INTRODUCTION

Tuberculosis is a major global health problem (WHO, 2009). The emergence of multidrug-resistant (MDR) tuberculosis and, more recently, of extensively drug-resistant tuberculosis, is widely considered to be a serious threat to global tuberculosis control (CDC, 2006; Migliori et al., 2007; Raviglione & Smith, 2007). The control of tuberculosis remains one of the more elusive goals in medicine. Rapid detection of drug resistance is essential to design appropriate treatment regimens, prevent treatment failure and thus reduce the further spread of drug-resistant isolates.

Ethambutol (EMB) [dextro-2,2′-(ethylenediimino)-di-1-butanol] is a narrow-spectrum antimycobacterial agent that is used for the treatment of tuberculosis. EMB is a first-line anti-tuberculous agent that is especially important when used in drug combinations to prevent the emergence of drug resistance or to treat single drug-resistant tuberculosis (WHO, 1997). Further, streptomycin has been replaced by EMB as a key drug in the intensive phase of tuberculosis chemotherapy as it is less expensive and patient compliance is better with this drug (Rabarijaona et al., 1999). This agent has been proposed to be an arabinose analogue; the specific target is likely to be an arabinosyltransferase, presumably a functionally important site. A two-gene locus (embAB) that encodes arabinosyltransferase has been studied to elucidate a potential mechanism of EMB resistance (Alcaide et al., 1997; Telenti et al., 1997).

Amino acid replacements at position 306 of EmbB have been shown in many studies to be present in EMB-resistant, but not EMB-susceptible, organisms (Alcaide et al., 1997; Ramaswamy & Musser, 1998; Sreevatsan et al., 1997). However, Mokrousov et al. (2002) detected mutations of embB306 in both EMB-susceptible and EMB-resistant strains. EMB is an important antimycobacterial drug as it enhances the effect of other companion drugs including aminoglycosides, rifamycins and quinolones. Moreover, this drug has also been shown to significantly decrease the levels of bacteraemia in patients with AIDS (Kemper et al., 1994).

Correlation of the phenotypic ethambutol susceptibility of Mycobacterium tuberculosis with embB gene mutations in Korea

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The phenotypic resistance to ethambutol (EMB) in Mycobacterium tuberculosis with embB gene mutations is still unclear. This study was designed to better understand EMB resistance due to embB gene mutation. Sequencing analysis of the embB gene was performed for 124 EMB-susceptible and 93 EMB-resistant M. tuberculosis strains isolated from South Korea. The MIC was determined for EMB-susceptible M. tuberculosis strains with the embB mutation and wild-type on Löwenstein–Jenson (LJ) solid medium in duplicate. Two (2.8%) of 72 pan-susceptible, two (9.1%) of 22 any-drug-resistant but EMB-susceptible, nine (30.0%) of 30 multidrug-resistant (MDR) but EMB-susceptible and 84 (90.3%) of 93 EMB-resistant M. tuberculosis strains possessed embB mutations at various codons including 306, 319, 354, 399, 405, 406, 459 and 497. Strains with embB mutations at codons 306, 354, 399, 405 and 497 had highly pronounced EMB resistance, while strains with mutations at codons 319 and 406 mutations were moderately resistant and those with an embB459 mutation were EMB-susceptible at the critical concentration (2.0 μg ml⁻¹) on LJ solid medium. However, the mean MIC for strains with embB mutations (1.42 μg ml⁻¹) was higher than that for strains without the embB mutation (1.0 μg ml⁻¹) in EMB-susceptible M. tuberculosis isolates (P=0.0052). Three novel embB mutations at codons 399, 405 and 495 were identified in this study. These results support the hypothesis that embB mutation except for a few specific mutation types may be the main cause of EMB resistance.

Abbreviations: EMB, ethambutol; MDR, multidrug-resistant.

A supplementary table is available with the online version of this paper.
Consequently, mutations in codon 306 have been suggested as molecular markers for the rapid detection of EMB resistance (Lee et al., 2004; Plinke et al., 2006; Van Rie et al., 2001). Furthermore, two recent studies demonstrated a clear causative relationship between embB306 point mutations and in vitro EMB resistance by allelic-exchange experiments (Safi et al., 2008; Starks et al., 2009). Identification of additional mutations occurring in EMB-resistant organisms will be useful in further understanding the mechanisms of resistance to this primary anti-tuberculosis agent. In our study, we tried to understand EMB resistance in detail by studying its relationship with mutations in the embB gene.

**RESULTS**

**Analysis of the embB mutation in EMB-susceptible *M. tuberculosis* strains**

A total of 124 EMB-susceptible and 93 EMB-resistant *M. tuberculosis* strains were analysed to determine a potential relationship between EMB resistance and embB mutation. Two (2.8 %) of 72 pan-susceptible *M. tuberculosis* strains possessed embB mutations at codons 360 (silent mutation, Val to Val) and 459 (Gly to Asp) (Table 1). These two strains revealed MICs of 1.25 µg ml⁻¹ and 0.75 µg ml⁻¹, which were not higher than the MICs for pan-susceptible *M. tuberculosis* strains without embB mutation (Table 2). Two strains (9.1 %) out of 22 any-drug-resistant EMB-susceptible *M. tuberculosis* strains had embB mutations (Table 1). These strains were rifampicin mono-resistant with an embB406 mutation. MICs for these strains were higher than those for pan-susceptible strains (Table 2).

Nine (30.0 %) of 30 MDR but EMB-susceptible *M. tuberculosis* strains revealed various embB mutations such as Met306Ile, Met306Val, Tyr319Ser, Gly406Asp, Gly406Ala and Gln497Lys (Table 2). One strain (3.8 %) was EMB-susceptible out of 26 strains with Met306Ile mutation (Table 1). Two (6.3 %) of 32 strains with Met306Val mutation were MDR but EMB-susceptible. One (50 %) of two strains with Tyr319Ser mutation was EMB-susceptible and had a higher MIC (1.5 µg ml⁻¹) than the MICs for pan-susceptible strains.

Four (66.7 %) of six Gly406Asp mutants (excluding embB405, 406 simultaneous mutant) and two (66.7 %) of three Gly406Ala mutants were EMB-susceptible. embB406 mutation seems to be less associated with EMB resistance at the LJ medium critical concentration (2.0 µg ml⁻¹) in this study. One (50.0 %) of two strains with Gln497Lys mutation was EMB-susceptible. However, all other Gln497Pro and Gln497Arg mutants revealed EMB resistance (Table 1).

**embB mutation rate according to drug resistance**

The embB mutation rate for 93 EMB-resistant *M. tuberculosis* strains was 90.3 %, but 2.8 % in pan-susceptible strains, 9.1 % in any-drug-resistant but non-MDR strains and 30.0 % in MDR but EMB-susceptible *M. tuberculosis* strains (Table 1). The proportion of strains with embB mutations increased with increasing anti-tuberculous drug resistance (i.e. in the order of pan-susceptible, any-drug-resistant EMB-susceptible, MDR EMB-susceptible and EMB-resistant *M. tuberculosis* strains) as shown by Bartholomew’s test (a statistical value of 141.65, higher than the critical value, which is in the range 3.805–4.354).

In the 93 EMB-resistant strains, the embB306 mutation was most prevalent (67.9 % of strains), followed by the embB497
mutation (14.3%) and the embB406 mutation (7.1%) (Table 3). The rate of embB mutation in EMB-resistant strains also increased in proportion to the number of drugs that the strains were resistant to (a statistical value of 76.21, higher than the critical value of 3.82 by Bartholomew’s test).

Comparison of MICs

The mean MICs for six pan-susceptible strains versus 11 EMB-susceptible strains but with other drug resistances and embB mutations at codons 306, 319, 406 or 497 were 1.0 μg ml⁻¹ and 1.42 μg ml⁻¹, respectively. This result shows that the embB mutation in the drug-resistant M. tuberculosis strains is associated with increased MICs of EMB (Wilcoxon statistic = 29.0, P-value = 0.0052), even though the critical concentration for EMB-susceptible strains was 2.0 μg ml⁻¹.

Novel embB mutations and EMB susceptibility

We identified novel embB mutations at codons 399, 405 and 459. We found two Asn399Thr and two Glu405Asp embB mutations in EMB-resistant M. tuberculosis strains. One Gly459Asp embB mutation was found in pan-susceptible M. tuberculosis strains.

Table 1. Distribution of the embB mutations

<table>
<thead>
<tr>
<th>Amino acid change</th>
<th>Nucleotide change</th>
<th>EMB-S (%)</th>
<th>EMB-R (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met306lle</td>
<td>ATG–ATC</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Met306lle</td>
<td>ATG–ATA</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>Met306lle</td>
<td>ATG–ATT</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>Met306Leu</td>
<td>ATG–CTG</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Met306Val</td>
<td>ATG–GTG</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tyr319Asp</td>
<td>TAT–GAT</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tyr319Ser</td>
<td>TAT–TCT</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Asp354Ala</td>
<td>GAC–GCC</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Val360Val</td>
<td>GTG–GTA</td>
<td>1*</td>
<td></td>
</tr>
<tr>
<td>Asn399Thr†</td>
<td>AAC–ACC</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Glu405Asp†</td>
<td>GAG–GAC</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Glu405Asp, Gly406Asp</td>
<td>GAG–GAC, GGC–GAC</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gly406Asp</td>
<td>GGC–GCC</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Gly406Ala</td>
<td>GGC–GGC</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Gly406Ser</td>
<td>GGC–AGC</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Gly406Gly</td>
<td>GGC–GGA</td>
<td>1*</td>
<td></td>
</tr>
<tr>
<td>Gly459Asp†</td>
<td>GGC–GAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gln497Lys</td>
<td>CAG–AAG</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gln497Pro</td>
<td>CAG–CCG</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Gln497Arg</td>
<td>CAG–CGG</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Mutation total</td>
<td>2 (2.8%)</td>
<td>2 (9.1%)</td>
<td>9 (30.0%)</td>
</tr>
<tr>
<td>None</td>
<td>70 (97.2%)</td>
<td>20 (90.9%)</td>
<td>21 (70.0%)</td>
</tr>
</tbody>
</table>

*Synonymous mutation.
†Novel mutation.

DISCUSSION

The ever-increasing burden of drug resistance is a serious concern in the world, particularly for patients with M. tuberculosis infection. This mycobacterium uses various mechanisms to evade killing by therapeutic drugs, including mutations in genes that encode drug target proteins (Cole et al., 1998; Morris et al., 1995; Musser, 1995; Shi et al., 2007). The objective of this study was to identify mutations in a drug target gene in strains of M. tuberculosis prevalent among the Korean population. embB gene mutation was believed to be the main cause of EMB resistance in M. tuberculosis. However, the relationship between embB gene mutation and EMB resistance is controversial because of embB mutation in EMB-susceptible M. tuberculosis strains (Mokrousov et al., 2002; Lee et al., 2004; Van Rie et al., 2001; Shi et al., 2007; Hazbón et al., 2005).

Hazbón et al. (2005) reported that 46 of 100 embB306 mutants were EMB-susceptible while 54 were EMB-resistant. After retesting, a few of the mutants were reclassified from susceptible to resistant. The EMB MICs for the embB306 mutants ranged from <1 to >32 μg ml⁻¹ (Hazbón et al., 2005). embB306 mutation was considered a common polymorphism with no relation to EMB resistance by Srivastava et al. (2009).
Depending upon mutation site or type, EMB resistance was found to be different. We found two \textit{embB} mutations among 72 pan-susceptible \textit{M. tuberculosis} strains. One of the two strains had a synonymous mutation at codon 360, which did not affect EMB resistance. The second pan-susceptible strain had a Gly459Asp mutation, which is a novel mutation. However, this mutation also did not affect EMB resistance. The \textit{embB} mutation in pan-susceptible strains in particular may not affect EMB resistance. Similar to our results, Hazbón et al. (2005) also could not find \textit{embB} mutation in 582 pan-susceptible \textit{M. tuberculosis} strains.

Our findings suggest that certain mutations of the \textit{embB} gene may not affect EMB resistance as had been postulated in previous reports (Hazbón et al., 2005; Sekiguchi et al., 2007).

Only three MDR isolates (5.0\%) out of 60 \textit{embB}306 mutants were EMB-susceptible in this study. Even though we also detected the \textit{embB}306 mutation in EMBSusceptible clinical isolates, an \textit{embB}306 substitution experiment from wild-type to mutation type proved that \textit{embB}306 mutation increased the EMB MIC (Safi et al., 2008; Starks et al., 2009). Safi et al. (2008) found that the substitution at \textit{embB}306 did not affect rifampicin or isoniazid MICs. Further, we found that six (54.5\%) out of 11 \textit{embB}406 mutant strains (excluding two strains, one with a synonymous mutation

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{ID} & \textbf{Phenotype of DST*} & \textbf{embB mutation codon and type} & \textbf{Mean MIC of duplicate retest (\textmu g ml}^{-1}) \textbf{)} \\
\hline
KMRC 00119-90955 & Pan-S & None & 1.25 \\
KMRC 00119-90995 & Pan-S & None & 0.75 \\
KMRC 00119-90807 & Pan-S & None & 1.0 \\
KMRC 00119-90808 & Pan-S & None & 1.0 \\
KMRC 00119-90999 & Pan-S & Val360Val (GTG–GTA) & 1.25 \\
KMRC 00119-90806 & Pan-S & Gly459Asp (GGC–GAC) & 0.75 \\
KMRC 00119-89635 & R & Gly406Asp (GGC–GAC) & 1.75 \\
KMRC 00119-90870 & R & Gly406Asp (GGC–GAC) & 1.63 \\
KMRC 00119-84132 & IR (MDR) & Met306Ile (ATG–ATA) & 1.5 \\
KMRC 00119-87595 & IR (MDR) & Met306Val (ATG–GTG) & 1.5 \\
KMRC 00119-89732 & IR (MDR) & Met306Val (ATG–GTG) & 0.88 \\
KMRC 00119-88533 & IR (MDR) & Tyr319Ser (TAT–TCT) & 1.5 \\
KMRC 00119-83962 & IR (MDR) & Gly406Asp (GGC–GAC) & 0.88 \\
KMRC 00119-84671 & IR (MDR) & Gly406Asp (GGC–GAC) & 1.38 \\
KMRC 00119-86964 & IR (MDR) & Gly406Ala (GGC–GCC) & 1.75 \\
KMRC 00119-89589 & IR (MDR) & Gly406Ala (GGC–GCC) & 1.5 \\
KMRC 00119-86862 & IRS (MDR) & Gln497Lys (CAG–AAG) & 1.38 \\
KMRC 00119-84185 & IE & None & 8 \\
KMRC 00119-89348 & IRE & Met306Ile (ATG–ATC) & 4 \\
KMRC 00119-90333 & IRE & Met306Ile (ATG–ATA) & 6 \\
KMRC 00119-88732 & IRE & Met306Leu (ATG–CTG) & 6 \\
KMRC 00119-90084 & IRE & Met306Val (ATG–GTG) & 6 \\
KMRC 00119-83615 & IRSE & Asn399Thr (AAC–ACC) & 12 \\
KMRC 00119-89393 & IRSE & Asn399Thr (AAC–ACC) & 6 \\
KMRC 00119-88791 & IE & Gly406Ala (GGC–GCC) & 4 \\
KMRC 00119-85859 & IRE & Gln497Lys (CAG–AAG) & 6 \\
KMRC 00119-89319 & IRSE & Gln497Pro (CAG–CGG) & 12 \\
\hline
\end{tabular}
\caption{EMB MIC for \textit{M. tuberculosis} strains retested in duplicate on LJ medium after 5 weeks culture}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Mutation} & \textbf{Drug resistance} & \textbf{Total} \\
\hline
\multicolumn{3}{|c|}{\textbf{EMB mono Non-MDR MDR}} \\
\hline
306 & 2 & 23 & 32 & 57 (67.9\%) \\
319 & 1 & 1 & 2 (2.4\%) \\
354 & 1 & 2 & 3 (3.6\%) \\
399 & 2 & 2 (2.4\%) \\
405 & 1 & 1 (1.2\%) \\
405, 406 & 1 & 1 (1.2\%) \\
406 & 1 & 5 & 6 (7.1\%) \\
497 & 12 & 12 (14.3\%) \\
\textbf{Total} & 3 (3.6\%) & 25 (29.8\%) & 56 (66.7\%) & 84 (100.0\%) \\
\hline
\end{tabular}
\caption{Distribution of mutation codons in 93 EMB-resistant strains}
\end{table}
and another with an additional mutation at embB405) were EMB-susceptible (Table 1). embB406 mutations have also been detected previously in EMB-susceptible isolates (Lee et al., 2004; Shi et al., 2011; Ramaswamy et al., 2004). We found only one EMB-susceptible (7.7 %) strain out of 13 embB497 mutants. In a recent study, embB406 and allelic embB497 exchange increased the EMB MIC 3–3.5-fold and 6-fold, respectively, over that for the wild-type embB gene and these allelic exchanges did not affect the MIC of other anti-tuberculous drugs such as isoniazid or rifampicin (Safi et al., 2010).

For the minor mutations, two of three embB319 mutants were EMB-resistant and another one was EMB-susceptible with MDR as shown in Table 2. Likewise, the Tyr319Ser and Tyr319Asp mutations were found in EMB-resistant M. tuberculosis strains in previous reports (Plinke et al., 2006; Sugawara et al., 2005). The Asp354Ala mutation revealed EMB resistance, which has been reported previously (Sekiguchi et al., 2007).

In this study, three novel embB mutations (Asn399Thr, Glu405Asp and Gly459Asp) were detected. Though all three of the codon sites were also found in another study, those mutations produced different amino acids (Srivastava et al., 2009). Among the three novel mutations, two (Asn399Thr and Glu405Asp) were associated with EMB resistance.

Our study further suggested that the proportion of strains with embB mutations increased with the increasing number of drug resistances in both EMB-susceptible and EMB-resistant strains. This finding also agreed with those in other studies (Lee et al., 2004; Shi et al., 2011; Shen et al., 2007).

Along with the above results, we found that the mean EMB MIC for embB-mutated EMB-susceptible strains was higher than that for pan-susceptible strains. This may be caused by the limitation of conventional drug susceptibility testing methods for EMB (Kim, 2005). Kim (2005) reported that 90 % of probably EMB-susceptible strains and 30–50 % of probably EMB-resistant strains were inhibited at the critical concentration of 2.0 μg ml⁻¹ on LJ solid medium.

However, we compared only the MICs for EMB-susceptible strains with those for pan-susceptible and other-drug-resistant strains with the embB mutation, so we need further studies to compare the MICs of EMB in EMB-susceptible strains with and without the embB mutations.

In conclusion, the exact mechanism of EMB resistance is not yet clearly understood. Our data support that the embB mutation, except for a few specific mutations such as Gly459Asp, may be the main cause of EMB resistance.

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