this drug remains suboptimally standardized (15). Current recommendations for testing in 7H10 medium call for an EMB concentration of 5 μg/ml (14). The critical concentration in BACTEC 12B medium has been recently decreased from 7.5 to 2.5 μg/ml as recommended by the manufacturer, as the best agreement between both susceptibility testing methods is reached with EMB at 2.5 μg/ml of BACTEC 12B (34). However, Lee and Heifets have proposed a screening concentration in BACTEC of 4 μg/ml based on data indicating that for a subset of wild-type isolates the MICs may be up to 3.8 μg/ml (16). Our genetic analysis indicates that all systems would detect those isolates with *embB* mutations. In contrast, the moderate decrease of susceptibility observed in a subset of clinical isolates without an ERDR mutation may be missed by those laboratories screening with BACTEC at 7.5 μg/ml, as the MIC for these strains lies between 5 and 10 μg/ml.

It is likely that isolates without an ERDR mutation likely represent the first level in the multistep phenomenon of EMB resistance acquisition. In these strains, moderate resistance may result from Emb overexpression or from mutations outside the ERDR (25, 31). What are the implications for patient management of the existence of such moderate-resistance organisms? The peak concentration in serum attainable in humans is 2 to 6 μg/ml after oral administration of 20 to 30 mg of EMB per kg (18). However, accumulation in macrophages and inflammation sites has been reported (12) and EMB enhances susceptibility to other antimycobacterial drugs (9) regardless of the actual MICs (20). Thus, in the absence of more precise information on EMB levels in tissue it can be hypothesized that moderately resistant isolates (those without an *embB* mutation) may be amenable to treatment with EMB.

Knowledge about the activity of EMB against NTM is limited. Among the slowly growing mycobacteria, *M. kansasii*, *M. xenopi*, *M. malmoense*, *M. szulgai*, *M. marinum*, *M. gordonae*, *M. terrae*, and *M. nonchromogenicum* are generally regarded as susceptible (9). Two-thirds of *M. avium-intracellulare* strains are tentatively classified as susceptible to EMB, while *M. simiae* and, perhaps, *M. asiaticum* and *M. haemophilum* are regarded as resistant (9). Because of its poor in vitro activity, EMB has not been considered to be of use for the treatment of infections caused by rapidly growing mycobacteria (6, 9).

Sequencing of the conserved ERDRs of 13 NTM strains allowed the identification of a unique variant sequence in three organisms—*M. leprae*, *M. abscessus*, and *M. chelonae*—which characteristically exhibit intrinsic high-level resistance to EMB (6, 11, 13, 19, 21, 26). Transfer of the *emb* region carrying the variant allele to the susceptible species *M. smegmatis* resulted in an increase in the MIC parallel to that achieved by introduction of a cosmid carrying the *M. smegmatis* mutant allele (≥500-fold increase) (31). This situation mirrors that encountered with the fluoroquinolones, in which the natural amino acid composition at a critical region in GyrA sets the baseline susceptibility within the mycobacterial genus and across bacterial species. Mutations in such critical regions also lead to acquired resistance to these drugs (7, 32). Whether the specific amino acid motifs found in EmbB represent the key factor determining the phenotype can only be inferred by analogy to *M. tuberculosis* and *M. smegmatis* acquired resistance data (25, 31). However, the possibility is not excluded that the overall structure of the *emb* operon or the baseline expression levels of the *emb* genes in the native host might influence the level of susceptibility to EMB. Our own data on *M. fortuitum emb* gene transfer to *M. smegmatis* suggest this possibility.

Is the ERDR part of a glycosyl transferase active site to which EMB binds? The stereospecificity of EMB—only the dextro isomer of EMB is active—indicates that the drug binds to a specific cellular target (33). As EMB has been proposed to act as an arabinose analog (17), the specific target could be an arabinosyl transferase active site. Indeed, overexpression of wild-type Emb proteins results in resistance, an effective mechanism to overcome direct target inhibition by a drug (24). The highly conserved features of the *embB* ERDR among all mycobacteria and among the various arabinosyl transferases identified (EmbC, EmbA, and EmbB) make it a potential active site. This notion is further supported by the observation that only some mutations are tolerated at codon 306—isoleucine, valine, and leucine, two of which are amino acids used at this position in other Emb proteins. Other residue substitutions generated by single nucleotide mutations (lysine, arginine, and threonine) have never been identified in *M. tuberculosis* (25, 31), as they might be incompatible for the function of the region. These observations led us to speculate that the ERDR constitutes the glycosyl transferase active site to which EMB directly binds.

The present data support the notion that the *emb* region determines both intrinsic and acquired resistance to EMB. In addition, these and previous results from gene disruption and subcloning experiments (1, 4) suggest that EmbB represents the most EMB-sensitive enzyme of the family of arabinosyl transferases. This information may represent useful structure-function information for understanding the biological basis of

the activity of glycosyl transferases and might help in the reassessment of current recommendations for the screening and treatment of infections with mycobacterial strains with low to moderate EMB resistance.

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