Indian J Med Res 132, August 2010, pp 176-188

jefA (Rv2459), a drug efflux gene in *Mycobacterium tuberculosis* confers resistance to isoniazid & ethambutol

Anuj Kumar Gupta[*], Vineel P. Reddy[+], Mallika Lavania[*], D.S. Chauhan[*], K. Venkatesan[**], V.D. Sharma[*], A.K. Tyagi[+] & V.M. Katoch[*,#]

Departments of [*]Microbiology & Molecular Biology & [**]Biochemistry, National JALMA Institute for Leprosy & Other Mycobacterial Diseases (ICMR), Agra & [+]Department of Biochemistry, University of Delhi, South Campus, New Delhi, India

Received August 17, 2009

***Background & objectives*: Drug efflux pumps have been contributing factor(s) in the development of multidrug resistance in various clinically relevant bacteria. During efflux pump gene expression studies on mycobacteria, we have found a previously uncharacterized open reading frame (ORF) Rv2459 to be overexpressed in drug stressed conditions. The objective of the present study was to investigate the role of this ORF as a drug efflux pump, which might add new information in our understanding about the alternative mechanisms of drug resistance in mycobacteria.**

***Methods*: The open reading frame Rv2459 of *Mycobacterium tuberculosis* encoding a probable drug efflux protein has been cloned using pSD5 *E.coli*-Mycobacterium shuttle vector and overexpressed in *M. tuberculosis* H_{37}Rv. This ORF was named as *jefA*. Overexpression of this gene in clones has been verified by real-time reverse transcription PCR. Minimum inhibitory concentrations (MICs) of recombinant as well as non-recombinant clones were determined by resazurin microtitre assay plate method (REMA) with and without efflux pump inhibitors carbonyl cyanide m-chlorophenylhydrazone (CCCP) and verapamil.**

***Results*: In recombinant strains of *M. tuberculosis*, the overexpression of this gene led to an increase in MIC of anti-tubercular drugs isoniazid and ethambutol when tested by REMA. In the presence of CCCP and verapamil, the recombinant strains showed decrease in MIC for these drugs. Bioinformatic analysis has shown a close relation of JefA protein with drug efflux pumps of other clinically relevant bacteria. In homology derived structure prepared from nearest available model, it was observed that amino acids forming TMH 1, 8 and 11 participated in ethambutol specificity and those forming TMH 2, 7 and 10 participated in isoniazid specificity in JefA.**

***Interpretation & conclusion*: The increased transcription of *jefA* leads to increased resistance to ethambutol and isoniazid in *M. tuberculosis* via efflux pump like mechanism and contributes in the development of resistance to these drugs. JefA amino acid sequence is well conserved among clinically important bacterial genera, which further provides evidence of being a potent drug efflux pump. The involvement in drug resistance and very little homology with any of the human proteins makes JefA important to be included in the list of potential drug targets.**

Key words Drug efflux - ethambutol - isoniazid - *jefA* - *Mycobacterium tuberculosis*

[#]*Present address*: Secretary to Govt. of India, Department of Health Research, Ministry of Health & Family Welfare & Director-General, Indian Council of Medical Research, Ramalingaswami Bhawan, Ansari Nagar, New Delhi 110 029, India

Efflux pump mediated multidrug resistance has become a significant complicating factor in the chemotherapy of bacterial infections. Efflux pumps are plasma membrane proteins having ability to extrude various antimicrobial substances outside the cell by an energy dependent manner. The main function of these efflux pumps is to extrude out environmental toxic substances entering the cell wall as well as metabolic wastes of the cell, thus providing protection to the cell from the toxic effect of these substances[1]. It is thought that these pumps arose so that noxious substances could be transported out of the bacterium allowing its survival, and their increased expression is associated with resistance to alien substances including drugs. These pumps are often called multidrug efflux pumps[2]. On the basis of bioenergetics and structural criteria multidrug efflux pumps are divided into two major classes. Secondary multidrug transporters utilize the trans-membrane electrochemical gradient of proton or sodium ion to drive the extrusion of drugs from the cell. These include transporters of major facilitator superfamily (MFS), small multidrug resistance family (SMR), resistance nodulation division family (RND) and multidrug and toxic compound extrusion family (MATE). On the other hand, ABC (ATP binding cassette) type multidrug transporters use the free energy of ATP hydrolysis to pump drugs out of the cell[3]. Though p-glycoprotein (P-gp) discovered by Juliano and Ling[4] is the most studied and characterized efflux pump in eukaryotes, involved in resistance to anticancer drugs[5], a number of efflux pumps have also been identified and characterized in bacteria, especially in *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus*, *etc*[6]. In tuberculosis, responses to chemotherapy have been greatly challenged by the increasing drug resistance. Multidrug resistance (MDR, resistance to at least isoniazid and rifampicin) has grown up into its more deadly form, extensive drug resistance (XDR), in which *Mycobacterium tuberculosis* isolates are resistant to isoniazid and rifampicin as well as any fluoroquinolone and at least one of three injectable second-line drugs *i.e.*, amikacin, kanamycin, or capreomycin[7]. Mutations in the genes associated with the mode of action of the drugs have been considered as the main mechanism for drug resistance in *M. tuberculosis*. Alternative mechanisms such as decreased cell wall permeability to drugs and active efflux pumping are likely to be important for several drugs specially in isolates in which no mutation in target genes are found. *M. tuberculosis*, being the causative agent of tuberculosis, has been studied for the presence and activity of a number of efflux pump genes and their encoded products[8-17]. One of the open reading frames (ORF) in the genome sequence of *M. tuberculosis*, Rv2459, annotated as gene encoding probable drug efflux protein (Welcome Trust Sanger Institute, *http://www.sanger.ac.uk/Projects/M_tuberculosis/Gene_list/functional_classes/III.A.6.shtml*), was studied in the present work. In our earlier microarray and real-time PCR based studies, this gene has been identified to be overexpressed in isoniazid and ethambutol induced cultures of multidrug resistant *M. tuberculosis*[18] (Indian Patent application No. 2071/DEL/2007) and we have named it as *jefA*. We further investigated the phenotypic effect of *jefA* overexpression in $H_{37}Rv$ in order to know its role in conferring resistance to common anti-tubercular drugs in *M. tuberculosis*.

Material & Methods

Bacterial strains, plasmids, media and reagents: Reference strain *M. tuberculosis* $H_{37}Rv$ (TMC 102) was obtained from Mycobacterial Repository Centre at National JALMA Institute for Leprosy & Other Mycobacterial Diseases, Agra. Competent cells of $H_{37}Rv$, *E. coli* XL1Blue[19] and replicative plasmid pSD5[20] were obtained from stocks earlier prepared by Dr A.K. Tyagi. For growth of *M. tuberculosis*, MiddleBrook 7H9 broth with ADC supplement (7H9-S) and 7H11 agar with OADC supplement (7H11-S; Difco, USA) were used. Luria Bertani (LB) medium was used for culturing *E. coli*. Resazurin sodium salt powder was obtained from Sigma, USA.

Antibiotics and inhibitors: Rifampicin, isoniazid, ethambutol, streptomycin, ofloxacin, norfloxacin, kanamycin and efflux pump inhibitors carbonyl cyanide m-chlorophenylhydrazone (CCCP) and verapamil were obtained from Sigma, USA. Rifampicin and CCCP were dissolved in dimethyl sulphoxide (DMSO, Sigma, USA), while rest of the drugs were dissolved in distilled water (D/w; Span Diagnostics, India). All drugs and inhibitors were filter sterilized through 0.22 µm filters (Millipore, USA).

Primer designing and amplification of Rv2459 (jefA): Forward and reverse primers (TBc1-F & TBc1-R) for whole gene amplification were designed using DNA sequence of Rv2459 taken from Tuberculist web server (*http://genolist.pasteur.fr/TubercuList/*). Primers were flanked with recognition sequences of restriction enzymes *NdeI* and *MluI* at their 5' end with six additional base pairs at extreme 5' end in both the

primers. PCR was set up in a 25 µl reaction volume for amplification of whole gene with 1X CERTAMP buffer, 1.5 units of CERTAMP enzyme (BioTools, Spain), 800 µM dNTP mix (Bangalore Genei, India), 3 mM $MgCl_2$ (Bioron GmbH, Germany), 1.5 µM each of forward and reverse primers and 2 µl $H_{37}Rv$ DNA (boiled and snap chilled). The cycle parameters of PCR included initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 60°C for 30 sec and 72°C for 2 min, followed by a final extension of 5 min at 72°C. This resulted in an amplification of 1551 bp (1527 bp Rv2459 + 12 bp sequence of recognition sites of restriction enzymes *NdeI* and *MluI* + 12 extra bp, 6 each at 5' end of both the primers). The sequences of primers are given in Table I. Primers for detection of *jefA* (Rv2459-F and Rv2459-R) and that of *polA* (polA-F and polA-R) were designed by using free online software Primer3 with default parameters[21]. The 1551 bp amplified PCR product (insert) having flanking restriction sequences was run on agarose gel electrophoresis and eluted from the gel by using QIAGEN kit (QIAGEN GmbH, Germany) or GFX DNA purification kit (Amersham Pharmacia, UK). Insert and vector pSD5, both were double digested with restriction enzymes *NdeI* and *MluI* (MBI Fermentas, USA). For restriction digestion, 1 µg of DNA was digested with 10 units of *MluI* and 20 units of *NdeI* and incubated at 37°C for 3-4 h. Restricted insert and vector were ligated by using 4 units of T4 DNA ligase (Bangalore Genei, India) with overnight ligation at 22°C. Ligation was also performed using Quick Ligase (New England Biolabs, UK) at room temperature for 10 min.

Transformation in E. coli: Ligation mixture was subjected to transformation in *E.coli* XL1Blue cells (efficiency 10^7 cfu/µg) by heat shock treatment and streaked on LB agar plates with kanamycin (25 µg/ml) and incubated at 37°C overnight. Single colonies thus obtained were inoculated in 5 ml LB broth with kanamycin (25 µg/ml) and incubated at 37°C overnight. Plasmid DNA from overnight culture of *E. coli* was isolated by alkaline lysis method with TEG (25 mM Tris-Cl *p*H 8, 10 mM EDTA, 50 mM glucose), NaOH and potassium acetate. Recombinant plasmids were verified by sequencing, restriction digestion with *MluI* and *NdeI* and further verified by PCR amplification of whole gene of *jefA* from plasmid DNA isolated from *E. coli* cells.

Electroporation in M. tuberculosis: Plasmid DNA (100 ng) containing *jefA* gene (pSD5-*jefA*) was mixed with 25 µl of electrocompetent cells of $H_{37}Rv$ (efficiency 10^4 cfu/µg). Electroporation was done in a Cell Porator (Gibco BRL, USA) at 330 µF, 8 KΩ and 375 volts. Cells were revived in 1 ml 7H9 medium at 37°C for 24 h and then streaked on 7H11 plates with kanamycin (25µg/ml), alongwith plates streaked with $H_{37}Rv$ as negative control, and incubated for 25 days at 37°C. Cells were also electroporated with only plasmid DNA (pSD5) as expression control. Colonies obtained were grown on 7H11 medium plates with kanamycin and adapted in 7H9 broth with kanamycin for 7 days at 37°C in a shaker incubator at 200 rpm. Growth was stored in 1 ml aliquots for further use. Recombinant clones were verified by sequencing.

RNA isolation: RNA from *M. tuberculosis* cultures were isolated by TRI Reagent (Sigma, USA). Briefly, two loopful of growth in 400 µl D/w was incubated with 100 µl lysozyme (20 mg/ml, Sigma, USA) for 15 min at 37°C. TRI Reagent (800 µl) was added and incubated at room temperature for 5 min. Suspension was passed through 1 ml (26 gauge) syringe needle 5 times and centrifuged at 12,000 *g* for 5 min. Deproteinization was done with 200 µl chloroform followed by centrifugation at 6000 *g* for 5 min. Upper aqueous layer was collected and RNA was precipitated with 0.75 per cent isopropanol and washed with 150 µl of 75 per cent ethanol with centrifugation at 10,000 *g* for 15 min. Pellet was air-dried and resuspended in 30 µl of diethyl pyrocarbonate (DEPC, Sigma, USA) treated D/w. RNAs were treated with DNaseI enzyme (1U/10 µl of RNA, Ambion, USA) prior to real-time RT-PCR.

Real-Time RT-PCR: Overexpression of *jefA* in recombinant $H_{37}Rv$ was confirmed by real-time reverse transcription PCR in a Light Cycler using RNA Amplification SYBR Green I kit (Roche Diagnostics, Germany) using the protocols of the manufacturer. For real-time RT-PCR, primers used were same as described in Table I (Rv2459 F & R and polA F & R). The annealing temperatures of both the primers were 60°C. The starting amounts of RNAs for *jefA* and *polA* amplifications were equalized for each sample. The crossing point (C_P) values of *jefA* amplification were recorded for each sample *i.e.*, clones harbouring recombinant plasmid pSD5 containing *jefA* gene ($H_{37}Rv$-pSD5-*jefA*), clones harbouring only plasmid pSD5 with no *jefA* insert ($H_{37}Rv$-pSD5) and reference strain $H_{37}Rv$. Mean ΔC_P values were calculated and normalized to that of a housekeeping gene *polA* by $2^{-\Delta\Delta Ct}$ method[22]. Relative quantification was done to

Table I. Bacterial strains, plasmids and oligos used in this study

Strains / Plasmids	Description	Source/Reference
Strains :		
XL1Blue	*E. coli* strain *recA1 endA1 gyrA96 thi-1 hsdR17 supE44*	Bullock *et al*, 1987[19]
XL1Blue-pSD5-*jefA*	XL1Blue harbouring pSD5-*jefA*	Present study
$H_{37}Rv$	*M.tuberculosis* reference laboratory strain	Mycobacterial Repository Centre, NJIL&OMD, Agra
$H_{37}Rv$-pSD5	$H_{37}Rv$ harbouring pSD5 vector	Present study
$H_{37}Rv$-pSD5-*jefA*	$H_{37}Rv$ harbouring pSD5-*jefA*	Present study
Plasmids:		
pSD5	*E. coli*-mycobacterium shuttle vector with mycobacterial hsp60 promoter, Kn^r, oriM, pl5A	DasGupta *et al*, 1998[20]
pSD5-*jefA*	pSD5 vector containing *jefA* gene in its multiple cloning site	Present study
Oligos:		
TBc1 (F)	5'>GAATTACATATGTCCCACACTGCAA TGACACCG<3'	Present study
TBc-1 (R)	5'>GAATTTACGCGTTTAGCTGGCGGGG GTCCGG<3'	Present study
Rv2459 (F)	5'>TGGACGTCAACATCGTCAAT<3'	Present study
Rv2459 (R)	5'>GTGACCCCGAACACAAAACT<3'	Present study
PolA (F)	5'> TTTCACTGCTCGATGACGAC<3'	Present study
PolA (R)	5'> TACCGGCACTTTCCATCTTC<3'	Present study

determine over-expression of *jefA* gene in $H_{37}Rv$-pSD5-*jefA* as compared to that of $H_{37}Rv$-pSD5. ΔC_P values were also calculated and normalized for clones and reference strain $H_{37}Rv$.

MIC determination by REMA: MICs of $H_{37}Rv$-pSD5-*jefA*, $H_{37}Rv$-pSD5 and of $H_{37}Rv$ were determined by Resazurin microtitre assay (REMA) plate method[23-26] with concentrations of drugs ranging from 0.125 to 16 µg/ml. MICs of the clones were also determined in the presence of efflux pump inhibitors CCCP (0.5 µg/ml), verapamil (5 µg/ml) and CCCP + verapamil. The concentrations of the inhibitors were decided after studying the effect of concentration dependent titration performed with these inhibitors on $H_{37}Rv$. Briefly, 100 µl 7H9-S medium was dropped in every well of 96-well microtitre plates (only medium, medium with CCCP, medium with verapamil and medium with CCCP + verapamil in separate rows) except peripheral wells where 250 µl D/w was dropped to prevent evaporation during incubation. Two-fold serial dilutions of drugs were made directly into the wells. 100 µl mycobacterial inoculum of turbidity resembling 0.1 MacFarland index were added to each well except peripheral wells containing D/w. Medium control (only medium without drug, inhibitor and inoculum), growth control (medium without drug and inhibitor but with inoculum) and inhibitor controls (medium without drug but with inhibitor/s and inoculum) were also made in each plate. Plates were covered and incubated for 7 days at 37°C. After 7 days 30 µl of 0.02 per cent resazurin sodium salt solution was added to each well and again incubated for further 24 h at 37°C. A change in colour of the resazurin dye from blue to pink was considered as positive growth and MIC was determined as corresponding concentration in the first blue colour in a row. All the experiments were repeated at least three times.

Phylogenetic and structural analysis: Distance relationship of JefA with homologous sequences within mycobacterial species, other organisms and human was studied. Protein sequence of JefA was derived from TubercuList web server (*http://genolist.pasteur.fr/TubercuList/*) and BLASTp was performed through NCBI server[27]. Multiple sequence alignment was done using ClustalW[28] with the related sequences obtained from BLASTp. Phenograms for distance relation analysis within mycobacterial species as well as within other organisms were generated using free online programme Phylodendron (© 1997 by DG Gilbert) as well as by NCBI web server using Neighbour-Joining

method. Trans-membrane regions in JefA protein were predicted by TMpred[29] and TMHMM server version 2.0 (*http://www.cbs.dtu.dk/services/TMHMM/*).

Results

Real-time RT-PCR analysis of clones: Real-time reverse transcription PCR using SYBR Green I format confirmed over-expression of *jefA* in *M. tuberculosis* $H_{37}Rv$ containing recombinant vector ($H_{37}Rv$-pSD5-*jefA*) with respect to $H_{37}Rv$ containing only pSD5 vector ($H_{37}Rv$-pSD5) as well as reference strain *M. tuberculosis* $H_{37}Rv$. A greater than 16 fold overexpression of *jefA* has been detected by real-time RT-PCR in clones with recombinant vectors as compared to those with non-recombinant vectors by the $2^{-\Delta\Delta Ct}$ method[22]. C_P and ΔC_P values in each case are shown in Table II. Relative normalized value $\Delta\Delta C_P$ for recombinant and non-recombinant clones was >4, while that of recombinant clones and $H_{37}Rv$ was >3, showing a greater than 16 fold over-expression of *jefA* gene in recombinant clones.

MIC determination of clones by REMA and effect of efflux pump inhibitors: There was no significant change in MICs of rifampicin, ofloxacin and norfloxacin in clones. However, in the case of other three drugs (ethambutol, isoniazid and streptomycin), the levels of resistance increased in *jefA* overexpressing clones. Whereas MIC of streptomycin was found increased by 8-fold only (from 0.125 to 1 µg/ml), MICs of isoniazid and ethambutol showed an increase of 64-fold (from 0.125 to 8 µg/ml) and 16 fold (0.5 to 8 µg/ml) respectively (Table III). When efflux pump inhibitors CCCP, verapamil and a combination of CCCP and verapamil were used in parallel wells in REMA, MICs showed a decrease in the case of these three drugs. CCCP lowered the MIC of streptomycin by 2-fold whereas verapamil singly as well as with CCCP lowered the MIC by 4-fold. While MIC of isoniazid was not lowered by verapamil, CCCP lowered the MIC by 2-fold. In case of ethambutol, CCCP lowered its MIC by 8 fold (from 8 to 1 µg/ml), which was greater than the decrease effected by verapamil (from 8 to 4 µg/ml). However, slight increase in MIC of rifampicin (from 0.125 to 0.25 µg/ml) was not decreased with pump inhibitors and therefore this increase was not considered as effect of efflux pump.

Phylogenetic analysis: Homology search and multiple sequence alignment of JefA with other bacterial sequences showed presence of close homologous regions (Fig. 1), whereas with human proteins showed very little identity *i.e.* <27 per cent with any of the human protein (Table IV) with rarer alignments (Fig. 2). It is evident from the phylogenetic tree drawn

Table II. Overexpression of *jefa* gene in recombinant clones as determined by real-time

gene	Crossing points (CP) values				
	Mean C_P (Cl # *jefA*)[a]	Mean C_P (Cl # pSD5)[b]	Mean C_P (H37Rv)[c]	ΔCP (*jefA* & pSD5)	ΔCP (*jefA* & Rv)
	(A)	(B)	(C)	(B)– (A)	(C) – (A)
jefA	19.18	24.62	25.55	5.44	6.37
polA	19.49	20.25	22.58	0.76	3.09
Relative normalized difference in expression (ΔΔCP)				4.68	3.28

[a]Clones containing recombinant vector ($H_{37}Rv$-pSD5-*jefA*); [b]Clones containing non-recombinant vector ($H_{37}Rv$-pSD5); [c]Reference strain $H_{37}Rv$. *polA* was used as reference gene

Table III. MIC of anti-tubercular drugs in bacterial strains harbouring foreign DNA

Drugs	MIC (µg/ml)		
	$H_{37}Rv$	$H_{37}Rv$-pSD5	$H_{37}Rv$-pSD5-*jefA*
RIF	<0.125	<0.125	0.25
RIF+CCCP	<0.125	<0.125	0.25
RIF+VER	<0.125	<0.125	0.25
RIF+CCCP+VER	<0.125	<0.125	0.25
INH	<0.125	<0.125	8
INH+CCCP	<0.125	<0.125	4
INH+VER	<0.125	<0.125	8
INH+CCCP+VER	<0.125	<0.125	4
EMB	0.5	0.5	8
EMB+CCCP	0.5	0.5	1
EMB+VER	0.5	0.5	4
EMB+CCCP+VER	0.5	0.5	1
STR	0.25	<0.125	1
STR+CCCP	0.25	<0.125	0.5
STR+VER	0.25	<0.125	0.25
STR+CCCP+VER	0.25	<0.125	0.25
OFL	0.25	0.25	0.25
OFL+CCCP	0.25	0.25	0.25
OFL+VER	0.25	0.25	0.25
OFL+CCCP+VER	0.25	0.25	0.25
NOR	0.25	0.25	0.25
NOR+CCCP	0.25	0.25	0.25
NOR+VER	0.25	0.25	0.25
NOR+CCCP+VER	0.25	0.25	0.25

RIF, rifampicin; INH, isoniazid; EMB, ethambutol; STR, streptomycin; OFL, ofloxacin; NOR, norfloxacin; CCCP, carbonyl cyanide m-chlorophenylhydrazone; MIC, minimum inhibitory concentration; VER, verapamil

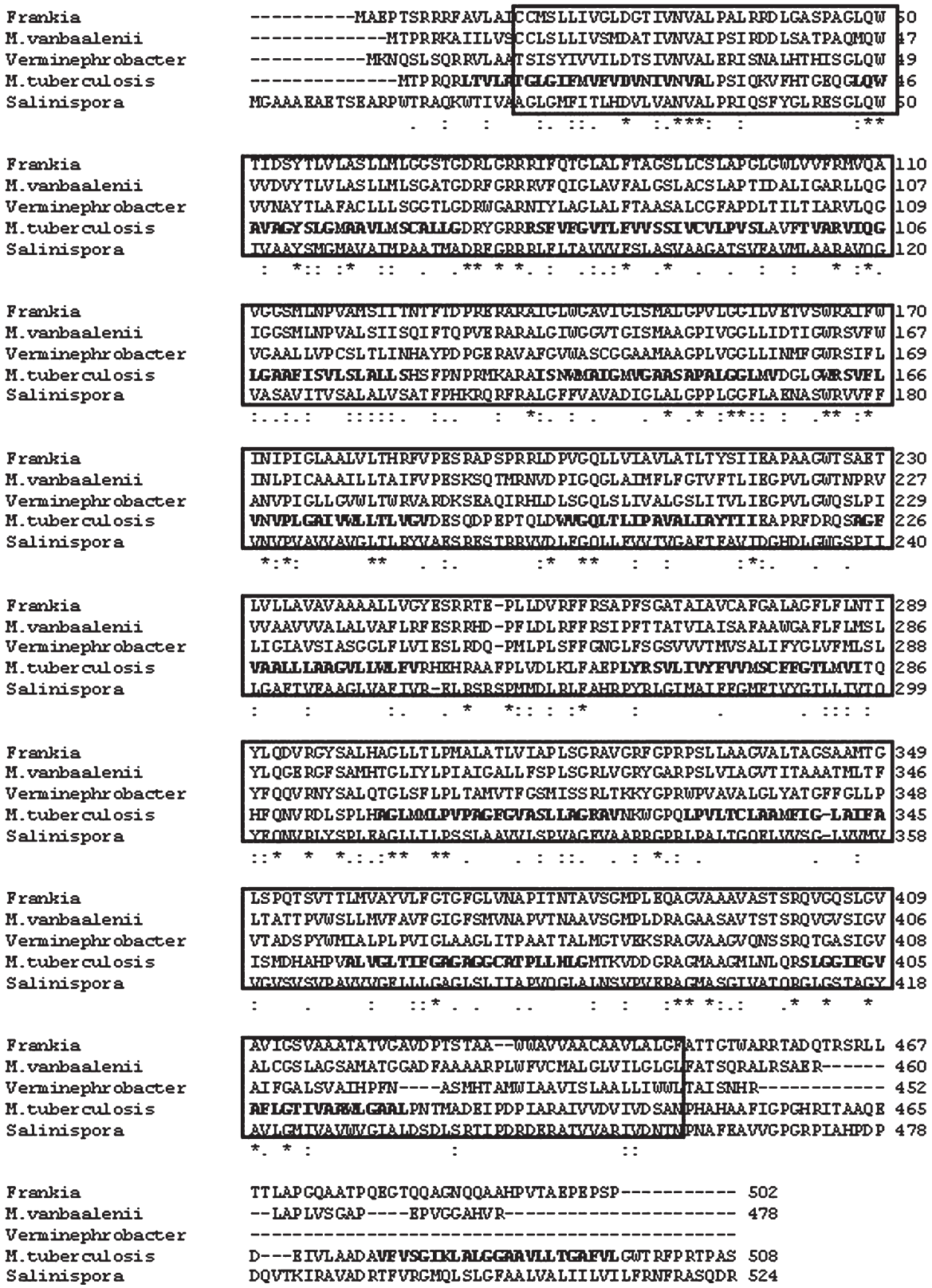


Fig. 1. Multiple sequence alignment of JefA with homologous bacterial proteins found with maximum identities. Block shows the sequence of *M. tuberculosis* forming major facilitator superfamily motif. Bold letters show amino acids forming transmembrane segments.

Fig. 2. Multiple sequence alignment of JefA with human proteins found with maximum identities. Very little identities can be seen within sequences as dots and asterisks. JefA: *M. tuberculosis* JefA, Vesicular: vesicular acetylcholine transporter (*Homo sapiens*), Sodium: sodium phosphate (*Homo sapiens*), Facilitated: facilitated glucose transporter (*Homo sapiens*), Inorganic: sodium-dependent inorganic phosphate cotransporter (*Homo sapiens*), SLC17A8: SLC17A8 protein (*Homo sapiens*).

within mycobacterial species that the amino acid sequence of JefA is well conserved in *M. tuberculosis* and *M. bovis*. Other mycobacterial species showing close relation to JefA sequence are *M. ulcerans* and *M. vanbaalenii* (Fig. 3a). Among other organisms, *Salinospora*, *Streptomyces*, *Verminephrovector*, *Frankia* and *Serratia* are the genera showing closely related homologues of JefA but distant than mycobacterium (Fig. 3b). All homologues are membrane drug transport/efflux proteins suggesting a common structural and functional behaviour of these sequences. Transmembrane region prediction analysis showed that it is a protein with 14 transmembrane helices (TMH) suggesting its location as a transmembrane protein in the cell (Fig. 4a). Due to unavailability of a modeled template with significant amino acid identity (>40%), structure of JefA could not be completely understood. However, homology derived structure prepared from nearest available model (Fig. 4b), suggests that amino acids, forming TMH 1, 8 and 11, participate in ethambutol specificity and those forming TMH 2, 7 and 10 participate in isoniazid specificity in JefA.

Discussion

Multidrug resistance in tuberculosis has become an increasing threat around the world. Various important antitubercular drugs like streptomycin, rifampicin, isoniazid, ethambutol and fluoroquinolones have become therapeutically ineffective in a section of cases due to development of multidrug resistance. This multidrug resistance is also important in the case of non-tuberculous mycobacteria (NTM), which are naturally resistant to a number of therapeutically relevant anti-tubercular drugs.

Mycobacterial drug efflux pumps have been known since the identification of LfrA in *M. smegmatis*[30,31]. Since its discovery, a number of genes have been identified and characterized in mycobacterial species. These genes have been shown to increase the MICs of antibacterial substances when overexpressed in a suitable host[8,10,14]. Several genes which are thought to encode efflux pump proteins are still unexplored. The ORF, Rv2459, which has been characterized in this

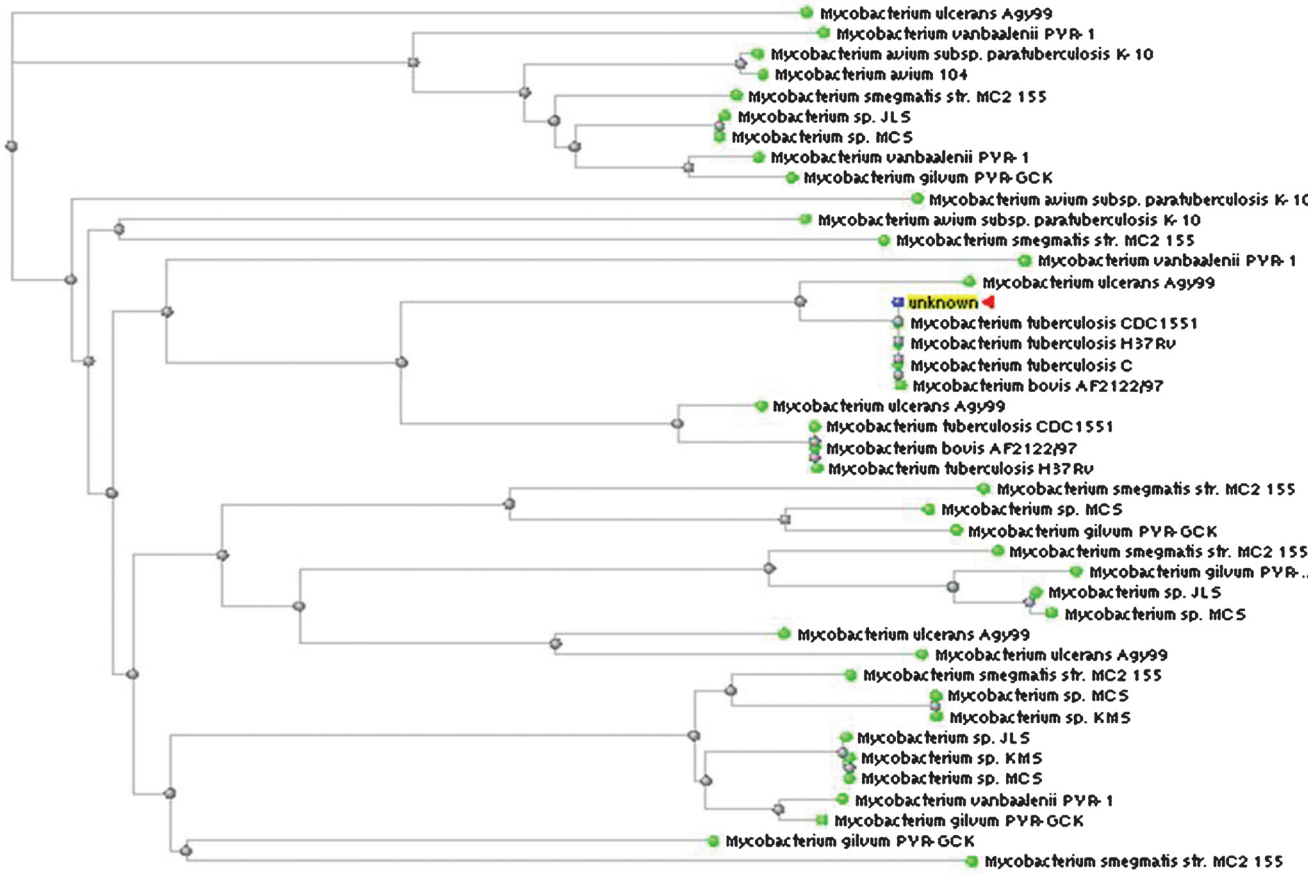


Fig. 3a. Phylogenetic tree showing distance relationship analysis of JefA within mycobacterial species.

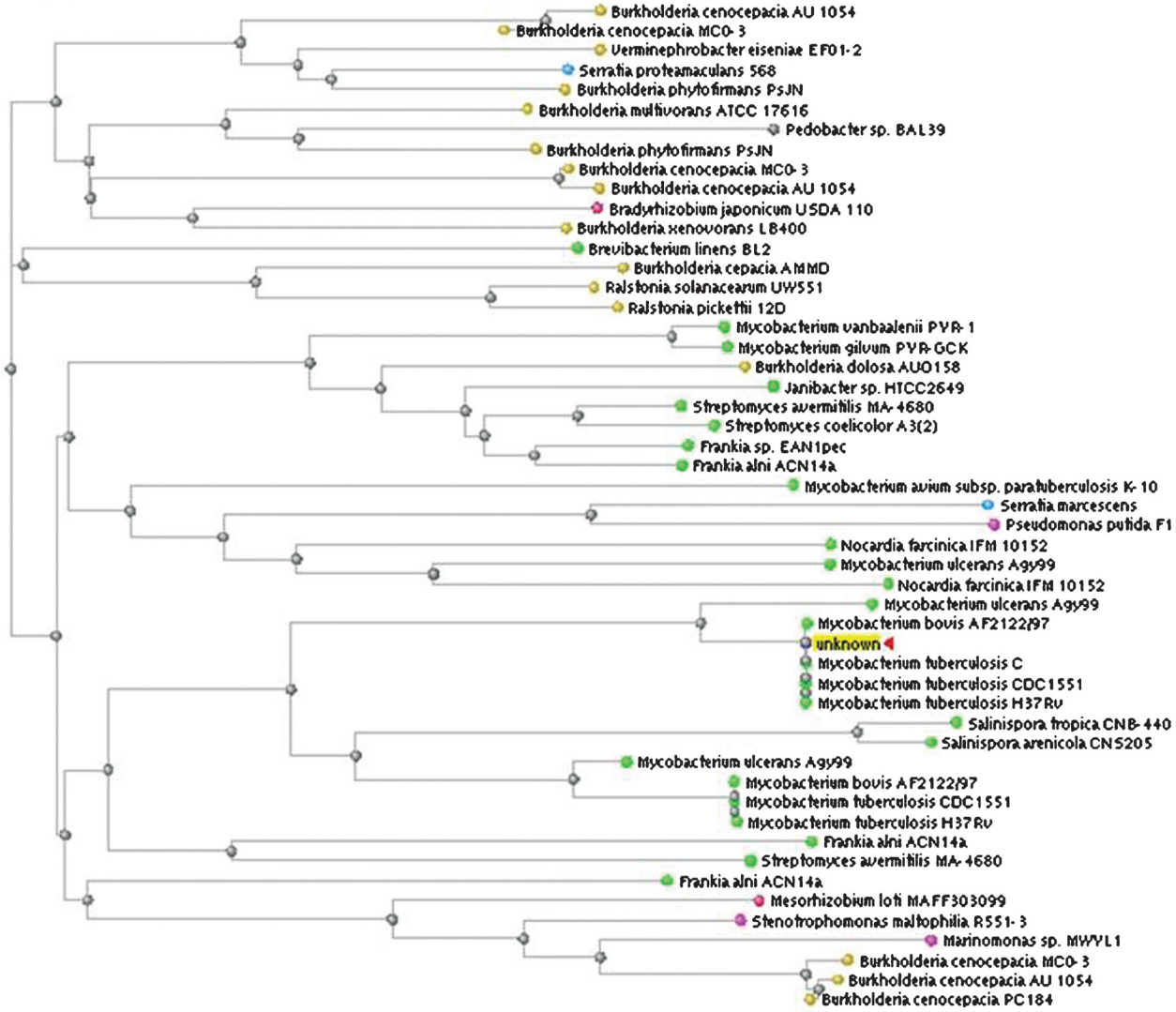
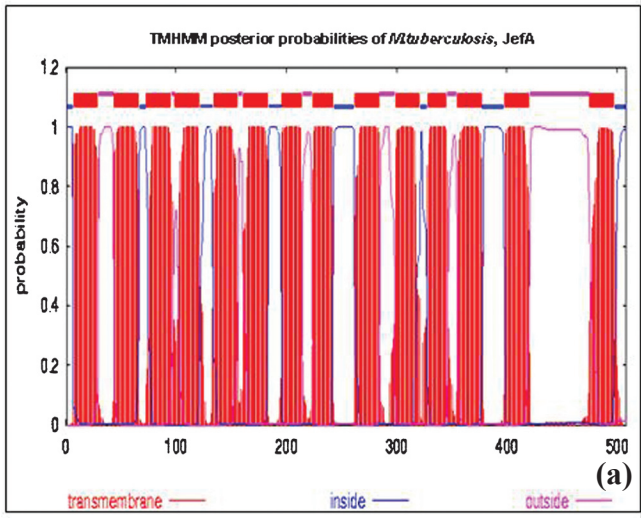


Fig. 3b. Phylogenetic tree showing distance relationship analysis of JefA within other organisms.

study, has been described in *M. tuberculosis* database as 'probable conserved integral membrane transport protein' (*http://genolist.pasteur.fr/TubercuList/*). After detecting its overexpression in multidrug resistant isolates of *M. tuberculosis* in our microarray and real-time PCR based studies[18] (Indian Patent application No. 2071/DEL/2007), it has been designated as '*jefA*'. In the present work, this gene has been amplified from standard laboratory strain *M. tuberculosis* $H_{37}Rv$, subcloned in *E. coli*, and finally cloned in $H_{37}Rv$ itself through a mycobacteria-*E. coli* expression shuttle vector pSD5[20] to make it overexpress its phenotype. Cloned $H_{37}Rv$ cells showed >16 fold increased expression of *jefA* as confirmed by real-time RT-PCR analysis. A comparison of MICs of drugs in recombinant versus non-recombinant clones by REMA showed that MICs for isoniazid and ethambutol were increased significantly (16 to 64 fold) in recombinant clones followed by, to a relatively lesser amount, that of streptomycin. An increase in MICs of isoniazid and ethambutol suggests that *jefA* is actively involved in extrusion of these drugs. This also reconfirms the results published with multidrug resistant isolates[18]. Experiments with protonophore CCCP and calcium channel blocker verapamil, singly or together, indicated that active efflux of these drugs from the bacterial cell was responsible for the increase in their MICs. The blocking effect of CCCP and verapamil brought down, although not fully, the increase in isoniazid and ethambutol MICs. The decrease in MIC of ethambutol by CCCP was greater than that for isoniazid. It may be presumed that the concentration of CCCP required to



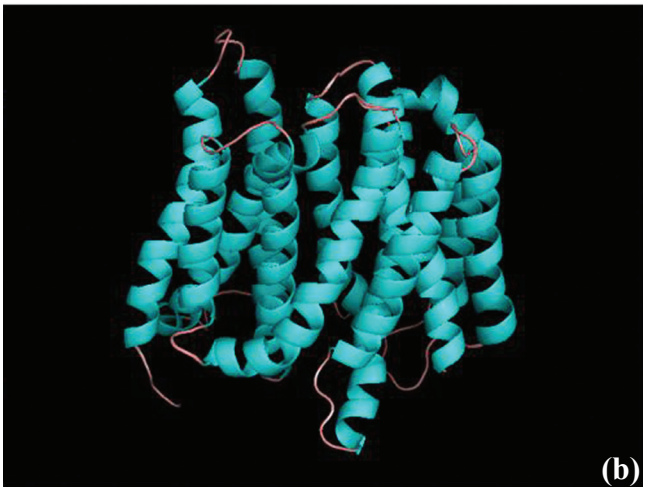


Fig. 4. Analysis of JefA protein structure. **(a)** Transmenbrane helix prediction of JefA protein by TMHMM server showing 14 trans membrane helices, **(b)** Homology derived model of JefA prepared using Swiss-model.

Table IV. Organisms, which show maximum homology with amino acid sequence of JefA

Identity within proteomes		
Protein description	Organism	Identity (%)
Mycobacteria:		
Putative integral membrane transport protein	*M. bovis* BCG pasteur	100
Putative transmembrane transport protein	*M. tuberculosis* H_{37}Ra	100
Probable conserved membrane transport protein	*M. bovis*	99
Conserved integral membrane transport protein	*M. ulcerans*	77
Drug resistance transporter, EmrB/QacA	*M. vanbaalenii*	30
Other bacteria:		
Drug resistance transporter, EmrB/QacA	*Salinispora arenicola*	39
Putative efflux membrane protein	*Streptomyces avermitilis*	36
Drug resistance transporter, EmrB/QacA	*Verminephovector eiseniae*	35
Drug resistance transporter EmrB/QacA	*Frankia* species	34
Major facilitator superfamily MFS_1	*Serratia proteamaculans*	34
Human:		
Vesicular acetylcholine transporter (VAChT)	*Homo sapiens*	27
solute carrier family 17 (sodium phosphate)	*Homo sapiens*	25
solute carrier family 2 (facilitated glucose transporter)	*Homo sapiens*	23
sodium-dependent inorganic phosphate cotransporter	*Homo sapiens*	21
SLC17A8 protein	*Homo sapiens*	21

lower isoniazid MIC should be greater than what was taken. However, increasing the CCCP concentration can rather affect the natural growth of the bacteria as observed in our parallel experiments to determine the optimum concentration of the inhibitors after performing concentration dependent titration of the wild type bacterial growth (data not shown). On the other hand, verapamil showed only little effect in both the cases. Inclusion of reserpine could also have added some information about decrease in MIC of drugs in *jefA* clones, which may be similar or little different. Small decrease in the MIC of streptomycin by both the inhibitors in the clones, compared to other drugs, suggests a mild involvement of *jefA* in streptomycin resistance. Moreover, the greater effect of verapamil on streptomycin MIC than that by CCCP suggests possible involvement of another ABC family pump in streptomycin resistance. Although no increase was found in the MICs of fluoroquinolones- ofloxacin and norfloxacin in the present study, these drugs are assumed to be potential targets for efflux mediated resistance[30-32]. As the drugs tested in the present study are used commonly in anti-TB therapy, we have concerned on clinically relevant drugs and not included other synthetic compounds in the study which may be possible efflux pumps substrates.

When JefA protein sequence was searched in databases for its homologous sequences, 39, 35

and 34 per cent amino acid identity was found with QacA family drug transporter proteins of *Salinispora arenicola*, *Verminephovector eiseniae* and *Frankia species* respectively. This suggests that JefA is closely related to drug transport proteins. Phylogenetic relationship of JefA showed that within mycobacterial species its amino acid sequence is well conserved especially within *M. ulcerans* and *M. bovis*. Relation of JefA with its homologues within other organisms shows that JefA amino acid sequence is conserved within high GC Gram positive organisms. The homology of JefA with membrane efflux proteins of other bacteria as well as its transmembrane region analysis indicated the nature and location of JefA as a transmembrane efflux pump containing 14 TMH. Although specificity of most of these sequences particularly for isoniazid and ethambutol is not known, homology in these sequences is suggestive of common specificity for other synthetic compounds, which may be the preferred substrates for these homologous pumps and which can be one of the natural defense mechanisms. Little identity of JefA with any of the human proteins and their rarer alignment suggests presence of non-homologous regions in JefA structure, which can be important as potential drug targets.

Although mutations in drug target genes are still thought to be the primary mechanism of resistance, they alone are unable to account for all cases of INH and EMB resistance in *M. tuberculosis*[33-37]. Hence, there is a need to look for alternative mechanisms such as efflux pumps in such situations. The role of efflux pumps in conferring INH and EMB resistance has been described in *M. tuberculosis*, where an efflux pump of resistance nodulation division (RND) family, mycobacterial membrane protein large (mmpL7), has been found to confer INH resistance[14], and another gene, *iniA*, encoding a pump component has been observed to assist an efflux pump to provide resistance to INH and EMB[13]. Microarray based studies have also identified an efflux pump gene *efpA* (Rv2846c) to be induced by INH treatment[38,39].

Our study provides significant new information about efflux mechanisms contributing in INH and EMB resistance through a MFS family efflux pump, JefA, which acts as alternative/additional mechanism of resistance to these drugs in *M. tuberculosis*. The increase in MICs of these drugs, when *jefA* was cloned in *M. tuberculosis* and the decrease in their MICs by efflux pump inhibitors CCCP and verapamil support its nature as a gene encoding efflux pump of major facilitator superfamily, which is also indicated by the transmembrane region prediction analysis of JefA as well as by its phylogenetic analysis. It can further be investigated in *jefA* knock-out strains to reinforce the observations. In conclusion, our results suggest that *jefA* gene contributes in isoniazid and ethambutol resistance in *M. tuberculosis* through an efflux pump mediated mechanism. Its actual epidemiological significance can be assessed only by the study of naturally occurring INH and EMB resistant isolates from patients. Since no known structural model of a close homologue of JefA with a significant sequence identity (>40%) has been identified based on BLASTp[27] and SWISS MODEL[40] analysis, further characterization of JefA requires alternative bioinformatics methods, X–ray crystallography or NMR spectroscopy analysis of JefA to explain on the basis of structure-function relationship, why it is preferentially associated with ethambutol and isoniazid resistance and not with other drugs tested.

Acknowledgment

Authors acknowledge Shri Yash Gupta for help in bioinformatic analysis. Technical help of Shriyuts Rajesh Singh, Dhaniram, Jitendra Yadav and Harishankar is also acknowledged. Authors acknowledge the Department of Biotechnology (Government of India), and Central TB Division (Government of India), New Delhi, for financial support. The first (AKG) and third authors (ML) acknowledge the Council of Scientific and Industrial Research and Indian Council of Medical Research, New Delhi respectively, for Senior Research Fellowship.

References

1. Piddock LJ. Multidrug resistance efflux pumps – not just for resistance. *Nat Rev Microbiol* 2006; *4* : 629-36.
2. Webber MA, Piddock LJV. The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother* 2003; *51*: 9-11.
3. Putman M, vanVeen HW, Konings WN. Molecular properties of bacterial multidrug transporters. *Microbiol Mol Biol Rev* 2000; *64* : 672-93.
4. Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* 1976; *455* : 152-62.
5. Kuo MT. Roles of multidrug resistance genes in breast cancer chemoresistance. *Adv Exp Med Biol* 2007; *608* : 23-30.
6. Poole K. Efflux mediated antimicrobial resistance. *J Antimicrob Chemother* 2005; *56* : 20-51.
7. Centers for Disease Control and Prevention (CDC). Revised definition of extensively drug-resistant tuberculosis. *MMWR Morb Mortal Wkly Rep* 2006; *55* : 1176.
8. DeRossi E, Branzoni M, Cantoni R, Milano A, Riccardi G, Ciferri O. *mmr*, a *Mycobacterium tuberculosis* gene conferring resistance to small cationic dyes and inhibitors. *J Bacteriol* 1998; *180* : 6068-71.

9. De Rossi E, Ainsa JA, Riccardi G. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. *FEMS Microbiol Rev* 2006; *30* : 36-52.
10. Ainsa JA, Blokpoel MC, Otal I, Young DB, De Smet KA, Martin C. Molecular cloning and characterization of Tap, a putative multidrug efflux pump present in *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*. *J Bacteriol* 1998; *180* : 5836-43.
11. Choudhuri BS, Bhakta S, Barik R, Basu J, Kundu M, Chakrabarti P. Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes *drrA* and *drrB* of *Mycobacterium tuberculosis*. *Biochem J* 2002; *367* : 279-85.
12. Siddiqi N, Das R, Pathak N, Banerjee S, Ahmed N, Katoch VM, *et al*. *Mycobacterium tuberculosis* isolate with a distinct genomic identity overexpresses a tap-like efflux pump. *Infection* 2004; *32* : 109-11.
13. Colangeli R, Helb D, Sridharan S, Sun J, Varma-Basil M, Hazbon MH, *et al*. The *Mycobacterium tuberculosis iniA* gene is essential for activity of an efflux pump that confers drug tolerance to both isoniazid and ethambutol. *Mol Microbiol* 2005; *55* : 1829-40.
14. Pasca MR, Guglierame P, De Rossi E, Zara F, Riccardi G. *MmpL7* gene of *Mycobacterium tuberculosis* is responsible for isoniazid efflux in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* 2005; *49* : 4775-7.
15. Pasca MR, Guglierame P, Arcesi F, Bellinzoni M, De Rossi E, Riccardi G. Rv2686c-Rv2687c-Rv2688c, an ABC fluoroquinolone efflux pump in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2004; *48* : 3175-80.
16. Gupta AK, Chauhan DS, Srivastava K, Das R, Batra S, Mittal M, *et al*. Estimation of efflux mediated multi-drug resistance and its correlation with expression levels of two major efflux pumps in mycobacteria. *J Commun Dis* 2006; *38* : 246-54.
17. Ramón-García S, Martín C, DeRossi E, Aínsa JA. Contribution of the Rv2333c efflux pump (the Stp protein) from *Mycobacterium tuberculosis* to intrinsic antibiotic resistance in *Mycobacterium bovis* BCG. *J Antimicrob Chemother* 2007; *59* : 544-7.
18. Gupta AK, Katoch VM, Chauhan DS, Sharma R, Singh M, Venkatesan K, *et al*. Microarray analysis of efflux pump genes in multidrug resistant *Mycobacterium tuberculosis* during stress induced by common anti-tuberculous drugs. *Microb Drug Resist* 2010; *16* : 21-8.
19. Bullock WO, Fernandez JM, Short JM. XL1-Blue: A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* 1987; *5* : 376-8.
20. DasGupta SK, Jain S, Kaushal D, Tyagi AK. Expression systems for study of mycobacterial gene regulation and development of recombinant BCG vaccines. *Biochem Biophys Res Commun* 1998; *246* : 797-804.
21. Rozen S, Skaletsky HJ. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, editors. *Bioinformatics methods and protocols: Methods in molecular biology*. Totowa, NJ: Humana Press; 2000. p. 365-86. Available at: *http://fokker.wi.mit.edu/primer3/input.htm*.
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. *Methods* 2001; *25* : 402-8.
23. Palomino JC, Martin A, Camacho M, Guerra H, Swings J, Portaels F. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2002; *46* : 2720-2.
24. Martin A, Camacho M, Portaels F, Palomino JC. Resazurin microtitre assay plate testing of *Mycobacterium tuberculosis* susceptibilities to second-line drugs: rapid, simple and inexpensive method. *Antimicrob Agents Chemother* 2003; *47* : 3616-9.
25. Nateche F, Martin A, Baraka S, Palomino JC, Khaled S, Portaels F. Application of the resazurin microtitre assay for detection of multidrug resistance in *Mycobacterium tuberculosis* in Algiers. *J Med Microbiol* 2006; *55* : 857-60.
26. Jadaun GP, Agarwal C, Sharma H, Ahmed Z, Upadhyay P, Faujdar J, *et al*. Determination of ethambutol MICs for *Mycobacterium tuberculosis* and *M.avium* isolates by resazurin microtitre assay. *J Antimicrob Chemother* 2007*; 60* : 152-5.
27. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; *215* : 403-10.
28. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; *22* : 4673-80.
29. Hofmann K, Stoffel W. TMbase- A database of membrane spanning protein segments. *Biol Chem Hoppe-Seyler* 1993; *374* : 166-70.
30. Liu J, Takiff HE, Nikaido H. Active efflux of fluoroquinolones in *Mycobacterium smegmatis*, mediated by LfrA, a multidrug efflux pump. *J Bacteriol* 1996; *178* : 3791-5.
31. Takiff HE, Cimino M, Musso MC, Weisbrod T, Martinez R, Delgado MB, *et al*. Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis*. *Proc Natl Acad Sci USA* 1996; *93* : 362-6.
32. Silva PE, Bigi F, Santangelo MP, Romano MI, Martin C, Cataldi A, *et al*. Characterization of P55, a multidrug efflux pump in *Mycobacterium bovis* and *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2001; *45* : 800-4.
33. Ahmad S, Mokaddas E. Contribution of AGC to ACC and other mutations at codon 315 of the katG gene in isoniazid-resistant *Mycobacterium tuberculosis* isolates from the Middle East. *Int J Antimicrob Agents* 2004; *23* : 473-9.
34. Ahmad S, Jaber AA, Mokaddas E. Frequency of *embB* codon 306 mutations in ethambutol-susceptible and -resistant clinical *Mycobacterium tuberculosis* isolates in Kuwait. *Tuberculosis (Edinb)* 2007; *87* : 123-9.
35. Hazbo´n MH, Bobadilla delValle M, Guerrero MI, Varma-Basil M, Filliol I, Cavatore M, *et al*. Role of *embB* codon 306 mutations in *Mycobacterium tuberculosis* revisited: a novel association with broad drug resistance and IS*6110* clustering rather than ethambutol resistance. *Antimicrob Agents Chemother* 2005; *49* : 3794-802.

36. Parsons LM, Salfinger M, Clobridge A, Dormandy J, Mirabello L, Polletta VL, *et al*. Phenotypic and molecular characterization of *Mycobacterium tuberculosis* isolates resistant to both isoniazid and ethambutol. *Antimicrob Agents Chemother* 2005; *49* : 2218-25.

37. Plinke C, Rusch-Gerdes S, Niemann S. Significance of mutations in *embB* codon 306 for prediction of ethambutol resistance in clinical *Mycobacterium tuberculosis* isolates. *Antimicrob Agents Chemother* 2006; *50* : 1900-2.

38. Fu LM. Exploring drug action on *Mycobacterium tuberculosis* using Affymetrix oligonucleotide genechips. *Tuberculosis (Edinb)* 2006; *86* : 134-43.

39. Wilson M, DeRisi J, Kristensen HH, Imboden P, Rane S, Brown PO, *et al*. Exploring drug-induced alterations in gene expression in *Mycobacterium tuberculosis* by microarray hybridization. *Proc Natl Acad Sci USA* 1999; *96* : 12833-8.

40. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis* 1997; *18* : 2714-23.

Reprint requests: Dr V.M. Katoch, Secretary, Department of Health Research, Ministry of Health & Family Welfare & Director-General, Indian Council of Medical Research, Ansari Nagar, New Delhi 110 029, India
e-mail: vishwamohan_katoch@yahoo.co.in