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H. 知的財産権の出願・登録状況

1. 特許取得 なし
2. 実用新案登録 なし
3. その他 なし

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（国際医学協力研究事業）

結核及び非結核性抗酸菌の迅速な菌種同定法の確立と治療法開発

分担研究報告書

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厚生労働科学研究費補助金（地球規模保健課題推進研究事業）
分担研究報告書

結核及び非結核性抗酸菌の迅速な菌種同定法の確立と治療法の開発

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研究要旨.
薬剤耐性結核菌は世界の深刻な社会問題の一つである。治療薬であるイソニアジド(INH)に対する耐性菌はよく分離されるが、耐性は *katG*、*ndh* などの遺伝子の変異による。これらの変異を標的とした DNA プローブによる迅速検出法が実用化されているが、その DNA プローブに含まれない変異も報告されている。これまでに、台湾で分離された INH 耐性結核菌で既存の耐性検出用 DNA プローブに含まれていない変異で、かつ耐性との関連が明らかにされていない変異 *ahpC* の C-10T、KatG の Y337C、Ndh の I68T を持つ株を複数見出した。今後これらの変異蛋白の機能を解析し、実際に耐性に関与しているかどうかを解析する。そして INH 耐性結核菌を検出する検査法の改良を目指す。台湾を始めアジア各国で結核罹患率の高い国で薬剤耐性結核の迅速診断に役立つことが期待される。また、ピラジナミドに対する耐性菌にも有効な新規薬剤の開発を目標に、ピラジナミドの作用メカニズムの解析を行っている。ピラジナミドの標的分子の候補であるニコチン酸フォスフォリボシルトランスフェラーゼのリコンビナント蛋白を作成し、機能解析を行った。ピラジナミドの活性型のピラジン酸はこの酵素の活性を阻害し、かつその状態で ATPase 活性を示すことが分かった。今後他の候補蛋白についても構造機能解析を進め、活性を阻害する化合物を探索して行く。

A. 研究目的

薬剤耐性結核菌は世界の深刻な社会問題の一つである。治療薬であるイソニアジド(INH)についても耐性菌がよく分離されるが、耐性は *katG* などの遺伝子の変異による。耐性菌の遺伝子の解析により、耐性に関与すると考えられる変異がいくつか報告されている。これらの変異を標的とした DNA プローブにより、耐性菌を迅速に検出する方法がいくつかのグループにより開発されている。しかしながら、その DNA プローブに含まれない変異も報告されており、その中には、実際に耐性に関与していることが証明出来ていないものがある。この研究では、台湾 CDC と共同研究で、台湾の医療機関で分離された INH 耐性結核菌で、見出された変異が実際に耐性に関与しているかどうかを確認することとした。

またピラジナミド（PZA）に対する耐性菌にも有効な新規薬剤の開発を目標に、ピラジナミドの作用メカニズムの解析を行った。PZA はニコチンアミドの構造類縁体であり、菌体内においてピラジナミダーゼ（ニコチンアミダーゼ）によって加水分解されて活性型のピラジン酸（POA）に変換される。POA の標的分子であるニコチン酸フォスフォリボシルトランスフェラーゼのリコンビナント蛋白を作成し、機能解析を行った。

B. 研究方法

台湾 CDC において、解析を行った結核菌で、INH 耐性のものについて遺伝子の変異

のスクリーニングを実施し、過去に耐性との関連が証明された変異部位以外の変異を持つもので、複数分離されたものについて、情報を蓄積した。感染研にて選びだした遺伝子を PCR で増幅し、クローニングして大腸菌で発現させ、リコンビナント蛋白を作成して機能解析を行い、変異により活性がどのように変化しているのか、またその変化が薬剤耐性と相関するかどうかを調べることとした。さらに、遺伝子に既知の変異を持たない耐性株も複数分離されたので、耐性を再度確認後、菌を感染研に送付してもらい、ゲノム解析を行うこととした。

ニコチン酸フォスフォリボシルトランスフェラーゼの解析には、この蛋白をコードする遺伝子 Rv1330c をクローニングし、大腸菌でリコンビナント蛋白を発現させて精製し、機能解析をおこなった。

倫理面への配慮　該当なし。

C. 研究結果

台湾 CDC 結核研究室と情報交換を行った。台湾 CDC ではこれまでに台湾全土から INH 耐性株を収集してきた。それらの分離株のなかで、既存の耐性検出用 DNA プローブに含まれていない変異で、耐性との関連が明らかにされていない変異 *ahpC* の C-10T、KatG の Y337C、Ndh の I68T を持つ株を複数見出した。うち、今回は KatG の Y337C、Ndh の I68T を解析対象とすることとした。結核菌の標準株 H37Rv 株のゲノム DNA を鋳型として、それぞれの遺伝子を増幅し、発現ベクターにクローニングし、さらに耐性株と同じ部位に変異を導入し、耐性株と同じ遺伝子配列を持つプラスミドを作成中である。今後、リコンビナント蛋白を精製し、酵素活性の測定、また INH とその代謝産物を基質としたときの活性を測定し、耐性との関連を明らかにする。

また、遺伝子に既知の変異を持たない耐性株については、全ゲノムを決定し、耐性に関与する遺伝子を探索する。

ニコチン酸フォスフォリボシルトランスフェラーゼは、生理的な基質であるニコチン酸と 5-phosphoribosyl-1-pyrohosphate をニコチン酸モノヌクレオチドとリン酸に変換するが、反応の際に ATP が消費されることが分かった。また、POA では、この酵素によりリボシル基の転移反応は起こらないが、ATP の消費は持続することが分かった。

D. 考察

台湾で分離された INH 耐性結核菌で、既存の DNA プローブで検出出来ない変異を見出した。今後これらの変異が実際に耐性に関与しているかどうかを明らかにする予定である。また、これまでに見出されていない耐性に関わる遺伝子変異を同定して行く。

ニコチン酸フォスフォリボシルトランスフェラーゼは POA の標的分子になっていて、ATP の消費により抗菌活性を示している可能性が示唆された。

E. 結論

INH 耐性結核菌を検出する検査法の改良が期待される。台湾を始めアジア各国で結核罹患率の高い国で薬剤耐性結核の迅速診断に役立つことが期待される。また、ピラジナミドの標的分子が明らかになることで、ピラジナミド耐性菌にも有効な新規抗結核薬の候補化合物の探索にもつながることが期待される。

G. 研究発表

1. 論文発表
   なし

2. 学会発表
   なし

H. 知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　なし

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

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雑誌

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Mutation Analysis of Mycobacterial *rpoB* Genes and Rifampin Resistance Using Recombinant *Mycobacterium smegmatis*

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Rifampin is a major drug used to treat leprosy and tuberculosis. The rifampin resistance of *Mycobacterium leprae* and *Mycobacterium tuberculosis* results from a mutation in the *rpoB* gene, encoding the β subunit of RNA polymerase. A method for the molecular determination of rifampin resistance in these two mycobacteria would be clinically valuable, but the relationship between the mutations and susceptibility to rifampin must be clarified before its use. Analyses of mutations responsible for rifampin resistance using clinical isolates present some limitations. Each clinical isolate has its own genetic variations in some loci other than *rpoB*, which might affect rifampin susceptibility. For this study, we constructed recombinant strains of *Mycobacterium smegmatis* carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without mutation and disrupted their own *rpoB* genes on the chromosome. The rifampin and rifabutin susceptibilities of the recombinant bacteria were measured to examine the influence of the mutations. The results confirmed that several mutations detected in clinical isolates of these two pathogenic mycobacteria can confer rifampin resistance, but they also suggested that some mutations detected in *M. leprae* isolates or rifampin-resistant *M. tuberculosis* isolates are not involved in rifampin resistance.

Leprosy and tuberculosis persist as important global public health concerns. Rifampin, a major drug used to treat these two infectious diseases, has a molecular mechanism of activity involving the inhibition of DNA-dependent RNA polymerase (15). In *Escherichia coli*, this enzyme is a complex oligomer comprised of four subunits, α, β, β′, and σ, encoded by *rpoA*, *rpoB*, *rpoC*, and *rpoD*, respectively. Rifampin binds to the β subunit of RNA polymerase and results in transcription inhibition (15). Mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase, reportedly result in resistance to rifampin in several mycobacterial species, including *Mycobacterium leprae* and *Mycobacterium tuberculosis* (9, 21). The former has not yet been cultured on artificial media; it requires 11 to 14 days to double in experimentally infected mice. Therefore, it is difficult to determine the rifampin susceptibilities of *M. leprae* isolates. The standardized method using a mouse footpad takes more than half a year to determine the rifampin susceptibility of *M. leprae* isolates and requires 5×10^3 *M. leprae* bacilli (3), which require almost a year to prepare. *In vitro* drug susceptibility testing for *M. leprae* using a radioactive reagent requires more (10^7) *M. leprae* cells (7). In contrast, mutations in the *rpoB* gene of *M. leprae* can be detected in a few days or less. It would be very helpful if mutations responsible for rifampin resistance could be determined without performing mouse footpad testing. The main mutations that confer rifampin resistance to *M. tuberculosis* are located in the 81-bp core region of the *rpoB* gene, encompassing codons 507 to 533, known as the rifampin resistance-determining region (RRDR) (17, 18). About 95% of rifampin-resistant *M. tuberculosis* strains have a mutation in this region (18, 20). Four mutations, D516V, H526Y, H526D, and S531L, are most commonly associated with the high-level rifampin resistance of *M. tuberculosis* strains (4, 10, 19), but some other mutations in the 81-bp region have not yet been confirmed completely as being responsible for rifampin resistance.

We have established a method to determine the mutations responsible for the dapsone resistance of *M. leprae* using recombinant *Mycobacterium smegmatis* strains (16). In the present study, we assessed the applicability of the determination of rifampin resistance for analysis. We then analyzed *rpoB* mutations conferring rifampin resistance to *M. leprae* and *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* DH5α was used for DNA cloning. *M. smegmatis* mc^2155 was used as a mycobacterial host to produce strains for drug susceptibility testing. Plasmids pYUB854 and phAE87 were kindly provided by W. R. Jacobs, Jr. (Department of Microbiology and Immunology, Albert Einstein College of Medicine, New York, NY). *M. smegmatis* mc^2155 and its transformants were grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) supplemented with 0.5% bovine serum albumin (fraction V), 0.2% glucose, 0.085% NaCl, 0.2% glycerol, and 0.1% Tween 80.

Site-directed mutagenesis. The wild-type *rpoB* genes of *M. leprae* and *M. tuberculosis* were amplified from *M. leprae* Thai-53 and *M. tuberculosis* H37Rv by PCR and cloned into pMV261. Site-directed mutagenesis was performed by using PCR with DNA polymerase (Takara PrimeStar HS; Takara Bio Inc., Kyoto, Japan) and the primers presented in Table 1. PCR products were purified and phosphorylated with T4 kinase and ATP and were then ligated to make them circular. The ligation mixture was used to transform *E. coli* DH5α cells, and kanamycin-resistant colonies were isolated. Plasmids were extracted from the transformants. The mutated sequences were then confirmed by sequencing. The inserts of the plasmids were also cloned into pNN301 (16). Mutations introduced into the *M. leprae rpoB* or *M. tuberculosis rpoB* gene are listed in Table 2.

Disruption of the *rpoB* gene on the *M. smegmatis* chromosome. *M. smegmatis* mc^2155 cells were transformed with plasmids carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without a point mutation. Recombinants were selected on LB medium containing kanamycin. Allel-

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TABLE 1 Primers used for this study

Primer	Sequence[a]	Application
M. smegmatis		
MSRBUF	GCCTTAAGGAGGAGAAGGACGAGGCCAC	*rpoB* disruption, upstream forward
MSRBUR	GCTCTAGACAAGATGCATCCTTCCAGCA	*rpoB* disruption, upstream reverse
MSRBDF	GCAAGCTTTCGCGCAACGAATCCGCGTC	*rpoB* disruption, downstream forward
MSRBDR	GCACTAGTAGCGCACGCAGCTTCTTCTG	*rpoB* disruption, downstream reverse
MSRBF	TGGTCAAGCAGTTCCTCAAC	Detection of *rpoB* disruption, forward
MSRBR	CGTTGTTGACGATGATCTCG	Detection of *rpoB* disruption, reverse
M. leprae		
MLRBWTF	GCGGATCCGTGCTGGAAGGATGCATCTT	Cloning of *M. leprae rpoB*, forward
MLRBWTR	GCGTTAACCTAAGCCAGATCTTCTATGG	Cloning of *M. leprae rpoB*, reverse
MLRBWTF1	CAGTTCATGGATCAGAACAACCCTC	Introduction of point mutation at codons 507 and 508
MLRBWTF2	TGTCGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation at codon 526
MLRBWTF3	TTCGCACTACGGCCGGATGTGCCCG	Introduction of point mutation at codon 547
MLRBWTR1	CGACAGCTGGCTGGTGCCGAAGAAT	Introduction of point mutation at codons 513, 516, and 517
MLRBWTR2	GCCGGCGCTTGTGGGTCAGGCCCGA	Introduction of point mutation at codons 531, 532, and 533
MLRB507GGG	CGACAGCTGGCTGGTCCCGAAGAAT	Introduction of point mutation GGC507→GGG
MLRB507AGC	CGACAGCTGGCTGGTGCTGAAGAAT	Introduction of point mutation GGC507→AGC
MLRB508ACA	CGACAGCTGGCTTGTGCCGAAGAAT	Introduction of point mutation ACC508→ACA
MLRB513GTG	GTGTTCATGGATCAGAACAACCCTC	Introduction of point mutation CAG513→GTG
MLRB516AAT	CAGTTCATGAATCAGAACAACCCTC	Introduction of point mutation GAT516→AAT
MLRB517CAT	CAGTTCATGGATCATAACAACCCTC	Introduction of point mutation CAG517→CAT
MLRB526TAC	GCCGGCGCTTGTAGGTCAGGCCCGA	Introduction of point mutation CAC526→TAC
MLRB531TTG	TGTTGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation TCG531→TTG
MLRB531TGG	TGTGGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation TCG531→TGG
MLRB532TCG	TGTCGTCGCTGGGCCCGGGTGGTTT	Introduction of point mutation GCG532→TCG
MLRB533CCG	TGTCGGCGCCGGGCCCGGGTGGTTT	Introduction of point mutation CTG533→CCG
MLRB547ATC	GGGTGCACGTCACGGATCTCTAGCC	Introduction of point mutation GTC547→ATC
M. tuberculosis		
MTRBWTF	GCGAATTCTTGGCAGATTCCCGCCAGAG	Cloning of *M. tuberculosis rpoB*, forward
MTRBWTR	GCAAGCTTTTACGCAAGATCCTCGACAC	Cloning of *M. tuberculosis rpoB*, reverse
MTRBWTF1	AATTCATGGACCAGAACAACCCGCT	Introduction of point mutation at codons 507, 508, 510, 511, 512, and 513 and deletion of codons 506-508
MTRBWTF2	CTGTCGGCGCTGGGGCCCGGCGGTC	Introduction of point mutation at codons 522, 523, 526, and 531
MTRBWTR1	GGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of mutation at codons 514, 516, 518, 519, and 521; deletion of codon 518; and insertion of TTC between codons 514 and 515
MTRBWTR2	TCGGCGCTTGTGGGTCAACCCCGAC	Introduction of point mutations TCG531→TTC and TCG531→TTG
MTRB507AGC	GGCTCAGCTGGCTGGTGCTGAAGAA	Introduction of point mutation GGC507→AGC
MTRB507GAT	GGCTCAGCTGGCTGGTATCGAAGAA	Introduction of point mutation GGC507→GAT
MTRB508CAC	GGCTCAGCTGGCTGTGGCCGAAGAA	Introduction of point mutation ACC508→CAC
MTRB508GCC	GGCTCAGCTGGCTGGCGCCGAAGAA	Introduction of point mutation ACC508→GCC
MTRB510CAT	GGCTCAGATGGCTGGTGCCGAAGAA	Introduction of point mutation CAG510→CAT
MTRB511CCG	GGCTCGGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CTG511→CCG
MTRB513AAT1	TGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CAA513→AAT
MTRB513AAT2	ATTTCATGGACCAGAACAACCCGCT	Introduction of point mutation CAA513→AAT
MTRB513GAA	CGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CAA513→GAA
MTRB516GAG	AATTCATGGAGCAGAACAACCCGCT	Introduction of point mutation GAC516→GAG
MTRB516CAC	AATTCATGCACCAGAACAACCCGCT	Introduction of point mutation GAC516→CAC
MTRB516GTC	AATTCATGGTCCAGAACAACCCGCT	Introduction of point mutation GAC516→GTC
MTRB521ATG	AATTCATGGACCAGAACAACCCGAT	Introduction of point mutation CTG521→ATG
MTRB522TTG	TCGGCGCTTGTGGGTCAACCCCAAC	Introduction of point mutation TCG522→TTG
MTRB523GCG	TCGGCGCTTGTGGGTCAACGCCGAC	Introduction of point mutation GGG523→GCG
MTRB523GGC	TCGGCGCTTGTGGGTCAAGCCCGAC	Introduction of point mutation GGG523→GGC
MTRB526CTC	TCGGCGCTTGAGGGTCAACCCCGAC	Introduction of point mutation CAC526→CTC
MTRB526TAC	TCGGCGCTTGTAGGTCAACCCCGAC	Introduction of point mutation CAC526→TAC
MTRB526GAC	TCGGCGCTTGTCGGTCAACCCCGAC	Introduction of point mutation CAC526→GAC
MTRB526TTC	TCGGCGCTTGAAGGTCAACCCCGAC	Introduction of point mutation CAC526→TTC
MTRB526AAC	TCGGCGCTTGTTGGTCAACCCCGAC	Introduction of point mutation CAC526→AAC
MTRB526CGC	TCGGCGCTTGCGGGTCAACCCCGAC	Introduction of point mutation CAC526→CGC
MTRB526CAA	TCGGCGCTTTTGGGTCAACCCCGAC	Introduction of point mutation CAC526→CAA
MTRB529AAA	TTTGCGCTTGTGGGTCAACC	Introduction of point mutation CGA529→AAA
MTRB531TTC	CTGTTCGCGCTGGGGCCCGGCGGTC	Introduction of point mutation TCG531→TTC
MTRB531TTG	CTGTTGGCGCTGGGGCCCGGCGGTC	Introduction of point mutation TCG531→TTG
MTRB506d	GGCTCAGCTGGCTGAACTCCTTGAT	Introduction of mutation 506-508del
MTRBin514TTC	AATTCTTCATGGACCAGAACAACCC	Introduction of mutation 514insTTC
MTRBd518	AATTCATGGACCAGAACCCGCTGTC	Introduction of mutation 518del

[a] Restriction sites are underlined.

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TABLE 2 Rifampin and rifabutin susceptibilities of the recombinant *M. smegmatis* strains

	Rifampin		Rifabutin		
Mutation	MIC (μg/ml)	Fold increase[a]	MIC (μg/ml)	Fold increase	Reference(s)
M. leprae					
Wild type	1		0.25		
GGC507→GGG (silent)	1	1	0.25	1	This study
GGC507→AGC (G507S)	0.5	0.5	0.125	0.5	3
ACC508→ACA (silent)	1	1	0.25	1	This study
CAG513→GTG (Q513V)	32	32	8	32	3
GAT516→AAT (D516N)	32	32	2	8	14
CAG517→CAT (Q517H)	1	1	0.25	1	11
CAC526→TAC (H526Y)	32	32	8	32	14
TCG531→TTG (S531L)	32	32	4	16	3, 14
TCG531→TGG (S531W)	32	32	8	32	14
GCG532→TCG (A532S)	1	1	0.25	1	11
CTG533→CCG (L533P)	32	32	4	16	14
GTC547→ATC (V547I)	1	1	0.25	1	This study
M. tuberculosis					
Wild type	1		0.25		
GGC507→AGC (G507S)	0.5	0.5	0.125	0.5	1
GGC507→GAT (G507D)	0.5	0.5	0.125	0.5	1
ACC508→CAC (T508H)	0.5	0.5	0.125	0.5	1
ACC508→GCC (T508A)	1	1	0.25	1	1
CAG510→CAT (Q510H)	1	1	0.25	1	22
CTG511→CCG (L511P)	16	16	1	4	1, 12
CAA513→AAT (Q513N)	8	8	0.5	2	1
CAA513→GAA (Q513E)	32	32	2	8	1
GAC516→GAG (D516E)	8	8	0.5	2	12
GAC516→CAC (D516H)	1	1	0.25	1	1
GAC516→GTC (D516V)	32	32	2	8	12, 21, 22
CTG521→ATG (L521M)	1	1	0.125	0.5	21
TCG522→TTG (S522L)	>32	>32	8	32	21
GGG523→GCG (G523A)	1	1	0.125	0.5	1
GGG523→GGC (silent)	1	1	0.25	1	1
CAC526→CTC (H526L)	32	32	4	16	12, 22
CAC526→TAC (H526Y)	>32	>32	8	32	12, 22
CAC526→GAC (H526D)	>32	>32	8	32	12, 22
CAC526→TTC (H526F)	>32	>32	4	16	1
CAC526→AAC (H526N)	32	32	2	8	8
CAC526→CGC (H526R)	32	32	8	32	12, 22
CAC526→CAA (H526Q)	8	8	0.5	2	1
CGA529→AAA (R529K)	32	32	4	16	22
TCG531→TTC (S531F)	32	32	4	16	1
TCG531→TTG (S531L)	32	32	8	32	21, 22
506-508del[b]	16	16	0.5	2	5
514insTTC[c]	>32	>32	8	32	12 22
518del[d]	32	32	2	8	22

[a] Fold increase in MIC compared to the wild-type sequence.
[b] Deletion of codons 506 to 508.
[c] Insertion of TTC between codons 514 and 515.
[d] Deletion of codon 518.

ic-exchange mutants were constructed by using a temperature-sensitive mycobacteriophage method described in a previous report (2). Using the *M. smegmatis* mc^2155 genome sequence (GenBank accession number CP000480), the upstream and downstream flanking DNA sequences were used to generate a deletion mutation in the *rpoB* gene (MSMEG_1367). To disrupt the *rpoB* gene, DNA segments from 1,119 bp upstream through 21 bp downstream of the initiation codon of *M. smegmatis rpoB* and from 39 bp upstream through 941 bp downstream of the termination codon were cloned directionally into the cosmid vector pYUB854, which contains a *res-hyg-res* cassette and a *cos* sequence for lambda phage assembly. The plasmids thus produced were digested with PacI and ligated into PH101 genomic DNA excised from the phage-plasmid hybrid (phasmid) phAE87 by PacI digestion. The ligated DNA was packaged (GigaPackIII Gold packaging extract; Stratagene, La Jolla, CA). The resultant mixture was used for the transduction of *E. coli* STBL2 cells (Life Technologies Inc., Carlsbad, CA) to yield cosmid DNA. After *E. coli* was transduced and the transductants were plated onto hygromycin-containing medium, phasmid DNA was prepared from the pooled antibiotic-resistant transductants and electroporated into *M. smegmatis* mc^2155. Bacterial cells were incubated at 30°C to produce the recombinant phage. The *M. smeg-*

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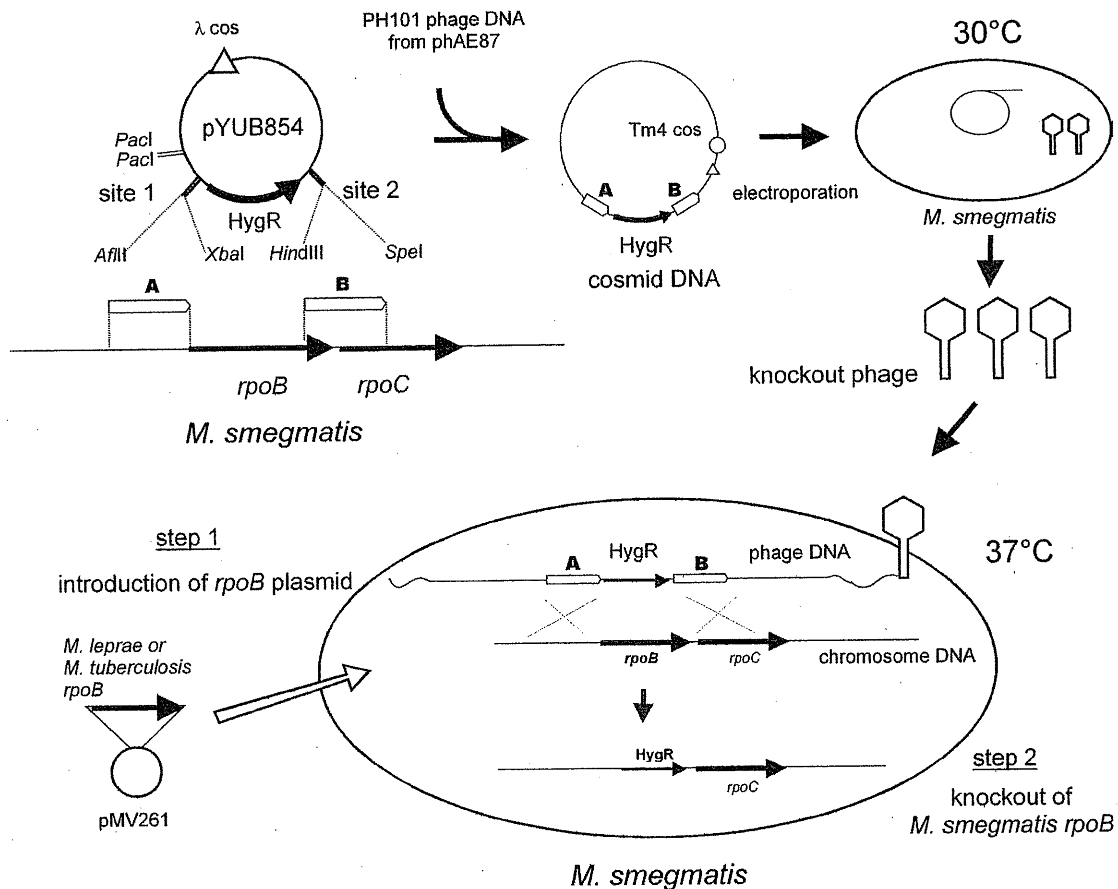

FIG 1 Construction of recombinant *M. smegmatis* strains for rifampin susceptibility testing.

matis transformant carrying the *M. leprae* or *M. tuberculosis rpoB* gene was infected with the produced temperature-sensitive phage at 37°C for allelic exchange, and kanamycin-resistant and hygromycin-resistant colonies were isolated. Two colonies for each point mutation were subjected to subsequent tests.

Drug susceptibility testing. The MIC values for *M. smegmatis* recombinant clones were determined by culture on Middlebrook 7H10 agar plates containing 2-fold serial dilutions of rifampin (0.25 to 32 μg/ml) or rifabutin (0.0625 to 8 μg/ml). The MIC value for each strain was defined as the lowest concentration of the drug necessary to inhibit bacterial growth.

RESULTS

Construction of recombinant *M. smegmatis* strains. In our previous study, we sequenced the *rpoB* regions of *M. leprae* clinical samples isolated in Vietnam and detected several mutations (11). In addition to these mutations, we detected some mutations (GGC→GGG at codon 507, ACC→ACA at codon 508, and GTC→ATC at codon 547) in clinical specimens from Vietnam and other countries (our unpublished data). We prepared plasmids with mutations in the *M. leprae* and *M. tuberculosis rpoB* genes. Each plasmid has one of 40 mutations (12 for *M. leprae rpoB* and 28 for *M. tuberculosis rpoB*) presented in Table 2. The mutated sequences were confirmed by sequencing. Plasmids carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without a point mutation were introduced individually into *M. smegmatis*. The *M. smegmatis* transformants were subjected to allelic exchange to disrupt the *rpoB* gene on their own chromosome (Fig. 1). The isolation of *rpoB*-disrupted mutants carrying the pNN301-*rpoB* constructs was unsuccessful. Consequently, the recombinant strains with pMV261-*rpoB* constructs were used for subsequent tests. PCR analysis confirmed that the *M. smegmatis rpoB* sequences in the recombinant strains with pMV261-*rpoB* constructs were replaced by hygromycin resistance gene sequences (see Fig. S1 in the supplemental material). All strains showed growth rates comparable to that of wild-type *M. smegmatis*.

Drug susceptibility. The rifampin susceptibilities and rifabutin susceptibilities of the recombinant *M. smegmatis* strains were tested (see Fig. S2 in the supplemental material). The MIC values of rifampin and rifabutin for the recombinant *M. smegmatis* strains and the fold increases in MIC compared to the wild-type sequences are presented in Table 2. It should be noted that the MIC values for the *M. smegmatis* strains might be shifted from those for *M. leprae* or *M. tuberculosis* because of their differences in cell wall permeability and other factors. The MIC value of rifampin for the recombinant *M. smegmatis* strain with the wild-type sequence of the *M. leprae rpoB* or *M. tuberculosis rpoB* gene was 1 μg/ml. Most strains that had a mutation at codon 511, 513, 516, 522, 526, 531, or 533 showed rifampin resistance. In contrast, strains that had a mutation at codon 507, 508, 517, 521, 523, or 532 showed MIC values of rifampin comparable to those for the wild-type sequence. The MIC values of rifabutin for the recombinant

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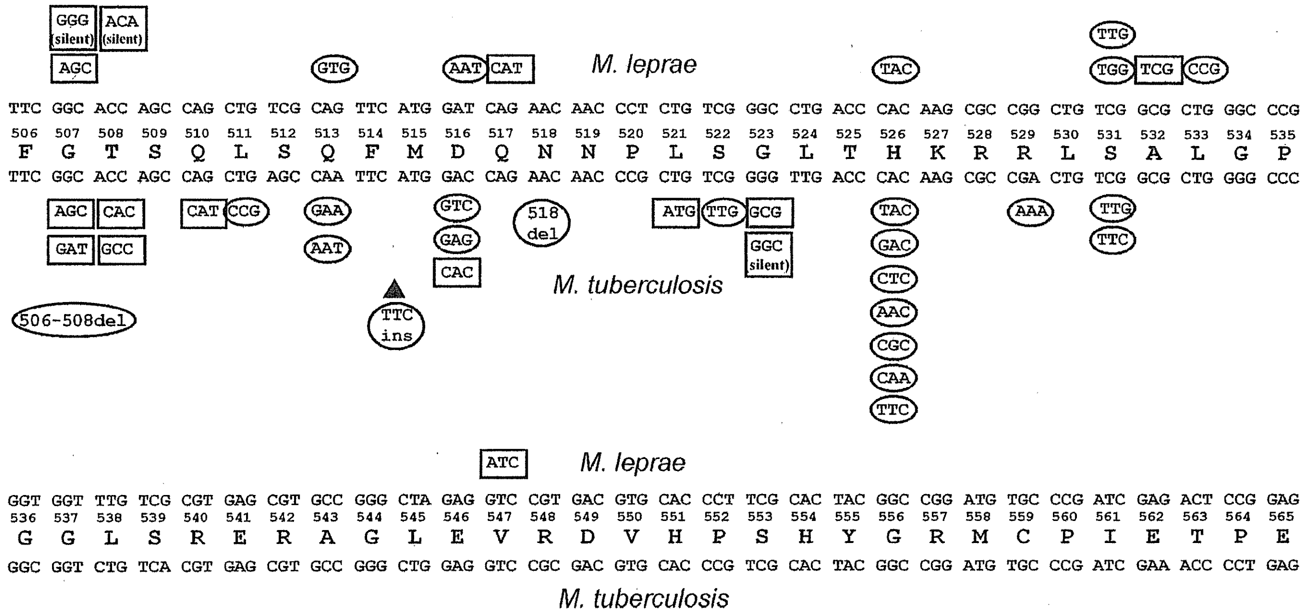

FIG 2 Mutations introduced into the *M. leprae rpoB* gene or *M. tuberculosis rpoB* gene and rifampin susceptibility. The consensus amino acid sequence of *M. leprae* RpoB and *M. tuberculosis* RpoB between codons 506 and 565 is shown. The *M. leprae rpoB* sequence and codons are shown above the consensus amino acid sequence. The *M. tuberculosis rpoB* sequence and codons are shown below the consensus sequence. Mutated codons that gave rise to rifampin resistance are surrounded by ovals. Mutated codons that showed levels of rifampin susceptibility comparable to those of the wild-type sequences are surrounded by rectangles.

M. smegmatis strains with the wild-type sequence of the *M. leprae rpoB* or *M. tuberculosis rpoB* gene were 0.25 μg/ml. Generally, rifabutin was more efficacious than rifampin in terms of concentration.

DISCUSSION

To functionally replace the *rpoB* gene of *M. smegmatis* with the *M. leprae* or *M tuberculosis* counterpart, we used a method established in our previous study (16). Because *rpoB* is a necessary gene for bacterial growth, this genetic locus cannot be disrupted without compensating for its activity. Therefore, we first introduced the *rpoB* gene of *M. leprae* or *M. tuberculosis* into *M. smegmatis* using vector plasmids of two types before disrupting the *rpoB* gene on the *M. smegmatis* chromosome. One vector was pMV261, a multicopy shuttle plasmid. The other was a single-copy integrative shuttle plasmid, pNN301. However, the isolation of *rpoB*-disrupted mutants carrying pNN301-*rpoB* constructs was unsuccessful, probably because of insufficient RpoB expression.

We tested 2 silent mutations and 10 mutations that change amino acid residues for *M. leprae* (Fig. 2). Codons 516, 526, 531, and 533 in the *M. leprae rpoB* gene are known to be codons responsible for rifampin resistance. However, it remains unclear whether or not mutations that have not been reported previously can confer rifampin resistance. Our results show that not all mutations in the *rpoB* gene detected in *M. leprae* clinical samples confer rifampin resistance. *M. leprae* is not cultivable. Therefore, it has been very difficult to analyze the mutation-susceptibility relationship. Using recombinant *M. smegmatis*, however, we can analyze it in a few weeks. We also tested 1 silent mutation, 24 mutations that change amino acids, 2 deletions, and 1 insertion for *M. tuberculosis*. Some mutations did not confer rifampin resistance, which is inconsistent with the susceptibility of the *M. tuberculosis* clinical isolates reported previously. Most mutations at codon 516, 526, or 531 showed rifampin resistance. It is interesting that the strains with the mutation GAC516→CAC for D516H were not rifampin resistant. All other mutations at codon 516 showed rifampin resistance. The mutation GAC516→CAC in *M. tuberculosis* was reported for a strain with multiple mutations and should not be involved in rifampin resistance.

Rifabutin, a spiropiperidyl rifampin, is a rifamycin derivative that is more active than rifampin against slow-growing mycobacteria, including *M. tuberculosis* and *M. avium-M. intracellulare* complex strains, *in vitro* and *in vivo*. It is also active against some rifampin-resistant strains of *M. tuberculosis* (6, 13). Our results indicate that some mutations (e.g., GAT516→AAT of *M. leprae* and GAC516→GAG of *M. tuberculosis*) show weak resistance to rifabutin.

Molecular methods designed to detect drug resistance have some limitations. In some cases, the identified mutations are not related to the acquisition of resistance. Caution is necessary when considering mutations, especially if the mutation detected in clinical isolates is not reported very often. For example, Q510H and L521M mutations were detected in rifampin-resistant *M. tuberculosis* isolates (21, 22), but our results suggest that these mutations are not responsible for rifampin resistance (Table 2). The method used for this study can directly assess the influence of designated mutations in *rpoB*. If the mutations can confer rifampin resistance, we can eliminate the possibility that genetic variation in some region other than *rpoB* on the chromosome of the clinical isolates is responsible for the resistance. Bahrmand et al. previously reported the high-level rifampin resistance of *M. tuberculosis* isolates with multiple mutations within the *rpoB* gene (1). Our method might also be useful for analyzing multiple mutations

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detected in the *rpoB* gene of clinical isolates to determine the contribution of each single mutation to rifampin resistance.

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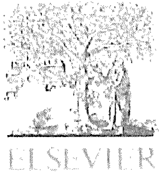

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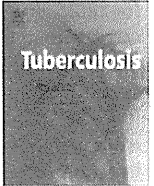

DRUG DISCOVERY AND RESISTANCE

Molecular mechanism of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* from Bangladesh

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SUMMARY

Despite having 100% coverage of directly observed treatment short-course, multi drug-resistant (MDR) tuberculosis (TB) is still increasing in Bangladesh. Early detection of MDR-TB by rapid molecular test and early initiation of treatment will effectively stop this trend. To develop rapid diagnostic tools, molecular characterization of genes conferring *Mycobacterium tuberculosis* resistance to rifampicin (RIF) and isoniazid (INH) will be required. Hence, this study elucidated the molecular mechanism RIF and INH resistance in 218 MDR strains from hospitalized ($n = 161$) and non-hospitalized ($n = 57$) TB patients in Bangladesh. Mutations in *rpoB* gene were detected in 207 (95.0%) with majority at codon 531 (52.3%). Mutations in *katG* or *inhA* or both were detected in 206 (94.5%) with majority at codon 315 of *katG* (83.9%). It was noteworthy that a novel C to T mutation at position −34 and G to A mutations at position −47 in *inhA* regulatory region were found, respectively, in combination with mutation at codon 315 of *katG*. This is the first comprehensive molecular analysis of *rpoB* and *katG* genes and *inhA* regulatory regions of MDR isolates from Bangladesh. This study provides basic data for the construction of low cost tailor-made molecular system for rapid diagnosis of MDR-TB in Bangladesh.

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1. Introduction

Tuberculosis (TB) is one of the important re-emerging infectious diseases. Due to its infectious nature, one third of the world's population is latently infected with the causative agent *Mycobacterium tuberculosis* and annual new cases of TB worldwide counts approximately 9 million. This disease is associated with more deaths than any other single infectious agents.[1] The World Health Organization (WHO) estimated prevalence of 277/100,000 in the South–East Asian Region (SEAR) with 90% of patients living in six high TB burden countries: India, China, Indonesia, Pakistan, Bangladesh and the Philippines. The target country of this study, Bangladesh, is one of the six high TB burden countries in SEAR and the 7th highest burden country worldwide. WHO estimated 330,000 new cases of TB with 64,000 deaths in this country in 2010.[2]

TB is a treatable infectious disease, however, its treatment fails with the emergence of multidrug resistance (MDR). The global rate of MDR-TB is increasing due to the lack of treatment compliance, supply of poor quality drugs and recent direct transmission from MDR patients. Hence, MDR-TB has become an issue of increasing importance in both developed and developing country.[3,4] In Bangladesh, MDR-TB increases from 2% to 5.5% with progress of time.[5,6] WHO estimated 2.2% and 14.7% MDR-TB, respectively, among new and previously treated TB cases.[4]

Mycobacteria adopt various mechanisms for its survival in humans under anti-TB treatment. It acquires mutations in genes encoding drug target protein to inhibit the binding of a drug,[7] encoding cell wall proteins contributing impermeability to reduce the entry of drugs, as well as encoding membrane protein that acts as efflux pump.[8,9]

Identification of mutations responsible for drug resistance seems to be a suitable approach for development of molecular tools

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for rapid drug susceptibility testing compared to time consuming conventional methods.[10,11] It has shown that association of rifampicin (RIF) resistance with mutations within the RIF resistance-determining region (RRDR) varies from 78 to 100% of cases[7,12–14] in different countries. On the contrary, *M. tuberculosis* acquires isoniazid (INH) resistance through mutations in several genes including the *katG* and *inhA* regulatory region. Mutations in *katG* induced resistance from 50 to 95% of INH resistance strains.[7,15,16] Mutations associated with up-regulation of the expression of *inhA* encoding a long-chain enoyl-acyl carrier protein reductase contributes to INH resistance via a titration mechanism in approximately 10–30% of strains.[7] Association of mutation with drug resistance differs in countries studied. Thus, it is important to study molecular mechanism of drug resistance in each country. Nevertheless, very limited information is available on molecular mechanism of drug resistance associated with MDR stains of *M. tuberculosis* in Bangladesh. Hence, we conducted this study to analyze mutations of *rpoB* and *katG* genes and the *inhA* regulatory region of MDR strains of *M. tuberculosis* of this country.

2. Materials and methods

2.1. Bacterial strains

M. tuberculosis strains were isolated from sputum samples at the Tuberculosis Laboratory of ICDDR,B from June 2001 to December 2010. Outdoor of the Shyamoli Chest Disease Clinic and the National Institute of the Diseases of Chest and Hospital (NIDCH) at Dhaka were the catchment areas for non-hospitalized TB patients (outpatient). Indoor wards of the NIDCH were the catchment area of hospitalized TB patients (inpatients).

2.2. Isolation of Mycobacterium and drug susceptibility testing (DST)

Isolation of *Mycobacterium* strains and DST were performed following the method already described.[17] Briefly; 5 ml sputum sample was collected from the TB suspects and sputum sample was decontaminated following the standard Petroff's method.[18] One loop-full (10 µl) of decontaminated sputum samples was inoculated on two Löwenstein-Jensen (L-J) slants. Then, well isolated characteristic colonies of *Mycobacterium* was taken from surface of the L-J slants for DST with respect to streptomycin (4 mg/L), INH (0.2 mg/L), RIF (40 mg/L) and ethambutol (2 mg/L) following proportion method of Canetti et al.[19] In each lot of DST, one strain of H37Rv was used as sensitive and known MDR strain was used as resistant control strains. DST result of a lot was considered valid when the sensitivity patterns of the control strains were consistent with previous sensitivity patterns.

2.3. Preparation of DNA

One loop full of *Mycobacterium* culture was scraped from the surface of the drug containing L-J slants and re-suspended in 500 µl sterile distilled water in a micro centrifuge tubes and the bacterial cells were lysed by boiling for 20 min. The cells were centrifuged (10,000× *g* for 10 min) and the supernatant was transferred into another micro centrifuge tube to use as template DNA.

2.4. Identification of strains

For the identification of MDR strains, an in-house multiplex PCR technique was used. This technique targeted three genetic regions (*cfp32*, RD9 and RD12) as described previously.[20]

2.5. Standard PCR for sequencing of rpoB and katG genes and inhA regulatory region

The target template DNA was amplified using the primer pairs listed in Table 1. PCR reactions were performed in a 20 µl mixture consisting of 0.25 mM each of dNTPs, 0.5 M betaine, 0.5 µM of each primer, 1 U of GoTaq DNA Polymerase (Promega, WI, USA), GoTaq buffer and 1 µl DNA template. The reaction was carried out in a thermal cycler (Bio-Rad Laboratories, CA, USA) under the following conditions: denaturation at 96 °C for 60 s followed by 35 cycles of amplification at 96 °C for 10 s, 55 °C for 10 s and 72 °C for 30 s with a final extension at 72 °C for 5 min. Desired PCR product was confirmed by agarose gel electrophoresis. PCR products were sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corp., CA, USA) in ABI PRISM 3130*xl* Genetic Analyzer (Life Technologies Corp.). The resulting sequences were compared with wild-type sequences of *M. tuberculosis* H37Rv using Bio-Edit software (version 7.0.9).[21]

3. Results

3.1. Bacterial strains

All the isolates in a collection of 218 MDR mycobacterial strains (161 from inpatients and 57 from outpatients) were identified by in-house multiplex PCR as *M. tuberculosis* because of their carriage of RD9 and RD12.

3.2. Mutations in the rpoB gene

To elucidate the molecular mechanism of RIF resistance, *rpoB* gene of MDR strains were sequenced. Mutated codons, amino acid and nucleotide substitution of these strains are shown in Table 2. Mutations at *rpoB* gene were detected in 95% (207 out of 218) of RIF resistant strains with majority having single mutations at codon 531 (57.3%). This was followed by mutation in codon 526 (20.6%) and 516 (7.3%). Low frequency mutations were detected at codons 513, 530 and 533. Deletion, insertion and double mutations were detected in a very few strains. Eleven (5.0%) phenotypic MDR strains were without known mutation in *rpoB* gene. Mutation conferring Asp to Val amino acid substitution at codon 516 (Asp516Val) was found only in strains from inpatient and mutation giving His526Arg was found only in strains from outpatients.

3.3. Mutations in the katG gene and inhA regulatory regions

For the molecular characterization of INH resistance, the nucleotide sequences of *katG* gene and *inhA* regulatory region were sequenced. Mutated codons, amino acid and nucleotide substitutions of these strains are shown in Table 3. Out of 218 strains, 206 (94.5%) had mutations in *katG* coding region or *inhA* regulatory region or both with the vast majority having the commonly

Table 1
Primers used for PCR amplification and sequencing of drug resistance associated genes in *M. tuberculosis*.

Locus	Primer name	Nucleotide sequence (5′–3′)	Target region	Product size (bp)
rpoB	TB rpoB S	CAGGACGTGGAGGCGATCAC	1519–1599*	278
	TB rpoB AS	GAGCCGATCAGACCGATGTTGG		
katG	TB katG S	ATGGCCATGAACGACGTCGAAAC	823–1140	392
	TB katG AS	CGCAGCGAGAGGTCAGTGGCCAG		
inhA	TB inhA S	TCACACCGACAAACGTCACGAGC	−40 to −1	231
	TB inhA AS	AGCCAGCCGCTGTGCGATCGCCA		

* Corresponding *Escherichia coli* numbering was used for rpoB.

Table 2
Distribution of mutations in the rpoB RRDR of 218 MDR- isolates from Bangladesh.

Mutated codon	Amino acid change		Nucleotide change		No. (%) of isolates		
	From	To	From	To	Inpatients ($n = 161$)	Outpatients ($n = 57$)	Total ($n = 218$)
513	Gln	Pro	CAA	CCA	1 (0.6)	1 (1.8)	2 (0.9)
		Lys		AAA	1 (0.6)	–	1 (0.5)
		Leu		CTA	1 (0.6)	–	1 (0.5)
516	Asp	Val	GAC	GTC	12 (7.5)	–	12 (5.5)
		Tyr		TAC	3 (1.8)	1 (1.9)	4 (1.8)
526	His	Tyr	CAC	TAC	13 (8.1)	4 (7.0)	17 (7.8)
		Asp		GAC	9 (5.6)	4 (7.0)	13 (6.0)
		Arg		CGC	–	6 (10.5)	6 (2.8)
		Leu		CTC	2 (1.2)	3 (5.3)	5 (2.3)
		Cys[a]		TGC	1 (0.6)	1 (1.8)	2 (0.9)
		Asn		AAC	1 (0.6)	–	1 (0.5)
		Gly[a]		GGC	–	1 (1.8)	1 (0.5)
530	Leu	Val	CTG	GTG	1 (0.6)	–	1 (0.5)
531	Ser	Leu	TCG	TTG	90 (55.9)	24 (42.1)	114 (52.3)
		Trp		TGG	9 (5.6)	1 (1.8)	10 (4.6)
		Phe[a]		TTT	1 (0.6)	–	1 (0.5)
533	Leu	Pro	CTG	CCG	2 (1.2)	3 (5.3)	5 (2.3)
510/526	Gln/His	His/Tyr	CAG/CAC	CAT/TAC	1 (0.6)	–	1 (0.5)
511/516	Leu/Asp	Arg/Tyr	CTG/GAC	CGG/TAC	1 (0.6)	–	1 (0.5)
513/526	Gln/His	Lys/Asp	GAC/CAC	GTC/CCG	1 (0.6)	–	1 (0.5)
516/517	Asp/Gln	Val/Pro	GAC/CAG	GTC/CCG	–	1 (1.8)	1 (0.5)
516/526	Asp/His	Glu/Asn	GAC/CAC	GAA/AAC	2 (1.2)	–	2 (0.9)
516/531	Asp/Ser	Tyr/Leu	GAC/TCG	TAC/TTG	1 (0.6)	–	1 (0.5)
526/533	His/Leu	Gln/Pro	CAC/CTG	CAA/CCG	1 (0.6)	–	1 (0.5)
514	Phe ins		TTC ins		1 (0.6)	–	1 (0.5)
514-516	Del		Del		1 (0.6)	–	1 (0.5)
516-517	Del		Del		1 (0.6)	–	1 (0.5)
Wild type[b]	None		None		4 (2.5)	7 (12.3)	11 (5.0)

[a]Double mutation in a codon.
[b]No mutations in the sequenced region.

described mutation in *katG* coding region conferring Ser315Thr amino acid substitution ($n = 178$; 83.9%). Seven and one strains respectively had Ser to Asn and Ser to Ile amino acid substitution at the same position. Tyr275Pro, Ala291Pro, Trp300Gly, Trp300Cys amino acid substitutions were observed in each one of INH-resistant strains. Three strains showed double mutations in two separate codons: Thr275Ala and Ser315Thr. One strain showed double mutations conferring Ser315Thr and Asp329Gly substitution. One strain showed frame shift mutation as a result of insertion of two base pairs after codon 300. In addition, simultaneous six amino acid deletions from 333 to 338 and Tyr339Asp amino acid substitution were noted. Thirty-nine (17.9%) INH-resistant strains

Table 3
Distribution of mutations in KatG gene and the inhA promoter region of 218 MDR- isolates from Bangladesh.

Mutated locus	Amino acid change		Nucleotide change		No. (%) of isolates		
	From	To	From	To	Inpatients ($n = 161$)	Outpatients ($n = 57$)	Total ($n = 218$)
KatG 275	Tyr	Pro	ACC	CCC	–	1 (1.8)	1 (0.5)
KatG 291	Ala	Pro	GCT	CCT	1 (0.6)	–	1 (0.5)
KatG 300	Trp	Gly	TGG	GGG	1 (0.6)	–	1 (0.5)
		Cys		TGC	1 (0.6)	–	1 (0.5)
KatG 315	Ser	Thr	AGC	ACC	100 (62.1)	36 (63.2)	136 (62.4)
		Asn		AAC	5 (3.1)	2 (3.5)	7 (3.2)
		Ile		ATC	–	1 (1.8)	1 (0.5)
KatG 275/315	Thr/Ser	Ala/Thr	ACC/AGC	GCC/ACC	3 (1.9)	–	3 (1.4)
KatG 315/329	Ser/Asp	Thr/Gly	ACC/GAC	GCC/GCC	1 (0.6)	–	1 (0.5)
KatG 300	frame shift		GC ins		1 (0.6)	–	1 (0.5)
KatG 333–338/339	Leu-Glu-Ile-Leu-Try-Gly/Tyr	Del*/Asn	CTCGAGATCCTGTACGGC/TAC	del/GAC	1 (0.6)	–	1 (0.5)
Deletion[†]	Deletion		Deletion		1 (0.6)	–	1 (0.5)
inhA −15	NA[§]		C	T	10 (6.2)	5 (8.8)	15 (6.9)
KatG 315/inhA −8[d]	Ser/NA	Thr/NA	AGC/T	ACC/A	1 (0.6)	–	1 (0.5)
				ACC/C	1 (0.6)	–	1 (0.5)
KatG 315/inhA −15[d]	Ser/NA	Thr/NA	AGC/C	ACC/T	16 (9.9)	5 (8.8)	21 (9.6)
KatG 315/inhA −34[d]	Ser/NA	Thr/NA	AGC/C	ACC/T	–	1 (1.8)	1 (0.5)
KatG 315/inhA −47[d]	Ser/NA	Thr/NA	AGC/G	ACC/A	10 (6.2)	2 (3.5)	12 (5.5)
Wild type**	None		None		8 (5.0)	4 (7.0)	12 (5.5)

[d]Mutations identified in both loci.
* Deletion.
† No amplification.
§ Not applicable.
** No mutations in sequenced regions of katG and inhA promoter.