MUTATIONS IN THE *RPOB* GENE OF RIFAMPICIN-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* STRAINS FROM THAILAND AND ITS EVOLUTIONARY IMPLICATION

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Abstract. Novel mutations in the *rpoB* gene are reported for 70 rifampicin-resistant (RIF^r) *M. tuberculosis* strains from Thailand. Sequence analysis of these strains revealed mutations in a 435 base-pair region of the *rpoB* gene. Twenty-eight strains (40%) had single mutations, and 26 of those strains had mutations at positions never before reported, of which, just one had a substitution at Val-432 (Asp), and the remaining 25, a silent mutation at Gln-517. All other strains had multiple mutations, of which 24 (34%) had mutations at two positions; 9(13%), at three positions; 2(3%), at five positions; and 1(1%) at six positions. Five strains (7%), reported to have the RIF^r phenotype, contained no mutation in the examined region of the *rpoB* gene. Surprisingly, one RIF^r strain had silent mutations at 29 positions. By far the dominant mutation was the silent mutations at Gln-517 (86%). This investigation demonstrates that mutations in the *rpoB* gene of *M. tuberculosis* strains from Thailand are more varied than previously reported for RIF^r *M. tuberculosis* strains. Screening by means of PCR-SSCP clearly separated RIF^r strains from rifampicin-susceptible (RIF^s) strains. There was no correlation between RIF^r mutations and random amplified polymorphic DNA (RAPD) types.

INTRODUCTION

A dramatic increase in the number of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) strains in human immunodeficiency virus-infected patients in the United States (Musser, 1995; Bifani *et al*, 1996), Europe (Fang *et al*, 1999), Australia (Sintchenko *et al*, 1999) and Asia (Hirano *et al*, 1999) has occurred. In the United States, for example, 3.2% of *M. tuberculosis* isolates are multidrug-resistant (Musser, 1995). Fortunately, however, increasing numbers of reports of MDR-TB are alerting the world that health and development gains will be jeopardized if action is not accelerated immediately. For these reasons, preventing the spread of MDR-TB strains, is of utmost importance. This requires early detection, not only because the disease can spread the longer it remains undetected within individuals, but also because patients infected with these strains are difficult to treat, and become increasingly difficult to treat the longer the clones are allowed to evolve within individuals. This often results in uncompleted treatment, and which is an important cause of increse in prevalence of MDR-TB strains.

In Thailand, the incidence of MDR-TB strains in 1998 was 2.3%, which also was the incidence of resistance to rifampicin (Payanandana *et al*, 1999). This suggests that rifampicin-resistance (RIF^r) can be an effective marker of MDR-TB, and that RIF^r detection by application of a rapid DNA-based technique can be a convenient method for revealing and tracking drug resistant *M. tuberculosis* strains (Iseman, 1999).

The mechanism of resistance to rifampicin (RIF), the main drug in the chemo-therapeutic regimens used to treat tuberculosis, has been well established, and has been associated with mutations in the *rpoB* gene, that encodes the ß subunit of RNA polymerase, an oligometric en-

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zyme responsible for RNA synthesis (Kunin, 1996). After Telenti *et al* (1993) first found mutations in the *rpoB* gene of *M. tuberculosis*, intensive attempts have been made to find sequence alterations in the *rpoB* gene of *M. tuberculosis* (Musser, 1995; Lee *et al*, 1998; Hirano *et al*, 1999; Iseman, 1999; Sintchenko *et al*, 1999). The most-encountered *rpoB* mutations in RIF^r strains are concentrated in a short 81-base-pair (bp) region of the gene (codons 507 through 533) (Telenti *et al*, 1993; Miller *et al,* 1994; Williams *et al*, 1994; Musser, 1995). These mutations can be detected, directly or indirectly, by different molecular techniques, such as polymerase chain reaction (PCR)-DNA sequencing, PCR-single strand conformation polymorphism (SSCP) (Kim *et al*, 1997; Lee *et al*, 1998), PCR-heteroduplex (Williams *et al*, 1994, 1998), PCR-reversed line blot hybridization (PLH) (Kremer *et al*, 1997), line probe assay (LIPA) (Hirano *et al*, 1999; Sintchenko *et al*, 1999), and dideoxy fingerprinting (Felmlee *et al*, 1995).

In this study, mutations in the *rpoB* genes of 70 RIF^r strains from Thailand were analyzed using automated DNA-sequencing. Also, the simple molecular techniques of single strand conformation polymorphism (SSCP) and random amplified polymorphic DNA (RAPD) (Williams *et al*, 1993) was used to screen for *rpoB* gene mutation and to assess possible clonality, respectively.

MATERIALS AND METHODS

M. tuberculosis strains

Seventy RIF^r strains were collected from pulmonary tuberculosis and AIDS patients who developed disseminated tuberculosis. The patients came from many provinces of Thailand, but mainly from Bangkok (Table 1). These patients were registered at the Central Chest Hospital in Nonthaburi, Thailand from 1997 to 1998. For use as control, 10 additional strains, known to be rifampicin-susceptible (RIF^s), were also collected. All 80 strains had been previously differentiated and identified by conventional culturing or biochemical test, and by a DNA hybridization assay (using commercial kit of Gen-Probe, USA). For examining drug resistance profiles (including resistance to rifampicin), the proportion method was carried out using Lowenstein-Jensen egg slants (WHO, 1997).

Preparation of chromosomal DNA from *M. tuberculosis* strains

For each strain, loopfuls of bacteria, cultured on Lowenstein-Jensen medium, were transferred to conical tubes containing 0.5 ml sterile water, one loopful per tube. The tubes were then vortexed for 1 minute and incubated at 95ºC for 5 minutes before being frozen at -80ºC for 30 minutes. Freezing and thawing was repeated 3 times before centrifuging at 13,000 rpm for 5 minutes in an Eppendorf centrifuge 5042. The DNA from the supernatant was purified by phenol: chloroform extraction and ethanol precipitation, described by Sambrook *et al* (1989). Afterwards, the resulting purified DNA pellet was dissolved in 100 ml sterile distilled water to determine its purity and concentration, spectrophotometrically, using Gene Quant RNA/DNA calculator (Pharmacia Biotech, Sweden).

PCR procedures for *rpoB* gene amplification

Aliquots of purified *M. tuberculosis* DNA were added to PCR reagents and primer RpoB-for and RpoB-rev with sequence 5′-TGG TCC GCT TGC ACG AGG GTC AGA-3′ and 5′-CTC AGG GGT TTC GAT CGG GCA CAT-3′, respectively. These two primers were prepared to completely match the *M. tuberculosis rpoB* gene sequence (primer sequences and PCR reaction conditions were obtained from Dr Dick van Soolingen, The Netherlands). This procedure produced a 435-bp product of the *rpoB* gene. The reactants were placed in a thermal cycler, which carried out the following PCR program (6-step):96ºC for 3 minutes, for 1 cycle, 60 seconds at 96ºC, 60 seconds at 72ºC, and 60 seconds at 72ºC, for 2 cycles, 60 seconds at 96ºC, 60 seconds at 71ºC, and 60 seconds at 72ºC, for 2 cycles, 60 seconds at 96ºC, 60 seconds at 70ºC, and 60 seconds at 72ºC, for 2 cycles, 60 seconds at 96ºC, 60 seconds at 69ºC, and 60 seconds at 72ºC, for 25 cycles, and a final 5-minute extension at 72ºC, for 1 cycle. This touchdown PCR started with a high annealing temperature for the first primer-annealing step, and then with reduction the annealing temperature by 1ºC for each later cycle. Touchdown PCR

reduces nonspecific amplification and the time for optimized PCR reaction, and improves specificity and product yield (Hecker and Roux, 1996).

DNA sequencing

The PCR products for direct DNA sequencing were purified to separate the unincorporated nucleic acid and primers from amplified DNA by filtration with the QIA quick Purification Kit (Qiagen, USA). Sequencing reactions with a DNA sequencing kit (dye terminator cycle sequencing ready reaction; Applied Biosystem, Foster City, CA,USA) were performed with 5 μl of purified PCR product and 3.2 pmol of the RpoB-for primer. Centri-sep spin columns (Applied Biosystems) were used to separate the unincorporated dye terminators and primers from the extension products. The products were dried in a vacuum centrifuge, resuspended in a loading buffer, heat-denatured for 2 minutes at 90ºC, and immediately loaded onto a 4% acrylamide gel in an automated DNA sequencer (Applied Biosystems Prism 377, Perkin Elmer).

SSCP analysis

For SSCP analysis, 1.5 μl aliquot of the 435-bp PCR products was mixed with 3 volumes of SSCP-loading buffer (90% formamide, 0.4M EDTA, 0.05% bromo-phenol-blue, 0.05% Xylene Cyanol FF; and 20mM NaOH). Samples were heated in a heating block to 95ºC for 3 minutes and immediately placed on ice. The resulting DNA conformations were analyzed by electrophoresis on a 20-cm 8% polyacrylamide gel (37.5:1 acrylamide: bisacrylamide) for 20 hours at 800 V in 0.5% Tris-borate-EDTA buffer (53 mM Tris, 53 mM boric acid, 1.5 mM EDTA-disodium) at room temperature. The gels were stained with an aqueous solution of silver stain containing acetic acid (10%), $AgNO_3$ (1g/l) and $Na_2S_2O_3$ (4.5 mg/l) (Bassam *et al*, 1991).

RAPD analysis

A "Ready-To-Go RAPD Analysis Beads" commercial kit (Amersham Pharmacia Biotech) consisting of standardized dry beads that are room-temperature-stable was used for RAPD analysis and molecular typing of the 70 RIF^r and 10 RIF^s strains. The beads contain thermostable polymerases (AmpliTaq DNA polymerase and Stoffel fragment), dNTPs (0.4mM each in a 25 μl reaction volume), BSA (2.5 μg), and buffer (3 mM $MgCl_2$, 30 mM KCl and 10 mM Tris pH 8.3 in a 25 μl reaction volume). Ten μl of 2.5 pmol/μl RAPD analysis primer 5 (5′-AACGCGCAAC-3′), 8 μl of template DNA (10 ng/μl), and 7 μl of sterile water were added. The reaction mixture was heated in a Gene Amp PCR system 2400 thermocycler (Perkin Elmer, USA) for 45 cycles (1 minute at 94ºC, 1 minute at 36ºC, and 2 minutes at 72ºC). After electrophoresis in a 2% Nusieve agarose gel, the amplicons were visualized by ethidium bromide staining. Photographs were digitalized and stored in tiff file format. To assure reproducibility of the results, PCR was done at least twice on all samples. *M. tuberculosis* H37Rv standard strain was used as an internal control (reference strain) for measuring variability of RAPD patterns among experiments. RAPD patterns for the individual strains were analyzed with respect to the presence or absence of electrophoresis DNA bands. The RAPD profiles were considered unrelated if they differed by more than one band.

RESULTS

With all 80 strains, the touch-down PCR program yielded a single band 435-bp PCR product of the *rpoB* gene that starts at Leu-420 codon and ends at Glu-565 codon that was used as a template for DNA sequencing. By increasing the chance of finding new sequence-altered codon positions, this longer PCR product allowed us to find unreported point mutations at positions outside the regions of the *rpoB* gene explored by other studies using shorter PCR products, such as 157-bp or 305-bp regions. Specifically, we found previously unreported point mutations at positions Val-432, Asp-443, Arg-454, and Glu-458; deletion mutations at positions Gly-434, Asp-443, and Asp-444; and silent mutations at positions Pro-433, Val-436, Phe-448 and Gln-517 (Table 2). Two mutations, Val-432 (Asp) and a silent mutation at Gln-517 were found in strains each of which had a single mutation in the explored region of the *rpoB* gene. However, in addition, we also found number of other strains with unreported forms of multiple mutations (Table 1) in the *rpoB* gene.

Table 1
Mutation combinations of 70 RIF^r and 10 RIF^s *M. tuberculosis* strains from Thailand.

Mutation combination	Resist profile	Number of strains/(%)	Disease/Patient domicile (no. patients)	RAPD type (no. strains)
Val-432(Asp)	R	1 (1.4)	*TB* / BK	A
517^s	R	22 (31.4)	*TB* / BK (9), NB (6), PT(2), SPK, AY, NP, SPR, CN	A (8) , B (6), C(4), D (1), G (1), H (2)
517^s	R S	1 (1.4)	*TB* / NB	A
517^s	R I	2 (2.8)	*TB* /AY, PR	B, E
Ser-531^a (Leu)	R	1 (1.4)	*TB* / BK	A
Leu-533(Pro)	R	1 (1.4)	*TB* / BK	A
Val-432(Asp) , 517^s	R	1 (1.4)	*TB* /NB	B
436^s, 517^s	R	1 (1.4)	*TB* /NB	B
444^d (GAC → -AC), 517^s	R	1 (1.4)	*TB* /NP	G
Arg-454 (Cys), Glu-458 (Asp)	R	1 (1.4)	*TB* / SPK	B
517^s, His-526^a(Cys)	R	3 (4.3)	*TB* / BK (2), RR	A, B, I
517^s, His-526^b(Tyr)	R	1 (1.4)	*TB* / PT	B
517^s, His-527 (Asp)	R	1 (1.4)	*TB* /NB	B
517^s, Ser-531^a(Leu)	R	1 (1.4)	*TB* /SPK	C
517^s, Ser-531^b(Trp)	R S	1 (1.4)	*TB* / BK	B
517^s, Ser-531^b (Trp)	R O	1 (1.4)	*TB* / BK	E
517^s, Leu-533 (Pro)	R	1 (1.4)	*TB* / PT	A
517^s, Arg-557^a (Gly)	R	4 (5.7)	*TB* /NB, BK (2), NW	A(3), F
517^s, 557^{In} (CGG→CGGG)	R	3 (4.3)	*TB* / BK, LP, PB	A, B, D
517^s, Pro-564 (Leu)	R	2 (2.8)	*AIDS, TB* / BK, NB	A, B
517^s, Pro-564 (Leu)	R I	1 (1.4)	*TB* / BK	E
Val-550 (Leu), Cys-559 (Phe)	R	1 (1.4)	*TB* / KK	B
433^s, Gln-513 (Thr), 517^s	RSIO	1 (1.4)	*TB* / BK	E
436^s, 517^s, 556^d (GGC→GG-)	R S	1 (1.4)	*TB* /NB	A
436^s , 517^s, Arg-557^a (Gly)	R	1 (1.4)	*AIDS* /BK	A
Asp-443^a (His), 517^s, Pro-564 (Leu)	R	1 (1.4)	*TB* /BK	C
444^d , 517^s, Arg-557^a (Gly)	R	1 (1.4)	*TB* / BK	A
444^d, 517^s, 557^{In} (CGG→CGGG)	R	1 (1.4)	*TB* / BK	A
448^s, 517^s, Ser-531^a (Leu)	R	1 (1.4)	*TB* /NB	B
Val-498 (Gly), 517^s, Arg-557^a	R	1 (1.4)	*TB* / KB	C
517^s, Ser-531^b (Trp), Pro-564 (Leu)	R	1 (1.4)	*TB* / BK	B
433^s ,436^s, Gln-513, 517^s, Pro-564	RIO	1 (1.4)	*TB* / BK	B
436^s, 443^b, 517^s, 556^s, Arg-557^a (Gly)	R I	1 (1.4)	*AIDS* / BK	D
434^d, 443^b, 444^d, 517^s, 531^a , 556^d	RS	1 (1.4)	*AIDS* / BK	A
29 Silent-mutation positions*	R	1 (1.4)	*TB* / BK	I
No mutation	R	5 (7.1)	*TB*/ BK (4), NB	A(2), B(2),G(1)
No mutation	None	10	*AIDS* / BK (10)	J (10)

Superscripts: s = silent mutation; d = deletion; In = Insertion; a, b indicate different substitutions for the same codon; * Table 3. R = rifampicin, I = isoniazid, S = streptomycin, O = ofloxacin, none = susceptible to all drug [44 patients from Bangkok (BK); 15 from Nonthaburi (NB); 4 from Prathumthani (PT); 3 from Samut Prakan (SPK); 2 from Ayutthaya (AY); 2 from Nakhon Pathom (NP); and 1 each from Suphan Buri (SPR), Phetchburi (PR), Chai Nat (CN), Phetchabun (PB), Rachburi (RR), Kanchanaburi (KB), Nakhon Sawan (NW), Khon Kaen (KK), Lop Buri (LR) and Samut Sakhon (SSK)].
The *rpoB* mutation position is based on *rpoB* gene of *E. coli* numbering system (Miller *et al*, 1994). Column 2 gives the drug resistance profile for each mutation combination, and column 3, the number and percent of strains carrying the combination. Column 4 gives the disease (*TB* = Patient with pulmonary tuberculosis, *AIDS* = AIDS patients who developed disseminated tuberculosis) of the patients from which the strains carrying a combination were isolated, and the provincial domiciles of those patients, and number of patients (strains) from each; and column 5 gives the RAPD types for the strains, and number of strains for each type.

Table 2
Pattern of *rpoB* mutations (from codon Val-432 to Pro-564) of 70 RIF^r *M. tuberculosis* strains from Thailand.

Codon*	Nucleotide Mutation	Type	Amino acid changes	Mutation frequency (strain)/(%)
432	GTT → GAT	Substitution	Val → Asp	2 (2.8)
433 ^{s}	CCG → CCA	Silent	Pro → Pro	2 (2.8)
434 ^{d}	GGC → -GC	Deletion	Gly →	1 (1.4)
436 ^{s}	GTC → GTA	Silent	Val → Val	5 (7.1)
443 ^{a}	GAC → CAC	Substitution	Asp → His	1 (1.4)
443 ^{d}	GAC → -AC	Deletion	Asp →	2 (2.8)
444 ^{d}	GAC → -AC	Deletion	Asp →	4 (5.7)
448 ^{s}	TTC → TTT	Silent	Phe → Phe	1 (1.4)
454	CGT → TGT	Substitution	Arg → Cys	1 (1.4)
458	GAG → GAT	Substitution	Glu → Asp	1 (1.4)
498	GTG → GGG	Substitution	Val → Gly	2 (2.8)
513	CAA → CCA	Substitution	Gln → Thr	3 (4.3)
517 ^{s}	CAG → CAA	Silent	Gln ' Gln	60 (85.7)
526 ^{a}	CAC → TGC	Substitution	His → Cys	1 (1.4)
526 ^{b}	CAC → TAC	Substitution	His → Tyr	3 (4.3)
527	CAC →GAC	Substitution	His → Asp	1 (1.4)
531^a	TCG → TTG	Substitution	Ser → Leu	5 (7.1)
531^b	TCG → TGG	Substitution	Ser → Trp	2 (2.8)
533	CTG → CCG	Substitution	Leu → Pro	2 (2.8)
550 ^{s}	GTG → TTG	Substitution	Val → Leu	1 (1.4)
556 ^{s}	GGC → GGG	Silent	Gly → Gly	2 (2.8)
556 ^{d}	GGC → GG-	Deletion	Gly →	1 (1.4)
557 ^{a}	CGG → GGG	Substitution	Arg ' Gly	9 (13)
557 ^{In}	CGG → CGGG	Insertion	Arg →	3 (4.3)
559	TGC → TTC	Substitution	Cys → Phe	1 (1.4)
564	CCT → CTT	Substitution	Pro ' Leu	6 (8.6)
None	No mutation	No mutation	No mutation	5 (7.1)

Superscripts: s = silent mutation; d = deletion; In = insertion; a, b indicate different substitutions for the same codon. Value shown in bold are the predominant mutation, respectively.
**E. coli* numbering system for the β-subunit of the RNA polymerase (Ovchinnikov *et al*, 1981) (excluded strain TB57 that had 29 silent mutations, and shown in Table 3).

Our DNA sequence analysis of the 70 RIF^r strains disclosed 26 different mutation positions within a 396-bp region of the *rpoB* gene (Table 2), and revealed that 28 of the 70 (40%) strains had a mutation at only one position (Table 1); 24 (34%), at two positions; 9 (13%), at three positions; 2 (3%), at five positions; just 1 (1%), at six positions. In addition, we found that 5 strains (7%), which had been reported to have the RIF^r phenotype, contained no mutation within the full 435-bp region of the *rpoB* gene examined in this study (Table 1 and Table 2). One RIF^r strain, surprisingly, had a silent mutation at twenty-nine positions. For this strain, strains with five and six positions of mutation and two selected RIF^s strains, we repeated the steps of *rpoB* gene PCR amplification, purification of PCR product, and DNA sequencing, and obtained the same results (Table1 and Table 3).

For the 70 RIF^r *M. tuberculosis* strains that carried a mutation, the silent mutation at the Gln-517 codon occurred in the largest proportion of the strains (60 strains, 86%); followed by the point mutation Arg-557 (Gly) in 9 strains (13%);

Table 3
Codon positions of twenty-nine silent mutations in *M. tuberculosis* RIF^r strain from Thailand.

No.	Codon*	Nucleotide mutation	Type	Amino acid changes
1	432	GTT → GTG	Silent	Val → Val
2	435	GGC → GGG	Silent	Gly → Gly
3	452	CGC → CGG	Silent	Arg → Arg
4	453	CTG → TTG	Silent	Leu → Leu
5	455	ACG → ACC	Silent	Thr → Thr
6	470	CGG → AGG	Silent	Arg → Arg
7	477	GAG → GAA	Silent	Glu → Glu
8	481	ACC → ACT	Silent	Thr → Thr
9	484	GTG → GTC	Silent	Val → Val
10	488	ACA → ACG	Silent	Thr → Thr
11	491	ACG → ACA	Silent	Thr → Thr
12	492	TTG → CTG	Silent	Leu → Leu
13	496	CGG → CGC	Silent	Arg → Arg
14	501	GCG → GCC	Silent	Ala → Ala
15	511	CTG → CTC	Silent	Leu → Leu
16	512	AGC → TCC	Silent	Ser → Ser
17	513	CAA → CAG	Silent	Gln → Gln
18	517	CAG → CAA	Silent	Gln → Gln
19	523	GGG → GGC	Silent	Gly → Gly
20	524	TTG → CTC	Silent	Leu → Leu
21	529	CGA → CGG	Silent	Arg → Arg
22	530	CTG → CTT	Silent	Leu → Leu
23	535	CCC → CCG	Silent	Pro → Pro
24	539	TCA → TCC	Silent	Ser → Ser
25	540	CGT → CGG	Silent	Arg → Arg
26	546	GAG → GAA	Silent	Glu → Glu
27	547	GTC → GTG	Silent	Val → Val
28	548	CGC → CGT	Silent	Arg → Arg
29	557	CGG → CGC	Silent	Arg → Arg

**E. coli* numbering system for the β-subunit of the RNA polymerase (Ovchinnikov *et al*, 1981).

point mutation Pro-564 (Leu) in 6 strains (9%); and the point mutation Ser-531(Leu) in 5 strains (7%) (Table 2).

When the 435-bp PCR products of the 65 RIF^r strains with *rpoB* mutations were analyzed by PCR-SSCP, three bands were found, whereas, when the 10 RIF^s strains were analyzed, together with the 5 RIF^r strains that contained no mutation within the 435-bp region of the *rpoB* gene, only 2 bands were found (Fig 1). The one RIF^s strain having a silent mutation at Gln-517 also yielded 2 bands, but the lower band was shifted down more than the 2 SSCP bands of other 9 RIF^s strains (figure not shown).

Using RAPD fingerprinting technique, all 80 strains (70 RIF^r and 10 RIF^s) could be typed and grouped genetically into 10 RAPD types (A-J) (Table 1). Only the 10 RIF^s strains (from the same group of AIDS patients) exhibited RAPD type J (Table 1). For the RIF^r strains, the predominant group is RAPD type A, represented by 26 strains (23 strains in profile R, and 3 strains in the multidrug-resistant profile RS); followed by RAPD type B, representing 22 strains (19 strains in profile R, and 3 strains in the multidrug resistant profiles RI, RS, and RIO), and then by RAPD type C, 7 strains; RAPD type D, 3 strains; RAPD type E, 4 strains; RAPD type F, 2 strains; RAPD type

Table 4
Comparison of mutations in *M. tuberculosis* RIF^r strains.

*rpo*B mutation position (based on *rpo*B gene of *E. coli* numbering system) (17)	Frequency of codon substitution [no. (%) of isolates]					
	Telenti (n = 66)	Williams (n= 110)	Hirano (n=90)	Sintchenko (n=21)	Pozzi (n =37)	This study (n=70)
Val-432 (Asp)	-	-	-	-	-	1 (1.4)
Leu-511 (point)	2 (3)	-	-	-	-	-
Gln-513 (point)	2 (3)	1 (0.9)	5 (5.6)	-	-	-
514-Phe^{ln}(insert)	-	1 (0.9)	2 (2.2)	-	-	-
Asp-516 (Val)	6 (9.1)	13 (14.4)	13 (14.4)	1 (4.5)	1 (2.7)	-
Asp-516 (Tyr)	-	-	-	1 (4.5)	-	-
Gln-517^s(silent)	-	-	-	-	-	25 (35.7)
Asn-518 (point)	1 (1.5)	1 (0.9)	-	-	-	-
Leu-521 (point)	-	1 (0.9)	-	-	-	-
Ser-522 (point)	1 (1.5)	2 (1.9)	1 (1.1)	2 (9)	-	-
His-526 (point)	18 (27.3)	37 (33.6)	15 (16.7)	4 (19)	11 (29.7)	-
Ser-531 (Leu/Trp)	35 (50)	46 (35.7)	48 (53.3)	11 (52)	21 (56.7)	1 (1.4)
Leu-533 (Pro)	1 (1.5)	3 (2.7)	-	-	-	1 (1.4)
Val-432, Gln-517^s	-	-	-	-	-	1 (1.4)
Val-436^s, Gln-517^s	-	-	-	-	-	1 (1.4)
Asp-444^d , Gln-517 ^{s}	-	-	-	-	-	1 (1.4)
Arg-454 (Cys), Glu-458 (Asp)	-	-	-	-	-	1 (1.4)
Ser-509, His-526	-	1 (0.9)	-	-	-	-
His-526, Lys-527	-	1 (0.9)	-	-	-	-
Gln-517^s, His-526^a (Cys)	-	-	-	-	-	3 (4.3)
Gln-517^s, His-526^b (Tyr)	-	-	-	-	-	1 (1.4)
Gln-517^s, His-527 (Asp)	-	-	-	-	-	1 (1.4)
Gln-517^s, Ser-531^a (Leu)	-	-	-	-	-	1 (1.4)
Gln-517^s, Ser-531^b (Trp)	-	-	-	-	-	2 (2.8)
Gln-517^s, Leu-533 (Pro)	-	-	-	-	-	1 (1.4)
Gln-517^s, Arg-557^a (Gly)	-	-	-	-	-	4 (5.7)
Gln-517^s, 557^{ln} (CGG → CGGG)	-	-	-	-	-	3 (4.3)
Gln-517^s, Pro-564 (Leu)	-	-	-	-	-	3 (4.3)
His-526(Asp), Ser 531^a(Leu)	-	-	-	-	1(2.7)	-
Val-550 (Leu), Cys-559 (Phe)	-	-	-	-	-	1 (1.4)
Pro-433^s, Gln-513 (Thr), Gln-517^s	-	-	-	-	-	1 (1.4)
Val-436^s, Gln-517^s, Gly-556^d	-	-	-	-	-	1 (1.4)
Val-436^s , Gln-517^s, Arg-557^a	-	-	-	-	-	1 (1.4)
Asp-443^a, Gln-517^s, Pro-564 (Leu)	-	-	-	-	-	1 (1.4)
Asp-444^d , Gln-517^s, Arg-557^a	-	-	-	-	-	1 (1.4)
Asp-444^d, Gln-517^s, Gln-557^{ln}	-	-	-	-	-	1 (1.4)
Phe-448^s, Gln-517^s, Ser-531^a(Leu)	-	-	-	-	-	1 (1.4)
Val-498 (Gly), Gln-517^s, Arg-557^a	-	-	-	-	-	1 (1.4)
Leu-511(Pro), Ser-512(Thr), Asp-516(Val)	-	-	-	-	1(2.7)	-
Thr-516(Ile), Gly-523(Trp), Asp-525(Tyr)	-	-	-	-	1(2.7)	-
Gln-517^s, Ser- 531^b, Pro-564 (Leu)	-	-	-	-	-	1 (1.4)
His-526(Asp), Glu-541(Gly), Ser-553(Ala)	-	-	-	-	1(2.7)	-
Five position of mutation*	-	-	-	-	-	2 (2.8)
434^d, 443^b, 444^d, 517^s, 531^a , 556^d	-	-	-	-	-	1 (1.4)
29 positions of silent mutation**	-	-	-	-	-	1 (1.4)
No mutation	2 (3)	8 (7.3)	5 (5.6)	3 (13.5)	-	5 (7.1)

Superscripts: s = silent mutation; d = deletion; ln = lnsertion; a, b indicate different substitutions for the same codon.
*See in Table 1: 433^s ,436^s, Gln-513, 517^s, Pro-564; 436^s, 443^b, 517^s, 556^s, Arg-557^a (Gly)
*See in Table 3.
Telenti *et al* (1993); Williams *et al* (1994); Hirano *et al* (1999); Sintchenko *et al*(1999); Pozzi *et al* (1999).

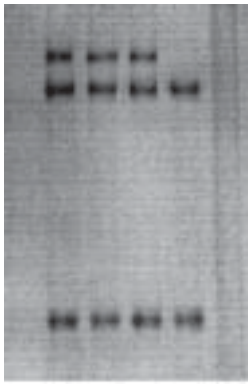

Fig 1–Photograph of Single Strand Conformation Polymorphism (SSCP). Numbers 1, 2, and 3 indicate three different rifampicin-resistant (RIF^r) *M. tuberculosis* strains, which show 3 bands; and "S" is a rifampicin-susceptible (RIF^s)"strain, which shows 2 bands.

G, 3 strains; RAPD type H, 2 strains; RAPD type I, 2 strains. RAPD type E was only found in 3 multidrug-resistant strains (RIF^r profiles RI and RSIO). There was no correlation between RIF^r mutations and RAPD types.

Although the 70 RIF^r strains came from many parts of Thailand (but mainly from Bangkok), we found no significant differences in the distribution of the mutations among them. Also, RAPD showed that strains classified together on the basis of RAPD fingerprinting patterns can have distinctly different *rpoB* alleles. Thus, strains that have RAPD type A were found in almost every group, when the strains were grouped by the number of mutated positions (zero, one, two, three, and six). Strains with the same *rpoB* alleles could have different RAPD profiles. For example, 3 strains in the double mutation group, which carried a silent mutation at Gln–517 and an insertion at Arg-557, had RAPD types A, B and D (Table 1).

DISCUSSION

In this study, silent mutation at the Gln-517 codon occurred in the largest proportion of the strains (60 strains, 86%); followed by the point mutation Arg-557 (Gly) in 9 strains (13%); the point mutation Pro-564 (Leu) in 6 strains (9%); and the point mutation Ser-531(Leu) in 5 strains (7%). This pattern of frequencies of *rpoB* mutations at the different positions was different from that reported in two previous studies using strains from Thailand: a first study of just 3 RIF^r strains and a second of 14 strains. The former study reported two new types of mutation, namely, Asp-516 (Tyr) and Ser-531 (Phe), together with a previously reported mutation Ser-531(Leu) (Vattanaviboon *et al*, 1995). The latter study found that Ser-531 (Leu) was the predominant mutation, representing 29%; followed by His-526(Arg), 14%; His-526(Asp) 14%; Asp-516(Val) 14%; His-526 (Tyr), 7%; and Gln-513 (Pro) 7% (Hirano *et al*, 1999).

In previous studies (not just in Thailand), most of the *rpoB* mutations found in *M. tuberculosis* strains were single amino acid substitutions (single point), at either position Ser-531 (Leu/Gln) or His-526 (Asp/Tyr); although for a few strains, double mutation (Pozzi *et al*, 1999, Williams *et al*, 1994), and even triple mutation existed (Pozzi *et al*, 1999). In contrast, in our study, 56% of the strains had mutations at more than one position in the *rpoB* gene, and the mutation frequencies at the different positions were also different from the earlier studies, as shown in Table 4.

MDR-TB outbreak is a major concern in many countries. in Thailand, even though the incidence of MDR-TB strains in 1998 was not high (2.3%), yet the incidence of MDR-TB in areas of high HIV infection, as in the Chiang Rai Province, is an alarming 3 times higher than that of the whole country (Payanandana *et al*, 1999). Understanding how this might occur requires understanding of the ecology and evolution of drug resistance for *M. tuberculosis*. Some aspects of our results pertaining to the evolutionary dynamics of drug resistance may be relevant to such understanding.

For the region of the *rpoB* gene that was studied, 10% of the 10 RIF^s strains and 36% of the 70 RIF^r strains had mutations all of which were silent. Since silent mutations are not subject to selection, one first expects the two above percentages to be the same. Hence, if the estimate for percentage of strains with only silent mutations is reasonably accurate, then the three-

fold difference begs explanation.

One explanation that seems plausible is as follows. When an asexual, clonal bacterial population finds itself under evolutionary stress, which occurs when a change in environmental conditions substantially reduces the average absolute fitness of individuals, then theoretically, the population can solve the problem by means of mutator alleles (Brooks, 1998). A mutator allele can increase mutation rates by 10- to 1,000-fold, and thereby can accelerate adaptation, even if the allele remains at a very low frequency (*eg*, 10^{-5}), as shown by Sniegowski *et al* (1997). The rate of adaptation is increased because the rate at which more fit individuals invade the population is increased (Taddei *et al*, 1997). Mutator alleles make no distinction between silent and selective mutation events. Hence, RIF^r clone has acquired mutator alleles, it can be expected to accumulate more silent mutations than a RIF^s clone. Consequently, our data appears to support Moxon and Thaler's (1997) proposal that mutators (or mutator alleles) are "a key aspect of adaptive evolution".

For RIF^s strains, the *rpoB* gene is a highly conservative gene (Lisitsyn *et al*, 1988) that has a basic role in the physiology of the bacteria. The gene encodes RNA polymerase (β-subunit) and this gene also participates with other genes, including *rpoA*, *rpoC* and *rpoD* genes, which produce proteins involved in gene transcription (Kunin, 1996). On the other hand, the *rpoB* gene for RIF^r strains is apparently pleiotropic, meaning that for such strains the *rpoB* gene provides not only the function required for RIF^s clones, but also the function of contributing to drug resistance.

This suggests that the mutated, pleiotropic *rpoB* gene in RIF^r strains is only able to succeed (become established) in populations and clones of *M. tuberculosis* because the gene contributes to drug resistance. That resistance apparently offsets a cost to the biochemistry of RIF^r *M. tuberculosis* cells, a cost that would lead to extinction of RIF^r clones if tuberculosis patients should cease to be treated with drugs. Such resistance to toxicants (in this case resistance of *M. tuberculosis* to drugs) has been a convenient model for investigating whether adaptive changes are, as appears to be true for RIF^r *M. tuberculosis* strains in this study, associated with pleiotropic fitness costs (Chevillon *et al*, 1997).

Our sequence analysis of the 70 RIF^r strains confirms that a high proportion of such strains have mutations in the *rpoB* gene, compared to a low proportion for RIF^s strains. The mutations of 57% of the RIF^r strains in our study include non-silent substitutions, deletions and insertions in the *rpoB* gene. These mutations are absent in the RIF^s strains, which are closer to the wild type.

This observed relative uniformity of RIF^s strains might be a consequence of a relatively recent sweep of a *rpoB* mutant throughout the RIF^s clones of a region in Eurasia that includes Thailand. But generally, sweeps across asexual clones that destroy large amounts of variation between clones within a region are more likely for clones that, for example, are participating in adaptive radiation within a newly available niche, as appears to be the case for RIF^r clones in Thailand, where, as throughout the world, drug treatment has created a new niche that is now in the process of exploitation. Asexual clones participating in adaptive radiation can quickly accumulate variation that leads to a next periodic sweep.

More likely, perhaps, the observed relative uniformity of RIF^s strains in Thailand reflects divergence in the evolutionary trajectories of RIF^s and RIF^r strains. Over a long evolutionary past, before drug treatment, RIF^s clones of the *M. tuberculosis* species presumably became well adapted to their natural circumstances, which was void of drug treatment for their host individuals. Therefore the action of natural selection on the *rpoB* gene apparently favored genotypes that improved or further optimized the effectiveness of the gene in carrying out its role in the production of RNA polymerase. In contrast, over a rather recent evolutionary past, RIF^r clones have acquired, through pleiotropy, evolutionary competency by becoming resistant to drugs used in treating tuberculosis patients, but quite possibly, as mentioned above, the competency has come at some cost to the evolutionary refinement of the *rpoB* gene's participation in the intricate biochemistry involving optimal production and quality of RNA polymerase.

Although many of the mutations found in the RIF^r strains but not in the RIF^s strains may be neutral, and therefore able to invade the population by means of genetic drift (regardless of existence or non-existence of drug treatment), at least some may be selective, and produce phenotypic changes that are unable to invade *M. tuberculosis* populations, were it not for drug treatment of patients. That is, the RIF^s strains may largely be the product of a long evolutionary history during which at least some, if not most, of the mutations found in our RIF^r strains were apparently unable to succeed, partly or wholly because host individuals did not, during that evolutionary history, receive drug treatment.

In support of this last explanation, for a RIF^r clone to succeed in competition with other RIF^r clones in Thailand, it may well have to evolve faster and more effectively than other clones. If so, this is an example of the Red Queen effect (Van Valen, 1973; Rosenzweig *et al*, 1987). The evolutionary principle called the Red Queen was first proposed in 1973 by Van Valen (1973), who stated that much of the evolution of a lineage consists simply of keeping up with environmental changes (particularly those changes that occur as a consequence simultaneous evolution of lineages), rather than occupying or adapting to new environments. In other words, what generally counts most for the evolutionary success of a clone or lineage may be that it evolves more rapidly and effectively than other clones with regard to overall environmental conditions. Hence, when for *M. tuberculosis* populations the overall environmental condition rather suddenly included drug therapy for tuberculosis patients, of those clones that acquired resistance to the drugs, those that most rapidly and effectively improved and refined their resistance, and most rapidly adjusted to changes in drug therapy, in competition with other such evolving clones, presumably have a long-term persistence advantage, and therefore tend to comprise the resistant clones that exist today in Thailand.

The consequence of such a race to adapt to a new available niche can be a "mutational" Red Queen (which can involve mutator alleles, as discussed above), and which in turn leads to mutation accumulation"(Rice, 1998). Hence, the Red Queen principle taking place under the circumstances of a need for rapid adaptation to a new difficult problem to be solved (*ie*, new difficult set of environmental circumstances imposed by man in the case of *M. tuberculosis*) may be a major reason for the observation of a high proportion of RIF^r strains having mutations in the *rpoB* gene compared to RIF^s strains.

Ideally, for any *M. tuberculosis* clone, superior fitness is a result of two fundamental capacities: the capacity to be able to multiply faster within hosts than other clones, and thus become prevalent within each host, and the capacity to keep human hosts alive and active, so that they serve well as infection agents. To solve this dilemma of somewhat conflicting requirements, *M. tuberculosis* clones may have evolved mechanisms that help to keep their chronically ill hosts alive, accomplished by freeing their hosts intermittently of symptoms.

Assuming that superior fitness for a *M. tuberculosis* clone is dependent on the two stated fundamental capacities mentioned above, humans can enhance the rate of evolution of drug resistance for *M. tuberculosis* in essentially two ways: by increasing the seriousness of the problem that drug treatment of patients poses for *M. tuberculosis* clones, and at the same time, by providing them the means of solving the problem stepwise instead of by a single grand solution. The seriousness of the problem for *M. tuberculosis* clones can be increased by increasing the proportion of infected humans that are treated with drugs, and by completing treatment for all patients. Providing *M. tuberculosis* clones a stepwise means of solving the problem of drug treatment can be accomplished by allowing uncompleted treatment to occur for some but not all patients. The first lower step for a clone then is to acquire resistance to drugs for patients whose treatments are uncompleted. The next and last step is to acquire further resistance that works even if treatment of patients would, apart from the acquired drug resistance of the clone, be completed.

Consequently, from the standpoint of control of MDR-TB, elimination of uncompleted treatments is critical. Since uncompleted treatment tends to facilitate the rate of evolution of in-

creased levels of drug resistance within MDR-TB clones (Kristski *et al*, 1997; Iseman, 1999), which facilitates increase in prevalence of such clones in *M. tuberculosis* populations, treatment becomes more difficult, which makes non-compliance more likely, and therefore uncompleted treatments more likely, which completes a vicious self-accelerating cycle. This possibility of uncompleted treatments resulting in escalating rise in prevalence and strength of MDR-TB means that every case of uncompleted treatment counts, and should be avoided if man is to stem the MDR-TB tide. Hence, early detection of the MDR-TB may well be critical to durable control of tuberculosis.

To our knowledge, this is the first paper to report mutations at more than 3 positions of the *rpoB* gene might be explained in several ways. Firstly, in this study, a larger region of the gene was examined. But it may also be that, from this study in Thailand, drug treatment for individuals infected with *M. tuberculosis* happens to be such that, compared with treatment in other countries studied, conditions are more optimal (as discussed above) for rapid evolution of drug resistance. Noteworthy, when traced back to clinical data, the RIF^r strains from this study with mutations at more than 3 positions of the *rpoB* gene were found to have come from patients experiencing chronic infection for years, and most had non-compliance treatment records (data not shown). Clearly, the longer *M. tuberculosis* is allowed to remain in a treated patient, the greater can be the accumulation of mutations for drug resistance.

Since rifampicin resistance (RIF^r) appears to be an effective marker of MDR-TB, and since mutation screening with PCR-SSCP and confirmation with PCR-DNA sequencing can detect the presence of RIF^r *M. tuberculosis* within 2 days, and can clearly differentiate them from RIF^s strains, the technique described in our study may prove to be useful not only for identifying MDR-TB, but also for further work towards the understanding of the ecology and evolution of *M. tuberculosis* evolution. For this, we need more sequence data and more understanding of structure-function relationships for the RNA polymerase ß-subunit. Further investigation of RIF^r *M. tuberculosis*, with larger sample sizes and a variety of mutation-screening techniques, and samples taken from different geographic regions, along with evaluation of the effects and roles of the different mutations in mechanisms of rifampicin resistance, are required.

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