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Mutations in the *embB* Locus among Korean Clinical Isolates of *Mycobacterium tuberculosis* Resistant to Ethambutol

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Resistance of *Mycobacterium tuberculosis* to ethambutol (EMB) has been assigned to an operon, *embCAB*, which has been proposed to be a structural gene for mycobacterial arabinosyl transferases. Recently, genetic events resulting in structural mutations at *embB* have been proposed as major contributors to the EMB-resistance of isolates whose minimum inhibitory concentration (MIC) level is higher than 20 μg/ml. On the contrary, isolates with a MIC level lower than 20 μg/ml do not seem to contain any sequence alterations. In this study, in an effort to understand the role of *embB* mutations at a low-level of EMB resistance, we investigated the sequence polymorphisms of clinical isolates whose MIC levels are lower than 10 μg/ml. Accordingly, the sequence alterations of a 312-bp region of the *embB* gene containing the 306th codon, which has been assigned as a hot-spot for EMB-resistance related mutations, were determined for 21 EMB-resistant and 5 EMB-susceptible clinical isolates. In brief, among 21 EMB-resistant isolates examined, 12 (57.1%) contained mutations in *embB* (10 at the 306th codon and 2 at other sites), and the remaining isolates 9 contained no mutations in any region of *embB*. The observed mutations included M306V, M306I, and M306L substitutions that have been reported previously. However, 3 were novel types, which included M306T, A313G and Y322C, D331Y double substitutions. On the other hand, all of the EMB-susceptible isolates were found to be free of mutations. In conclusion, our findings suggest that sequence polymorphism of *embB* may play a pivotal role in the EMB-resistance of *M. tuberculosis*.

Key Words: *M. tuberculosis*, ethambutol resistance, *embB* mutations

INTRODUCTION

Despite the availability of an efficient case-findings and treatment program, tuberculosis remains a major public health problem in developing countries.[1,2] Moreover, the spread of human immunodeficiency virus (HIV) poses a serious threat to tuberculosis control.[3] Even though susceptible *M. tuberculosis* can be successfully treated with modern antituberculosis drugs, the increased frequency of multidrug-resistant strains of *M. tuberculosis* (MDR-TB) and the highly contagious nature of the disease[4-6] call for better surveillance systems for the detection of MDR-TB.[7-9] Currently, enthusiastic efforts to define the genetic basis of antimicrobial resistance in mycobacteria have been made. The basic assumption underlying much of this interest is the development of a rapid and simple but accurate detection system of resistant strains; however currently progress is slow.

Ethambutol [2,2'-(ethylenediimiino)-di-1-butanol, (EMB)] is a synthetic compound, known for its antimycobacterial activity.[10] It is one of first-line antituberculosis drugs and is recommended for

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the treatment of diseases caused by *M. tuberculosis* as well as opportunistic infections caused by *M. avium* complex.[11,12] The mechanism of EMB and the genetic basis for EMB resistance are not clear.

The effects of EMB seem to be pleiotropic, and thus, several hypothesis have been proposed concerning its mode of action.[13-18] The primary target of EMB is known to be the mycobacterial cell wall that consists of an outer layer of mycolic acids covalently bound to peptidoglycan via arabinogalactan.[19] Since EMB inhibits the polymerization of cell wall arabinan, which causes an accumulation of the lipid carrier decaprenol phosphoarabinose, it has been suggested that the drug interferes with the transfer of arabinose to the cell wall acceptor.[2,20]

Recently, the cellular target for EMB was sought using EMB resistant strains of *M. avium, M. leprae, M. smegmatis* and *M. tuberculosis* and these efforts led to the identification of an operon rendering EMB-resistance, now named emb.[21,22] Even though the genetic organization of these loci in each organism is differs, their involvement in EMB-resistance is generally accepted. The embCAB genetic locus of *M. tuberculosis,* for instance, has been proposed to encode for mycobacterial arabinosyl transferases.[22] In addition, it has been shown that the EMB-resistance of *M. tuberculosis* is related to genetic alterations of the *embB*.[22-24] According to the results of these studies, genetic alterations were found exclusively at the 306th codon of *embB*, which results in amino acid substitutions. On the other hand, mutations at other sites in the *embB* region were found at much less frequent levels. Even though the results of studies vary, 47 - 69% of EMB resistant *M. tuberculosis* had an amino acid change in the region of *embB* investigated and the majority of alterations occurred at position 306 (89%), whereas none of the EMB-susceptible strains contained sequence alterations. Furthermore, sequence alterations seemed to be closely co-related with EMB-resistant isolates with MIC levels higher than 20 μg/ml, as clinical isolates with an MIC level of below 20 μg/ml did not contain any sequence alterations at the *embB* locus.[22-24]

This study was initiated to find out the molecular basis of EMB-resistance in clinical isolates of *M. tuberculosis* with an MIC level lower than 20 μg/ml, that is between 2 μg/ml and 10 μg/ml. For this purpose, the sequence polymorphism of the *embB* region containing the 306th codon were investigated to identify the relationship between this structural gene and the mechanism of EMB-resistance in *M. tuberculosis.*

MATERIALS AND METHODS

M. tuberculosis isolates

Twenty-six clinical isolates of *M. tuberculosis* were obtained from the Korean Institute of Tuberculosis, The Korean National Tuberculosis Association, Seoul, Korea. The drug resistance of isolates was determined using the proportion method described by Lowenstein-Jensen.[8] All of the isolates were obtained from Korean patients in TB clinics. No genetic relatedness was found between any of the isolates by IS*6110*-based RFLP analysis (see below). The critical concentrations for antituberculosis drug susceptibility tests were as follows: INH (0.2 μg/ml), ETH (40 μg/ml), rifampin (RIF, 20 μg/ml), emviomycin (EVM, 30 μg/ml), cycloserine (CS, 30 μg/ml), para-aminosalicylic acid (PAS, 1.0 μg/ml), ofloxacin (OFX, 2.5 μg/ml), streptomycin (STR, 10 μg/ml), kanamycin (KM, 40 μg/ml), and ethambutol (EMB, 2.0 μg/ml) in Lowenstein-Jensen (L-J) medium. For isolates resistant to 2.0 μg/ml EMB, the MIC's of bacterial samples VS. EMB were determined (12), and isolates with MIC levels $\leq$ 10 μg/ml were used.

Outward-PCR

The genetic relatedness of isolates was tested by molecular fingerprinting using the IS*6110*-based outward PCR method.[18,25-27] Briefly, outward-PCR employs a single primer based on the invert-repeat fragment located at the ends of IS*6110* whose sequence was 5'-GACIIICCGGG GCGGTTCA-3', where "I" was inosine. The 3' end of the primer was directed outwardly from both sides of the inverted repeats present in IS*6110*, amplifying the flanking sequences between two copies of IS*6110*. PCR was carried

out using approximately 10 ng of genomic DNA, which had been prepared by the freeze-boiling technique,[28] in a final volume of 50 μl and in a reaction buffer containing 0.4 pmol of primers, 2 mM $MgCl_2$, 200μM of deoxynucleotide triphosphates, and 2.5 units of *Taq* polymerase. DNA samples were denatured at 95℃ for 3min before amplification over 35 cycles at 94℃ for 1 min, 58℃ for 1 min, and 72℃ for 2 min using a Thermocycler (model 9600, Perkin Elmer). After amplification, the samples were incubated for 7 min at 72℃ to complete the elongation of the PCR intermediate products. Positive and negative controls were used for each reaction. The reference strains *M. tuberculosis* H37Rv and H37Ra were used as positive controls, and the negative control was the PCR mix without DNA. PCR products were analyzed by electrophoresis through a 1.2% agarose gel and stained with ethidium bromide. Each sample was tested at least three times in order to ensure reproducibility.

PCR, cloning, and sequencing

The primer sets used to amplify *embB* (from codon 233 to codon 337) were 5′-CTGAAACTGCTG GCGATCAT -3′ and 5′-CTGACATGGGTCATCAG CGC-3′) and these resulted in a 312-bp PCR product.[22] PCR was conducted as described previously, except that 50 ng of template DNA and 10 pmol of each set of oligonucleotide primers were used. The DNA amplification cycle included (a) a denaturation stage at 94℃ for 1 min, (b) a primer annealing stage at 58℃ for 1 min, and (c) a DNA extension stage at 72℃ for 1 min. PCR products amplified with each set of PCR primers were then purified using a Geneclean kit (BIO101, Vista, Calif., USA) and cloned into pT7blue (Novagen, Madison, Wis., USA). At least 3 individual clones were obtained and subjected to sequence analysis to ensure the accuracy of the PCR amplification. Escherichia coli strain, XL1-Blue (Strategene, La Jolla, Calif., USA) was used to transform and purify the plasmid DNA.

DNA sequencing was carried out using the dideoxy nucleotide-chain termination method[29] using an ARL automatic sequencer (Pharmacia Biotechs, Uppsala, Sweden).

RESULTS

At total of 26 clinical isolates were used in this study to characterize the molecular mechanism of EMB resistance of *M. tuberculosis*. Among 26 isolates, 21 were resistant to EMB with an MIC level of less than (8μg/ml; the remainder of the 5 isolates were susceptible to EMB. Of the 21 isolates resistant to EMB, 18 (85.7%) were multidrug resistant, as they were resistant to both RMP and INH. The relatedness of each isolate was shown by PCR-based typing (Fig. 1) and by comparing the drug resistant profiles (Table 1). Sometimes, several isolates seemed to belong to certain type of IS*6110*-based outward-PCR profile, however, these strains could be further differentiated using their drug resistance profiles.

In order to characterize the molecular basis of EMB resistance, all of the samples were subjected to PCR using specific primers (EMB4 and EMB5), which amplified the 312-bp region of the *embB* gene (codons 233 to 337). This region includes the 306th codon of the *embB* gene, which has been associated exclusively with mutation among EMB-resistant *M. tuberculosis* isolates with MIC levels higher than 20μg/ml. PCR product were then cloned and sequenced, and the results of this

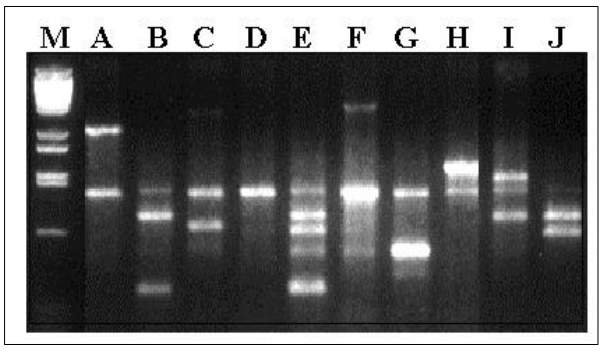

Fig. 1. Types of clinical isolates of *M. tuberculosis* as classified by the IS*6110*-based outward-PCR. Lanes: M, DNA size marker (λ DNA digested with Bst EII); A-J, profiles of outward-PCR with 21 clinical isolates of *M. tuberculosis* resistant to EMB. The outward-PCR profiles of 5 EMB-susceptible clinical isolates were also analyzed and each isolate shown to be genetically unrelated (data not shown). DNA fragments amplified by outward-PCR were run on a 1.2% agarose gel and the banding patterns compared. PCR was repeated at least three times to confirm its reproducibility. Letters represent the IS6110 DNA fingerprinting types based on the outward-PCR profiles.

Table 1. Characteristics of EMB-resistant Clinical Isolates of *M. Tuberculosis* Based on Antibiotics Susceptibility Test and Outward-PCR Typing

Isolate No.	Resistance to										IS6110-based Outward-PCR Types
	EMB	INH	RIF	SM	KM	CS	OFX	EVM	PTH	PAS	
1	+	+	+	+	+	-	+	-	-	+	A
2	+	+	+	-	-	-	+	-	+	-	B
3	+	+	+	+	+	-	+	+	-	+	C
4	+	+	+	+		-	+	-	-	+	A
5	+	+	+	+	+	-	-	+	-	+	A
6	+	+	+	+	+	-	-	-	-	+	E
7	+	+	+	+	-	-	-	-	-	-	F
8	+	+	+	-	-	+	+	-	+	+	G
9	+	+	+	-	-	-	+	-	-	-	H
10	+	+	+	-	-	-	-	-	-	-	I
11	+	+	+	+	-	-	-	-	-	+	B
12	+	+	+	+	+	+	-	-	-	+	F
13	+	+	+	+	-	-	-	-	-	-	A
14	+	+	+	-	+	-	-	-	-	-	D
15	+	+	+	+	-	-	-	-	-	+	J
16	+	+	-	-	+	-	+	-	-	-	C
17	+	-	-	+	+	-	+	-	-	+	B
18	+	+	+	-	-	-	+	-	+	-	E
19	+	-	+	+	+	+	+	-	+	+	J
20	+	+	+	+	+	+	-	-	+	+	A
21	+	+	+	+	-	-	-	-	-	+	A

The '+' indicates the resistance to drug, while the '-' indicates the susceptibility.

sequence analysis are summarized in Table 2.

In brief, of 21 EMB-resistant isolates investigated, sequence alterations were detected in 12 (57.1%) isolates. These isolates included point mutations at the 306th codon of *embB*, and M306V, M306I, M306L, and M306T substitutions. Of these, M306V and M306I, M306L, had been previously reported, whereas M306T had not. In addition, other types of substitutions were also identified at sites other than the 306th codon, such as, the D331Y and A314G/Y322C double substitutions. EMB-susceptible isolates, on the other hand, did not contain any sequence alterations.

DISCUSSION

As has been previously reported, sequence alterations were detected among EMB-resistant isolates. Furthermore, the mutation rate at the 306th codon was found to dominate with a frequency of 10/12 (83%), indicating the importance of structural alterations of this site in EMB resistance.

Among the mutations found at the 306th codon, substitutions such as methionine to valine or methionine to isoleucine were the most common types found in the *embB* gene, whereas methionine to leucine was found to be rare. On the other hand, methionine to threonine substitution is a novel substitution, as was substitution at 331st (D331Y) and the double substitutions at the 314th and the 322nd (A314G and Y322C) codon. These findings suggest that sequence polymorphisms of the *embB* gene are relate to EMB resistance and that these may be more extensive than previously expected.

The difference between our current result and previous reports is the finding of sequence alterations in EMB-resistant clinical isolates, with MIC levels lower than 8 μg/ml. Evidence showing the association between sequence alterations and high levels of EMB-resistance can be found in several

Table 2. The Genetic Characteristics of Clinical Isolates of *M. Tuberculosis* Resistant to EMB with Mutations in *embB* Locus

Sample No.	EMB MIC (ug/ml)	Base changes	Amino acid changes
1	2	ATG → GTG	Met306Val
2	4	ATG → GTG	Met306Val
3	4	ATG → GTG	Met306Val
4	4	ATG → GTG	Met306Val
5	8	ATG → GTG	Met306Val
6	2	ATG → ATA	Met306Ile
7	4	ATG → ATA	Met306Ile
8	4	ATG → ATA	Met306Ile
9	2	ATG → TTG	Met306Leu
10	2	ATG → ACG	Met306Thr
11	4	GAT → TAT	Asp331Tyr
12	4	GCC → GGC, TAT → TGT	Ala314Gly, Tyr322Cys
13	2	None	None
14	2	None	None
15	4	None	None
16	2	None	None
17	8	None	None
18	2	None	None
19	4	None	None
20	4	None	None
21	8	None	None

Shaded area illustrates sequence changes that were not reported in the previous studies.

studies.[22-24] In particular, the notion of an association between the presence of M306V and high level EMB-resistance originates from an analysis of isogenic *M. tuberculosis* isolates from a single patient before and after the development of EMB resistance.[23] This study demonstrated the occurrence of M306V mutation associated with an increase of EMB resistance, associated with an MIC increase from 5 to 40 μg/ml. Thus, our result seemed to support the role of M306V mutation in EMB-resistance.

However, contrary to previous studies, our results show that clinical isolates whose MIC level is less than 20 μg/ml also contain sequence alterations in *embB*. In addition, major types of substitutions, such as, M306V and M306I were dominant mutations in the *embB* region. Moreover, the rate of substitutions (83%) found at the 306th codon of *embB* in EMB-resistant clinical isolates with a low MIC level (less than 8 μg/ml) was as high as that found in isolates with a high MIC level (more than 20 μg/ml) (89%).

Nevertheless, several isolates, did not contain any sequence alterations in the *embB* region as determined by this study. This may suggest the existence of a sequence polymorphism in an *embB* region, which we did not investigate. On the other hand, it is also possible that another mechanism is operative, which explains the EMB-resistance of *M. tuberculosis* isolates with intact *embB* regions. It has been previously noted that in addition to the structural mutations found in the open reading frames of the emb locus, that the overexpression of emb proteins seems to be associated with the low level of EMB resistance in *M. smegmatis* and the high level of EMB-resistance in *M. tuberculosis*. However, no sequence alteration at promoter of the emb operon, which may explain the mecha-

nism of the overexpression of emb in such organisms has been found, suggesting the need for further investigation of the emb locus in order to gain a clear understanding of EMB-resistance mechanisms.

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