Molecular characterization of drug resistance of *Mycobacterium tuberculosis* strains with different origins can generate information that is useful for developing molecular methods. These methods are widely applicable for rapid detection of drug resistance. A total of 166 rifampin (RIF)- and/or isoniazid (INH)-resistant strains of *M. tuberculosis* have been isolated from different parts of Vietnam; they were screened for mutations associated with resistance to these drugs by sequence analysis investigating genetic mutations associated with RIF and INH resistance. Seventeen different mutations were identified in 74 RIF-resistant parts of Vietnam; they were screened for mutations associated with resistance to these drugs by sequence analysis investigationing genetic mutations associated with RIF and INH resistance. Seventeen different mutations were identified in 74 RIF-resistant strains, 56 of which (approximately 76%) had mutations in the so-called 81-bp “hot-spot” region of the *rpoB* gene. The most common point mutations were in codons 531 (37.8%), 526 (23%), and 516 (9.4%) of the *rpoB* gene. Mutations were not found in three strains (4.05%). In the case of INH resistance, five different mutations in the *katG* genes of 82 resistant strains were detected, among which the nucleotide substitution at codon 315 (76.83%) is the most common mutation. This study provided the first molecular characterization of INH and RIF resistance of *M. tuberculosis* strains from Vietnam, and detection of the *katG* and *rpoB* mutations of the INH and RIF-resistant strains should be useful for rapid detection of the INH- and RIF-resistant strains by molecular tests.

*Mycobacterium tuberculosis* is one of the most harmful human pathogens worldwide, causing about 9.4 million incident cases of tuberculosis (TB) and 14 million prevalent cases and the deaths of 1.3 million HIV-negative and an additional 0.38 million HIV-positive people (31). Since the reemergence of TB in the mid-1980s, there have been an increasing number of drug-resistant strains throughout the world, in particular, an upsurge of *M. tuberculosis* strains that are resistant to one or more of the primary anti-TB drugs.

Early diagnosis of the disease and the rapid identification of resistance to primary anti-TB agents are essential for the efficient treatment and control of multidrug-resistant (MDR) strains. It is known that resistance to isoniazid (INH) and rifampin (RIF) is a key factor in determining the effectiveness of the currently recommended standard treatment regimens. The elucidation of the mechanism of action of these drugs, which was accomplished only recently, has led to the development of new rapid diagnostic methods (5, 8, 10, 12, 21). The rapid detection of RIF resistance is of particular importance, since it also represents a valuable surrogate marker for multidrug resistance (resistance to at least INH and RIF), which is a tremendous obstacle to TB therapy (9, 10).

Previous studies have found that about 96% of epidemiologically unrelated RIF-resistant strains have mutations in the “81-bp hot spot” core region of the *rpoB* gene of *M. tuberculosis*, which includes codons 507 to 533, encoding 27 amino acids (5). The most commonly seen mutations that occur from amino acid replacements are at codons 516, 526, and 531 (5, 6, 9, 29), and DNA sequencing of this region could be used as a clinical marker for probe assay of mutation and treatment. For INH resistance, while mutations in several genes of *M. tuberculosis* have been found to be associated with INH resistance, mutations in the *katG* gene, which encodes the catalase-peroxidase enzyme, have been the most commonly observed (26.0 to 93.6%) (7, 16, 19, 24).

Vietnam is one of the high-burden countries for *M. tuberculosis* infection globally, with a smear-positive tuberculosis prevalence of 89 per 100,000 population. In addition, Vietnam is one of the 22 countries in which 80% of the world’s new TB cases occur (31). Primary drug resistance has been monitored since 1978, with decreasing frequency up to 1998. In 2006, a 12.52% increase in the primary resistance rate was observed in comparison with that in 1978 (30.2% versus 18.18%). At the same time, an increased level of primary multidrug resistance was also observed (19.3% versus 2.3%). The Beijing genotype, which was found to be strongly genetically associated with drug and multidrug resistance, comprised at least half of the strains isolated in Vietnam (2, 4, 12). Vietnam has one of the most successful directly observed therapy short-course programs, with a cure rate of approximately 90% (89% in 1995 and 92% in 2007) and a case detection rate estimated at 37% in 1995 and at >50% since then (31). In recent years, the reported rate of resistance to at least one drug, i.e., INH, is 16% to 25.0%, and the MDR-TB rates are at 2% to 4% among the first and 23% to 27% among subsequent treated TB patients, respectively (11, 23). However, so far, only a limited number of studies have been carried out to characterize the genetic changes associated with the drug resistance of *M. tuberculosis* strains obtained from Vietnam.

In this paper, we present an investigation to profile genetic mutations associated with the RIF and INH resistance of *M. tu-
**MATERIALS AND METHODS**

**Mycobacterial growth.** *M. tuberculosis* strains were grown simultaneously in solid Ogawa medium (Korean Institute of Tuberculosis, South Korea) and liquid MGIT 960 (Becton Dickinson, Sparks, MD) at 37°C for approximately 3 to 4 weeks with occasional agitation.

**Mycobacterial strains and drug susceptibility testing.** The *M. tuberculosis* strains examined for this study were isolated from TB patients in Vietnam in 2007 to 2009. Strains obtained in different regions of the country were provided by the National Lung Disease Hospital in the north, Hue Central Hospital in the central part, and Pham Ngoc Thach Hospital in the south of Vietnam. In this source, 166 drug-resistant *M. tuberculosis* strains examined for this study were isolated from TB patients in approximately 3 to 4 weeks with occasional agitation.

**DNA preparation.** A rapid DNA extraction procedure for the direct testing of *M. tuberculosis* grown on Ogawa medium (Korean Institute of Tuberculosis, South Korea) was performed. Bacteria were suspended in 1 ml of sterile water and inactivated at 80°C for 30 min. The cells were harvested after centrifugation (12,000 rpm for 5 min) and subjected to DNA extraction. Briefly, 1 ml lysis buffer (1 ml 1 M Tris–HCl, pH 8.5, 0.1 ml 0.5 M EDTA, 0.2 ml 10% SDS, 0.4 ml 5 M NaCl in 10 ml deionized water) and 40 μl lysozyme (25 mg/ml) were added to the cell pellets. The lysate was vortexed, kept on ice for 30 min, and then vigorously vortexed after adding 40 μl proteinase K (10 mg/ml). After incubation at 56°C with medium agitation for 3 h, the lysate was heated at 100°C for 20 min; then, 1,080 μl phenol-chloroform-isoamyl alcohol (25:24:1) was added and vortexed. The supernatant was harvested after centrifugation at 13,000 × g for 10 min and transferred to a Falcon tube that was kept on ice and that contained 2 ml absolute ethanol and 200 μl 3 M sodium acetate (NaOAc). The content was mixed by inversion of the tube several times, kept at −20°C for 2 h, and centrifuged at 13,000 × g for 10 min. The supernatant was carefully decanted, and the DNA pellet was dried and then resolved in 50 μl water and transferred into an Eppendorf tube. The DNA was kept at −20°C until use.

**Standard PCR.** The DNA extract was used as a template for PCR with the primers listed in Table 1. Primer pairs rpoB_F/rpoB_R and katG_F/katG_R were used to amplify the regions within the *rpoB* and *katG* genes, respectively. The PCR was carried out for 35 cycles of denaturation at 94°C for 60 s, annealing at 50°C (for *rpoB*) or 56°C (for *katG*) for 45 s, and extension at 72°C for 60 s in a GeneAmp PCR System 9700 thermal cycler. The PCR products were examined by gel electrophoresis and purified by use of a QIAquick PCR purification kit (Qiagen).

**Cloning and DNA sequence analysis.** Purification of PCR products was used for cloning, and then recombinant plasmids were sequenced with an automated ABI Prism 377 DNA sequencer and corresponding kits from the same manufacturer (Applied Biosystems, Foster City, CA). The BLAST 2 Sequences computer program was used for DNA sequence comparisons (http://www.ncbi.nlm.nih.gov/BLAST/).

**RESULTS**

**Detection of mutations in the rpoB gene.** Seventy-four RIF-resistant *M. tuberculosis* strains (including 57 MDR strains) were subjected to DNA-sequencing analysis of the hypervariable (hot-spot) *rpoB* region. Seventeen different types of mutations were identified (Table 2) (mutations at codons 490 and 531 were not identified).
counted as one type). Table 2 shows more details on the distribution of the \textit{rpoB} mutations. Most of them were single-nucleotide mutations involving seven codons. The codons most frequently affected by point mutations were codons 531, 526, and 516, with frequencies of 37.8%, 23%, and 9.46%, respectively. However, a strain having mutations at codon 531 appeared to be associated with an additional mutation at codon 490, while mutations at codons 526 and 516 were not associated with any additional mutations at any other codons. Interestingly, 15 (20.3%) strains had a novel mutation at codon 490. Moreover, 94.7% (54/57) of MDR strains were found to harbor a mutation in the \textit{rpoB} hot-spot region, and the mutation at codon 531 also occurred at a remarkably high frequency of 49.1% (28/57) (Table 2). Three RIF-resistant strains (4.05%) contained no mutations in the sequenced region, although these strains were phenotypically resistant to RIF. No mutation was found in any of the 10 RIF-susceptible strains. Of the total of 71 RIF-resistant strains, only 56 (approximately 76%) had mutations in the 81-bp hot-spot region.

\textbf{Detection of mutations in the \textit{katG} gene.} Eighty-two \textit{M. tuberculosis} strains that were resistant to INH (including 49 MDR strains) were investigated by DNA sequencing of a 684-bp \textit{katG} fragment, including codon 315, the codon at which mutations are most frequently associated with INH resistance. Sixty-three INH strains (76.83%) had mutations at codon 315 (Table 3). The wild-type codon AGC (Ser) was altered to ACC (Thr) in 60 strains, to AAC (Asn) in 2 strains, and to ACG (Asn) in one strain. In addition, 8 strains (9.76%) had a novel mutation at codon 309 or codon 327. In 11 cases (21.87%), no mutation was found in the analyzed 684-bp \textit{katG} fragment, although these strains were phenotypically resistant to INH. Ten INH-susceptible strains had no mutation in the region (Table 3).

\section*{Discussion}

Our findings of mutations in the \textit{rpoB} and \textit{katG} genes were comparable to those reported for strains from other parts of the world, especially the common mutations, which reflect a global pattern (24). The \textit{rpoB} codons 531, 526, and 516 are the most frequently mutated codons worldwide, although variations in the relative frequencies of mutations in these codons have been described for \textit{M. tuberculosis} strains from different geographic locations (5). In our study, most of the strains with the RIF-resistant phenotype contained such missense mutations, which led to amino acid substitutions in the Ser531 (37.8%), His526 (23.0%), and Asp516 (9.46%) residues. Compared with results observed in other studies, our results showed that the mutations in codon 531 are also predominant in Vietnam. High frequencies of changes in this codon were also described recently for MDR \textit{M. tuberculosis} isolates from Poland (41%) (25), Germany (75.7%) (9), Turkey (47.6%) (1) and Beijing, China (59.2%) (13). The high incidence of these less common changes in strains from Vietnam may be explained by either geographic variations in the frequencies of particular \textit{rpoB} mutations or sample bias.

Moreover, two novel mutations were also recognized in this study. A mutation from CTG (Leu) to ATG (Met) at codon 530 and a mutation from TCG (Ser) to TGG (Trp) at codon 531 that appeared to be associated with an additional mutation from CAG (Gln) to CGG (Arg) at codon 490 in one strain have not been reported previously. One new mutation from CAG (Gln) to CAT/CGG (His/Arg) at codon 490 that is outside the 81-bp hot-spot region of the \textit{rpoB} gene was also identified in 15 RIF-resistant strains and was almost equally distributed, i.e., 3 strains from Hue Central Hospital in the central part, 4 strains from Pham Ngoc Thach Hospital in the south, and 8 strains from National Lung Disease Hospital in the north of Vietnam. Previous studies (5, 22, 26, 32) had also reported mutations outside the hot-spot region: CAG to CAT at codon 490, CCC to CAC at codon 535, GAG to GAT at codon 504, GAG to GAT at codon 541, TCG to GCG at codon 553, and ATC to TTC at codon 572.

Sequence analysis identified no mutation in three (4.05%) of the strains tested, although they were resistant to RIF as determined by the proportion method. Several recent studies revealed that mutations associated with RIF resistance can also be located outside the 81-bp \textit{rpoB} core region, although this does not occur frequently. Examples are ATC to TTC at codon 572 (1, 32), CAG to CAT at codon 490 (5), GGG to GAG at codon 534, and CCC to CAC at codon 535 (18). Another possibility is that for this resistant strain, changes have occurred in genes whose products participate in antibiotic permeation or metabolism (14).

In INH-resistant strains, neither insertions nor deletions (complete or partial) of \textit{katG} were found, which is evidence of the rare occurrence of these mutations in clinical strains, although they were reported previously by other authors (27, 28). In this study, most mutations in the 684-bp \textit{katG} fragment were observed in codon 315, indicating three types of mutations, AGC→ACC, AGC→ACG, and AGC→AAG, in 63 strains, corresponding to 76.83% of all strains. However, the frequency of mutation from AGC (Ser) to ACC (Thr) in 60 strains (73.17%) was higher than the 70% of Polish strains but was lower than the 93.6% of strains in northwestern Russia (19), 91% of strains in Latvia (28), and 85.7% of strains in Lithuania (3). In accordance with the previous reports, the most frequent mutation was AGC→ACC at \textit{katG} codon 315 (73.17%). However, mutations from GGT (Gly) to TGT (Cys) at codon 309 in four strains and from AAA (Lys) to AGA (Arg) at codon 327 in four strains have not been reported previously. These results of our study may indicate that different rates of mutation were observed in codons 315, and other new mutations may reflect geographic and epidemiologic status in Vietnam.

Among isoniazid resistance cases, 14 strains demonstrated no mutation in the \textit{katG} gene. Other studies showed that there are additional genes responsible for isoniazid resistance, such as \textit{inhA}, \textit{ahpC}, \textit{oxyR}, and \textit{kasA} (15, 20); however, they have not been included in this study. The absence of mutation in a 684-bp fragment of the above-mentioned 14 strains does not preclude \textit{katG} alteration, since any frameshift would result in loss of \textit{katG} activity (the entire \textit{katG} gene was not sequenced in this study); therefore, we cannot clarify the nature of these 14 INH-resistant strains.

The information generated from this study will be useful for

\begin{table}[h!]
\centering
\caption{DNA-sequencing data for \textit{katG} mutations in INH-resistant \textit{M. tuberculosis} strains from Vietnam}
\begin{tabular}{llll}
\hline
\textbf{Altered codon} & \textbf{Nucleotide change(s)} & \textbf{Amino acid change} & \textbf{No. and rate (%) of strains with mutations} \\
\hline
309 & GGT→TGT & Gly→Cys & 4/82 (4.87) \\
315 & AGC→ACC & Ser→Thr & 60/82 (73.17) \\
 & AGC→AAC & Ser→Asn & 2/82 (2.44) \\
 & AGC→ACG & Ser→Asn & 1/82 (1.22) \\
327 & AAA→ATA & Lys→Ile & 4/82 (4.87) \\
None & None & None (wild type) & 11/82 (13.41) \\
\hline
\end{tabular}
\end{table}
the rational design of molecular tests for rapid screening for INH and RIF resistance-related mutations in Vietnam, which will, in turn, contribute to the control of MDR TB in Vietnam and worldwide.

The prevalence of RIF-resistant mutations within the *rpoB* core region (~76%), as well as INH-resistant mutations at *katG* codon 315 (76.83%), in *M. tuberculosis* strains from Vietnam indicated the potential for a rapid diagnostic test for the detection of drug-resistant *M. tuberculosis*. Different genotypic assays have been proposed for the detection of mutations involved in drug resistance in *M. tuberculosis*.

ACKNOWLEDGMENTS

This work was supported by the project “Study on Rapid Detection of *M. tuberculosis* and Drug-Resistant *M. tuberculosis* by Biomolecular Techniques” of the Ministry of Science and Technology (Vietnam).

We thank our colleagues at Hospital 103, Military Medical University, for providing clinical strains and valuable advice.

REFERENCES