The Journal of Antibiotics (2015) 68, 431–435

www.nature.com/ja

ORIGINAL ARTICLE

Study of efflux pump gene expression in rifampicin-monoresistant *Mycobacterium tuberculosis* clinical isolates

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Rifampicin (RIF) resistance is a risk factor for poor outcome in tuberculosis (TB). In *Mycobacterium tuberculosis*, both target gene mutation and efflux pumps have major roles in the resistance to anti-TB drugs. This study aimed to determine whether RIF induces efflux pump activation in RIF-monoresistant *M. tuberculosis* strains. Here, we took advantage of 16 RIF-monoresistant *M. tuberculosis* clinical isolates to evaluate the expression of 27 putative drug efflux pump genes and measured the influence of four drug efflux pump inhibitors, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), verapamil (VP), thioridazine (TZ) and chlorpromazine (CPZ), on the RIF MICs of these strains. Eight of the 16 RIF-monoresistant isolates carried mutations in *rpoB* and overexpressed one or two of the following putative efflux pump genes: *Rv2333*, *drrB*, *drrC*, *Rv0842*, *bacA* and *efpA*. CCCP, VP, TZ and CPZ lowered the RIF MICs greater than fourfold in 6, 12, 9 and 12 isolates, respectively. The lowered RIF MICs by VP and CPZ were identical and stronger than CCCP (*P*-values were all 0.033). In conclusion, the efflux pumps Rv2333, DrrB, DrrC, Rv0842, BacA and EfpA may have a role in RIF resistance in addition to classical mutations in the *rpoB* gene, and the addition of VP and CPZ could significantly increase RIF susceptibility in RIF-monoresistant *M. tuberculosis*.
The Journal of Antibiotics (2015) **68,** 431–435; doi:10.1038/ja.2015.9; published online 18 February 2015

INTRODUCTION

Tuberculosis (TB) is still a major public health problem, accounting for 8.6 million new cases and 1.3 million deaths each year.[1] Moreover, the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB have hampered the effective TB treatment and control. Rifampicin (RIF) is one of the most important anti-TB antibiotics; it exerts its bactericidal activity by arresting DNA-directed RNA synthesis of *Mycobacterium tuberculosis* by interacting with the β subunit of RNA polymerase.[2,3] Previous research has demonstrated that >95% of RIF-resistant isolates are associated with mutations in the 81-bp region (codons 507–533) called the RIF resistance-determining region of the *rpoB* gene. However, several studies demonstrated that RIF induced differential expression of efflux genes in MDR-TB isolates based on large-scale transcriptional data.[4–6] Some efflux pumps selectively extrude specific antibiotics, whereas MDR pumps expel a variety of structurally diverse compounds with differing antibacterial modes of action. For *M. tuberculosis*, the *in vivo* activation of both drug-specific and broadly active efflux pumps has been demonstrated for several anti-TB drugs, including isoniazid, ethambutol and fluoroquinolone.[7,8] A previous study has demonstrated that efflux pumps contribute to RIF resistance in RIF-monoresistant *M. tuberculosis* without mutations in *rpoB*.[9] By eliminating the effect of polyresistance, the choice of RIF-monoresistant strains may allow us to identify RIF-specific efflux pump genes. The aim of this study was to determine the efflux pump genes contributing to RIF resistance in RIF-monoresistant clinical *M. tuberculosis* isolates carrying mutations in *rpoB* in China for the first time.

MATERIALS AND METHODS

Ethical approval

This study obtained approval from the Ethics Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. The patients with TB included in this research were given a subject information sheet, and all of them gave written informed consent to participate in the study.

Bacterial strains

Sixteen RIF-monoresistant (resistant to RIF but sensitive to seven other drugs tested) *M. tuberculosis* clinical isolates that were isolated from 16 adult patients with pulmonary TB during the period of 2004–2006 were collected in seven

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Received 27 September 2014; revised 28 October 2014; accepted 19 January 2015; published online 18 February 2015

provincial TB hospitals in China including Anhui, Guangxi, Henan, Hunan, Sichuan, Shanxi and Tibet. H37Rv (ATCC 27294) was included as a control.

Antibiotics and chemicals

Middlebrook 7H9 broth and albumin-dextrose-catalase supplement were purchased from Difco (Detroit, MI, USA). Isoniazid (INH), RIF, ethambutol (EMB), streptomycin (STR), ofloxacin, kanamycin, capreomycin, amikacin, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), verapamil (VP), thioridazine (TZ) and chlorpromazine (CPZ) were purchased from Sigma–Aldrich Company (St Louis, MO, USA). All solutions were prepared on the day of the experiment. Trizol was purchased from Invitrogen (Carlsbad, CA, USA). Alamar blue was purchased from AbD Serotec (Oxford, UK).

Conventional drug susceptibility testing and *Mycobacterium* species identification

Clinical isolates were tested for susceptibility to four first-line anti-TB drugs (INH, RIF, STR and EMB), and four second-line anti-TB drugs (kanamycin, ofloxacin, capreomycin and amikacin) using a proportion method with Lowenstein–Jensen plates as described by the World Health Organization.[10] The concentrations of drugs in medium were: 0.2 μg ml^{-1} INH, 40 μg ml^{-1} RIF, 4 μg ml^{-1} STR, 2 μg ml^{-1} EMB, 30 μg ml^{-1} kanamycin, 2 μg ml^{-1} ofloxacin, 40 μg ml^{-1} capreomycin[10] and 30 μg ml^{-1} amikacin.[11] Lowenstein–Jensen medium containing paranitrobenzoic acid (500 μg ml^{-1}) was used to identify *M. tuberculosis* complex species from non-TB mycobacteria, and medium containing thiophen-2-carboxylic acid hydrazide (5 μg ml^{-1}) was used to exclude *Mycobacterium bovis* from the *M. tuberculosis* complex. This study included the *M. tuberculosis* complex but did not include *M. bovis* clinical isolates.

PCR amplification and DNA sequencing of the *rpoB* gene

Genomic DNA was extracted from freshly cultured bacteria by the cetyltrimethylammonium bromide method. A 450-bp region of the *rpoB* gene containing the 81-bp resistance-determining region was amplified by polymerase chain reaction (PCR). The forward primer was 5′-ACCGACGACA TCGACCACTT-3′, and the reverse primer was 5′-GTACGGCGTTTCGAT GAACC-3′. Using a 50-μl PCR mixture (Kangwei Biotechnology, Beijing, China), the PCR reactions were denatured at 94 °C for 5 min and subjected to 35 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. Partial PCR sequence was characterized by DNA sequencing using the specific primers on an ABI Prism 3730 automated DNA sequencer (ABI Prism). The resulting DNA sequences were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The specific mutations in protein sequences of individual isolates were identified.

Spoligotyping and data analysis

Spoligotyping was performed using 43 covalently bound oligonucleotides derived from the spacer sequences of *M. tuberculosis* H37Rv and *M. bovis* BCG P3 as previously described by Kamerbeek *et al.*[12] The results in binary format were entered in an Excel spreadsheet and compared with the spoligotyping database SpolDB4 (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp).

IS6110 in the NTF region

All Beijing family strains identified by the spoligotyping were amplified by PCR to detect the presence or absence of *IS6110* in the NTF region[13] using the following primers: F-6110 (5′-CCAGATATCGGGTGTGTCGAC-3′) and R-6110 (5′-TGCCGTTGTCGAAATCTAAACCC-3′). Strains with the insert yielded an amplified product of ~1100 bp, whereas those without the insert yielded an ~300-bp PCR product.

Determination of MICs

To determine the RIF MICs of 16 clinical isolates and H37Rv, the microplate alamar blue assay was performed according to the protocol of Leonard *et al.*[14] The effect of the efflux pump inhibitors (EPIs) CCCP, VP, TZ and CPZ on the RIF MIC was also studied by incorporating the inhibitors at subinhibitory concentrations in the *M. tuberculosis* cultures in the assay. Twofold serial dilutions of RIF (resulting in a range of concentrations from 0.001 to 256 μg ml^{-1}) were made directly in the wells in the absence or presence of 1.6 μg ml^{-1} CCCP, 64 μg ml^{-1} VP, 2 μg ml^{-1} TZ or 4 μg ml^{-1} CPZ. The above concentrations for four EPIs were half the MIC values against 92 *M. tuberculosis* isolates (data not shown). The MIC was defined as the lowest drug concentration that prevented a change in color. Strains with an MIC $\leqslant$ 1 μg ml^{-1} were considered to be susceptible to RIF.[14] All tests for each strain were carried out at least in duplicate to calculate a mean MIC for each strain.

Expression of efflux pump genes

Selected RIF-monoresistant isolates and H37Rv were cultured in duplicate in enriched 7H9 medium in the presence or absence of RIF (1/4 the MIC of each strain) for 4 weeks. Total RNA was extracted by Trizol according to the instructions of the manufacturer. After treatment with DNase I (Invitrogen), the complementary DNAs were reverse-transcribed from 1.5 μg of total RNA with a TransScript First-Strand cDNA Synthesis SuperMix Kit (Transgen Bio-Technology Company, Beijing, China). Quantitative reverse transcription-PCR was performed in a 20-μl system containing 10 μl 2× mixture supplied with SYBR Green, 10 ng complementary DNA template and 3 pmol each primer set. Primers for quantitative reverse transcription-PCR analysis are listed in Table 1. The ΔCT values were obtained by subtracting the CT value of the *secA1* gene from the CT value obtained for each gene, and ΔΔCT was calculated by subtracting the ΔCT values of the non-induced strain from the corresponding RIF-induced ΔCT for each gene tested. The quantity of messenger RNA in RIF-induced strains relative to the non-induced strain was calculated by the $2^{-\Delta\Delta Ct}$ method. When compared with the non-induced control, an expression equal to 1 indicated identical expression levels, and an expression $\geqslant$4 indicated overexpressed.[15–18]

Data analysis

SPSS 14.0 (SPSS Inc., Chicago, IL, USA) was used to perform X^2 analysis, and the difference was considered to be statistically significant when $P<0.05$.

RESULTS

Identification of *rpoB* mutations

The *rpoB* sequence of 16 clinical isolates was examined. Only one RIF-monoresistant isolate did not have a mutation in the resistance-determining region of *rpoB*. Of the other RIF-monoresistant isolates, 10 carried mutations of 531 TCG-TTG (Ser–Leu), 1 carried 531 TCG-TTC (Ser–Phe), 2 carried 526 CAC-GAC (His–Asp), 1 carried 522 TCG-ATG (Ser–Met) and 1 carried 516 GAC-TTC (Asp–Phe) (Table 2).

Genotyping results

Spoligotyping results for the 16 RIF-monoresistant isolates showed that a total of 12 isolates belonged to the Beijing family, whereas 4 isolates belonged to non-Beijing families, which included the U family (2 isolates), T1 family (1 isolate) and Orphan (1 isolate) (Table 2). On the basis of the presence of *IS6110* upstream of the NTF region,[19,20] 10 out of 12 Beijing family strains were confirmed as modern Beijing strains and two were ancient Beijing strains (Table 2).

RIF MICs and the effect of EPIs on the RIF MICs of *M. tuberculosis*

As demonstrated in Table 2, levels of resistance to RIF as measured by MICs varied among RIF-monoresistant isolates. These data suggest that biological mechanisms in addition to the *rpoB* mutation and the genetic background of the strains are responsible for defining the respective RIF MIC values.

To test the hypothesis that efflux pumps were involved in modulating MICs, RIF-monoresistant strains were cultured in the presence of RIF together with the EPIs CCCP (1.6 μg ml^{-1}), VP

Table 1 Primers used to quantify gene expression

Gene	Primer	Sequence (5′–3′)	Amplicon size (bp)
efpA	*Rv2846c F*	CGCCCTACGGGAAACCAACAAAGA	226
	Rv2846c R	GCGGAACAAGTGGAACGGCACGAC	
emrB	*Rv0783c F*	ACCGCACAGAACATCCGCTCATAG	148
	Rv0783c R	GATTGGTGCAACACTTGCTGGAGG	
Rv0849	*Rv0849 F*	GTCGTTCGCAACCGTCCGTTTCTG	94
	Rv0849 R	CCTGCATGGGCAGAGCCAGATAGA	
Rv1250	*Rv1250 F*	GCAGCCTTGGATTTGGGCGGTGAT	133
	Rv1250 R	GGACAAGCTGAAGTTCCGGTCGTT	
tap(Rv1258c)	*Rv1258c F*	CGTCTGGAACCTGCGGGTATTGCG	118
	Rv1258c R	CGGTTGCTGGTGGTCGGTGAAGTA	
P55(Rv1410c)	*Rv1410c F*	ATCCCGACGGCAAACACGTACTGC	205
	Rv1410c R	ACATCAACCAGCGTCACCATCAGC	
Rv1634	*Rv1634 F*	TCGATACCTACGTGCCGCTGTTCG	157
	Rv1634 R	GCTGCCACGACATGCCCGATAACT	
Rv2994	*Rv2994 F*	ATGCGTCCCGTCCGCCTGAT	147
	Rv2994 R	GGTGGCTTCTAGCCCGTTGTCC	
bacA	*Rv1819c F*	GCGTCGTAGTTGTTGCGGAAG	165
	Rv1819c R	TGGATGGAATCTGTCGGTGAGC	
stp	*Rv2333c F*	TCCGATGATGGATCTGACCCTG	217
	Rv2333c R	GCCAACCAGGTGCCCAACA	
jefA(Rv2459)	*Rv2459 F*	CGTCGCCCTGATCGCATACA	213
	Rv2459 R	CAGGACATCACCACGAAGTAGACG	
Rv2265	*Rv2265 F*	CGGTTGTCCTCGGTAATCCT	112
	Rv2265 R	AACCCGAACGTGCCAAAC	
Rv2456c	*Rv2456c F*	CAGCGAACCCCACCAAA	140
	Rv2456c R	GCACAATCGAGACGAAGGAA	
Rv3239c	*Rv3239c-F*	GCCGATTCCTGGCACTTTT	146
	Rv3239c R	ATGTGGATGGCGGTGTGTT	
mmpL13a	*Rv1145 F*	GACGACCTGCTGGTGATGGAGTTG	242
	Rv1145 R	CGACTGACGATGAGCAGCGTGTAG	
mmpL13b	*Rv1146 F*	ATGTTCGGCCTCGGCCTGACTTTA	182
	Rv1146 R	GAACGTCTCCTCGAAACCGGCTCT	
pstB	*Rv0933 F*	CTGGACCCGACTACCACCGAGAA	95
	Rv0933 R	GCCTGGGCAAGGTTATGGGTC	
drrA	*Rv2936 F*	TAGACATCGCGTGCGGATTGGT	147
	Rv2936 R	GCGTGGTCAACAACGTGGCAAT	
drrB	*Rv2937 F*	TCGCCAGCAACTTAGGGCAATACA	233
	Rv2937 R	TCCGATGACGTAGCCGCAAACTAG	
drrC	*Rv2938 F*	GTTTGGTGCCGCTCAACTCGTATC	171
	Rv2938 R	GGTACGGCGCATACGACGCAGATA	
mmr	*Rv3065 F*	TAGTGGGTTATGGCATCGCTTTCG	167
	Rv3065 R	GACGCCAACCACCTTCATCACAGA	
Rv0037c	*Rv0037c F*	GCGAAGAACAGCAGTGCGGTA	105
	Rv0037c R	GCATCGGATGGTGGTCGGTATC	
Rv0191	*Rv0191 F*	GCTGCCATGAGCCTGATGTG	164
	Rv0191 R	CGAGGATTACGGTGGTGACGAG	
Rv1672c	*Rv1672c F*	CCGTTGTTGGCAGTGTGATATGG	189
	Rv1672c R	CGCTGTATGCGTTGCAGTTCTT	
Rv0842	*Rv0842 F*	GCCGCTGTATACCTGCCGATGT	100
	Rv0842 R	TTGTCCGAGAGTGCCTGCCGATA	
Rv0876c	*Rv0876c F*	GGACCGATGAGTGGAGCGATCA	133
	Rv0876c R	ACTCGGCAATGGCGGTAGCA	
Rv2209	*Rv2209 F*	CTGGGCACCACGTTCTTCAGC	220
	Rv2209 R	GCGTGAACCCACTGCCCACA	
secAl	*secAl-F*	AGAGGTGTTCACGCCACTTACG	146
	SecAl-R	GCTGGAGGCACTACTCAAGGAC	

(64 μg ml^{-1}), TZ (2 μg ml^{-1}) or CPZ (4 μg ml^{-1}). CCCP, VP, TZ and CPZ decreases RIF MICs greater than or equal to fourfold in 6, 12, 9 and 12 isolates, respectively (Table 2). The ability to decrease RIF MICs more than fourfold by VP and CPZ was identical and stronger than CCCP (*P*-values were all 0.033) (Table 2). The RIF MIC of 14 RIF-monoresistant isolates was decreased by at least eightfold by at least one out of four EPIs. We also found that the increased RIF susceptibility was independent of both the *rpoB* mutation and the genetic background (Table 2). However, the RIF susceptibility of none of the 16 RIF-resistant isolates was restored by the EPIs. For strain H37Rv, the RIF MIC of 0.0625 μg ml^{-1} decreased to 0.0156 μg ml^{-1} by CPZ and to 0.0313 μg ml^{-1} by CCCP; TZ and VP had no effect on MIC.

Effect of RIF induction on the expression of drug efflux pumps

For strain H37Rv, RIF did not increase expression for 24 of 27 genes, but resulted in a ± twofold increase in expression for three genes. The expression of *drrC* showed the highest induction (2.81-fold).

Of the 16 RIF-monoresistant isolates, 8 isolates overexpressed (⩾ fourfold induction) one or two of the following genes: *Rv0842*, *bacA*, *Rv2333*, *efpA*, *drrB* and *drrC*. Among the eight isolates with overexpressed drug efflux pump genes, seven carried mutations of *rpoB* 531 TCG-TTG (Ser–Leu), six belonged to the modern Beijing family, six had RIF MICs ⩾128 μg ml^{-1} and six had ⩾ 8-fold decrease in the RIF MICs with at least one of the four EPIs (Table 2).

Of 27 efflux pump genes tested, 15 (*Rv0037c*, *Rv0191*, *Rv0783*, *Rv0876c*, *pstB*, *mmpL13a*, *Rv1672c*, *bacA*, *Rv2209*, *Rv2294*, *Rv2459*, *P55* (*Rv1258c*), *drrA*, *drrB* and *drrC*) were induced two- to threefold by RIF in at least one RIF-monoresistant *M. tuberculosis* isolate (Table 3). Thirteen of 16 isolates showed two- to threefolds induction in at least one of the 15 induced genes. Three (SHX05124, XZ06125 and SC06157) of 16 isolates (18.8%) showed no induction in any of the 27 efflux pump genes under RIF stress.

DISCUSSION

Previous research indicated that mutations in the resistance-determining region of *rpoB* are responsible for up to 95% of RIF-resistant *M. tuberculosis* strains. In this study, resistance-determining region mutations were detected in 15 of 16 clinical isolates (93.8%). However, half of the RIF-monoresistant isolates that carried mutations in *rpoB* overexpressed one or two of the following putative efflux pump genes: *Rv2333*, *drrB*, *drrC*, *Rv0842*, *bacA* and *efpA*. In addition, the level of RIF resistance varied independently of the mutations in the *rpoB* gene and the genetic background of the clinical isolates of *M. tuberculosis*. One possible explanation for this is that classical mutations in *rpoB* and efflux pumps both confer RIF resistance in RIF-monoresistant *M. tuberculosis*.

Four EPIs (CCCP, VP, TZ and CPZ) were used to determine the efflux pump contribution to RIF resistance. Our observation that the inhibition of VP and CPZ significantly increased RIF susceptibility in RIF-monoresistant strains suggests the importance of efflux pump proteins in defining the intracellular concentration of anti-TB drugs. This finding is concordant with findings of previous studies, which also showed that EPIs increased susceptibility to RIF.[6,21] It also implies that EPIs have the potential to improve the efficacy of anti-TB drug treatment.[6] In this study, the effect of VP and CPZ on RIF susceptibility was identical and showed stronger inhibition than CCCP, suggesting that differences in the efficacy of EPIs may be associated with the drug mode of action. CPZ and VP are ion channel blockers,[22,23] whereas CCCP is an uncoupler of proton transport that

Table 2 Mutations, genotypes, MICs of RIF, effect of efflux inhibitors on the RIF MIC and overexpressed efflux pump genes in RIF-monoresistant *M. tuberculosis* clinical isolates

				MIC ($\mu g\,ml^{-1}$)[b]						
Strain ID	n[a]	*Mutations in* rpoB	*Genotypes*	*RIF*	*RIF+CCCP*	*RIF+VP*	*RIF+TZ*	*RIF+CPZ*	*Maximum fold decrease of RIF MIC by inhibitors*	*Genes overexpressed (fold change)*
SHX05124	11	531 TCG-TTG(Ser–Leu)	U	256	64	16	32	8	32	No
SHX05178		531 TCG-TTG(Ser–Leu)	U	256	16	32	32	16	8	*Rv0842* (10.25)
AH04017		531 TCG-TTG(Ser–Leu)	Modern Beijing	256	16	4	16	8	16	*bacA* (7.82)
GX06183		531 TCG-TTG(Ser–Leu)	Modern Beijing	128	128	32	32	16	8	*stp* (4.34), *drrB* (5.54)
HN06075		531 TCG-TTG(Ser–Leu)	Modern Beijing	128	32	64	16	16	8	*drrC* (5.45)
HN04076		531 TCG-TTG(Ser–Leu)	Modern Beijing	128	32	16	32	16	8	*drrC* (5.45)
HN04054		531 TCG-TTG(Ser–Leu)	Modern Beijing	64	256	16	128	16	16	*drrB* (4.72), *drrC* (6.80)
XZ06115		531 TCG-TTG(Ser–Leu)	Modern Beijing	32	64	32	32	32	2	*efpA* (5.05)
XZ06013		531 TCG-TTG(Ser–Leu)	Modern Beijing	128	16	4	8	8	8	No
HeN05030		531 TCG-TTC(Ser–Phe)	Modern Beijing	64	64	8	64	16	8	No
SC06157		531 TCG-TTG(Ser–Leu)	Orphan	32	64	8	16	16	32	No
HN04143	2	526 CAC-GAC(His–Asp)	Modern Beijing	256	128	8	128	128	16	No
HN06095		526 CAC-GAC(His–Asp)	Ancient Beijing	64	256	32	128	128	8	No
XZ06125	1	516 GAC-TTC(Asp–Phe)	Modern Beijing	2	2	1	2	1	2	No
SC06161	1	522 TCG-ATG(Ser–Met)	T1	16	32	32	16	4	16	*drrB* (4.35)
GX06026	1	—	Ancient Beijing	64	4	2	4	1	8	No

Abbreviations: CCCP, carbonyl cyanide m-chlorophenyl hydrazone; CPZ, chlorpromazine; RIF, rifampicin; TZ, thioridazine; VP, verapamil.
[a]*n*, the number of strains tested.
[b]The ability to decrease the RIF MIC more than fourfold; verapamil (VP) compared with carbonyl cyanide m-chlorophenyl hydrazone (CCCP): $\chi^2=4.571$, $P=0.033$; chlorpromazine (CPZ) compared with CCCP: $\chi^2=4.571$, $P=0.033$; thioridazine (TZ).

Table 3 Number of rifampicin-monoresistant isolates that showed increased expression (fold change) of 27 efflux pump genes

Gene	*~0*	*~1*	*~2*	*~3*	*>4*	*Total*
drrA	1	9	5	1	0	16
drrB	1	8	1	3	3	16
drrC	2	3	5	3	3	16
efpA	11	4	0	0	1	16
mmr	11	5	0	0	0	16
emrB	5	7	2	2	0	16
Rv0849	14	2	0	0	0	16
mmpL13a	11	3	2	0	0	16
mmpL13b	11	5	0	0	0	16
Rv1250	13	3	0	0	0	16
tap (Rv1258c)	12	3	1	0	0	16
P55 (Rv1410c)	6	10	0	0	0	16
Rv1634	9	7	0	0	0	16
bacA	9	4	2	0	1	16
Rv2209	4	8	1	3	0	16
Rv2294	8	7	1	0	0	16
Rv0191	11	4	1	0	0	16
stp	11	4	0	0	1	16
Rv2459	7	8	1	0	0	16
pstB	4	9	2	1	0	16
Rv2456c	9	7	0	0	0	16
Rv2265	9	7	0	0	0	16
Rv3239c	10	6	0	0	0	16
Rv1672c	7	8	0	1	0	16
Rv0842	13	2	0	0	1	16
Rv0876c	7	8	1	0	0	16
Rv0037c	1	11	4	0	0	16

can inhibit the proton concentration gradient of the active efflux system.[24]

In this study, a few efflux pump genes were induced by RIF in RIF-monoresistant *M. tuberculosis*. *drrA* (*Rv2936*), *drrB* (*Rv2937*) and *drrC* (*Rv2938*) were combined in the open reading frame of *drrABC* of the ABC family. *drrAB* expressed in *Mycobacterium smegmatis* can lead to resistance to EMB, STR, norfloxacin, erythromycin, tetracycline and chloramphenicol.[25] Gupta *et al.* reported that *drrC* was induced 4.1- and 7.7-fold under EMB and STR stress, respectively; however, they did not find that *drrC* was induced under RIF stress in MDR *M. tuberculosis*.[4] In this study *drrC* was overexpressed in three RIF-monoresistant isolates that belonged to the modern Beijing family and carried the *rpoB* 531 TCG-TTG (Ser–Leu) mutation. *drrB* was also overexpressed in three RIF-monoresistant isolates; however, only one of these three isolates overexpressed *drrC*. Pang *et al.*[9] reported that *drrA* was overexpressed among high-level RIF-resistant clinical isolates. Our study showed that RIF induced a low level (two- to threefold) increase in *drrA* expression in six *M. tuberculosis* isolates with RIF MICs that ranged from 16 to 128 $\mu g\,ml^{-1}$. More studies should be performed to determine whether expression differences of *drrABC* lead to differences in RIF MIC.

Gupta *et al.*[4] showed that the *efpA* expression was 4.5-fold greater under INH stress in five MDR isolates, but there was no induction of *efpA* expression under RIF stress in five MDR isolates. They hypothesized that the EfpA efflux pump presents itself as an alternative/additive mechanism of INH resistance in *M. tuberculosis*. Wilson *et al.*[26] also reported a 2.5-fold induction of this gene by INH treatment along with a twofold induction by ethionamide treatment in an INH-sensitive isolate of *M. tuberculosis*. In our study, one RIF-monoresistant strain (XZ06115) overexpressed *efpA*; this strain belonged to the modern Beijing family and carried the 531 TCG-TTG (Ser–Leu) mutation of *rpoB*. Previous studies[4,26,27] did not

provide the molecular background of strains; therefore, the molecular background should be examined to determine whether this is the reason for expression differences.

Stp and Rv0842 are probable conserved integral membrane proteins that are similar to many antibiotic and drug efflux proteins.[28–32] No further information about these proteins has been reported. *stp* and *Rv0842* were overexpressed with RIF stress in one RIF-monoresistant isolate each; both strains carried the 531 TCG-TTG (Ser–Leu) mutation in *rpoB*, but the former belonged to the modern Beijing family and the latter belonged to the U family.

BacA is thought to be involved in the active transport of drugs across the membrane. Gupta *et al.*[4] showed that *bacA* was induced in two of five MDR isolates in the present study, only one high-level RIF-monoresistant *M. tuberculosis* isolate overexpressed *bacA*.

In the present study, two RIF-monoresistant *M. tuberculosis* isolates (SHX05124 and SHX05178) belonged to the U family, carried an identical mutation of *rpoB*, and showed identical RIF MICs. However, in isolate SHX05124, none of the 27 examined efflux pump genes had a greater than twofold induction because of RIF stress. In contrast, in strain SHX05178, *Rv0842* was overexpressed 10-fold under RIF stress. In strain GX06026, which has no *rpoB* mutations, and a RIF MIC value of 64 $\mu g\,ml^{-1}$, none of the 27 efflux pump genes was overexpressed (more than a fourfold change), but the expression of three genes (*drrA*, *drrB* and *drrC*) was increased threefold, this result was not consistent with the result of a previous report.[9] Therefore, more research should be undertaken to determine the cause of the difference.

CONCLUSION

In conclusion, in addition to classical mutations in *rpoB*, the efflux pumps Rv2333, DrrB, DrrC, Rv0842, BacA and EfpA may have a role in RIF resistance. *M. tuberculosis* with knockout genes (such as those belonging to certain genotypes or carrying certain mutations in *rpoB*) should be used to repeat experiments to determine their contributions to RIF resistance. In addition, the EPIs VP and CPZ significantly increased RIF susceptibility in RIF-monoresistant *M. tuberculosis*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was financially supported by projects 2013ZX10003002-001 of the National Key Programme of Mega Infectious Diseases and the key project 2014SKLID104 of the State Key Laboratory for Infectious Disease Prevention and Control. The funders had no role in the study design, data collection and analysis, decision to publish or manuscript preparation.

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