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Efflux Pumps of *Mycobacterium tuberculosis*: Expression analysis and in-silico characterization

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Abstract

Multidrug resistant tuberculosis, a global threat to the world community, involves high cost and long duration of treatment. Emergence of drug resistance is attributed to activity of efflux pumps as one of the earliest cause in mycobacterium. Here we show the real time expression profiles of 20 probable efflux pumps of Mycobacterium tuberculosis under the two most important drugs of antiTB regime – rifampin and isoniazid. Under rifampin pressure 10 efflux pump genes were over expressed (Rv1258c, Rv1410c, Rv1819c, Rv1145, Rv2936, Rv2937, Rv0849, Rv1877, Rv2209, and Rv0783). Under isoniazid pressure 4 efflux pump genes were induced (Rv1258c, Rv1819c, Rv2938, and Rv3065). The three dimensional structure of Rv1410c was modelled that shares significant structural similarity with MFS transporter proteins. Docking with rifampin revealed the drug's binding pattern. It is hoped that current study will provide significant information regarding efflux mediated drug resistance of mycobacterium and can facilitate the development of new potent efflux pump inhibitors against tuberculosis.

Keywords: *Mycobacterium tuberculosis*, Efflux pumps, Rifampin, Isoniazid, qRT-PCR, Docking.

Introduction

Tuberculosis once close to eradication in the second half of the 20th century has resurged and has become a major global threat. It claimed 1.23 million human lives with an enormous 10.7 million cases in 2024[1]. This resurgence can be explained by the worldwide emergence of drug resistant *Mycobacterium tuberculosis* strains. Multidrug resistant (MDR) tuberculosis is caused by bacteria resistant to at least, rifampin (RIF) and isoniazid/isonicotinic acid hydrazide (INH). A more severe form is extensively drug resistant (XDR) tuberculosis which has additional resistance to fluoroquinolones and at least to one of the second line injectables[2]. Totally drug resistant (TDR) tuberculosis, a yet to be defined infection, believed to be resistant to all forms of therapy was also reported[3].

M.tuberculosis exhibits both intrinsic and acquired resistant mechanisms. The intrinsic resistance prevents the drug to attain the required concentration necessary for killing the bacterium. The two most prominent features involved are the unusual mycolic acid rich waxy cell wall and action of efflux pumps[4]. The cell wall provides a permeability barrier that largely hampers the entry of toxic substances and efflux pumps actively export toxic metabolites out of the cell. Acquired drug resistance in *M.tuberculosis* is caused by spontaneous chromosomal mutations in target or regulatory domains[5-9]. The absence of plasmid in *M.tuberculosis* rules out the possibility of resistance acquisition by horizontal gene transfer[10]. Additional mechanisms of resistance like production of drug-modifying and drug-inactivating enzymes are also reported for mycobacterium[11].

RIF and INH are the backbone of short-course chemotherapy regime for treating sensitive *M. tuberculosis*. RIF was introduced in 1960s because of its high bactericidal activity against both actively growing and slowly metabolizing bacilli[12]. The drug binds to the β subunit (coded by *rpoB* gene) of RNA polymerase and results in transcription inhibition. Alteration of the binding site of the target results in decrease in the binding affinity of the drug conferring resistant phenotype. The alteration involves point mutations, short insertions and deletions in a well defined region of 81-bp core known as rifampin resistance-determining region (RRDR) of *rpoB* (codons 507–533, containing 27 amino acids)[13]. INH, introduced in 1950s, is activated by the catalase-peroxidase, KatG, and produces isonicotinic-acyl radicals that react with NADH to form an INH-NADH adduct. The adduct binds to its main target, enoly-acyl carrier protein reductase (InhA), and inhibits mycolic acid biosynthesis. Resistance to the drug is known to be caused by (a) structural mutations in *katG* (which leads to decrease or total loss of catalase/peroxidise activity[14]), *inhA* (prevents binding of the active INH-NADH adduct to InhA[15]), *kasA* and *ndh* (reduces the activity of NADH dehydrogenase[16]) (b) mutation in the regulatory region of *inhA* (causing over expression of the target[17]), *oxyR-aphC* intergenic region (reduce the level of expression of *inhA*[18]) and *furA-katG* intergenic region (decrease the expression of *katG*[19]). Interestingly around 20 to 30% of clinical INH-resistant isolates

and about 5% of clinical RIF-resistant *M. tuberculosis* isolates does not harbour the above classical gene mutations[20] suggesting other mechanisms at work.

Efflux pumps are known to be associated with cell division[21,22], alkali tolerance[23], and cellular homeostasis as part of their natural physiological role. In mycobacteria, these are also found to be involved in conferring resistance to many drugs[24]. Moreover evidences suggest that induction of multi-drug efflux pumps is the very first step in the chain of evolution driven events for acquiring high level resistant MDR and XDR strains[25,26]. Hence it is extremely necessary to understand the molecular basis of efflux mechanism in order to prevent high level resistance development and ultimately fight MDR/XDR/TDR tuberculosis. Sequencing of the complete genome of *M. tuberculosis* had identified several putative/ probable efflux pumps[27], many of which still require characterisation. Therefore the aim of the present investigation was to study the effect of the two most important drugs of short-course chemotherapy regime, RIF and INH on the 20 putative efflux pumps genes. Furthermore bioinformatics analysis was undertaken to understand more about the important features of an over-induced efflux pump protein because these may represent good targets for limiting the emergence of resistance against the above said drugs and an effective treatment of TB.

Materials and Methods

Bacterial strains, growth conditions and chemicals: *Mycobacterium tuberculosis* strain H37Rv (ATCC 27294) was purchased from American Type Culture Collection (ATCC; Manassas, VA) and handled in the BSL-3 facility of Clinical Microbiology Division, Indian Institute of Integrative Medicine, Jammu. The cultures were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, USA) supplemented with 0.5% (v/v) glycerol, 0.25% (v/v) Tween 80 (Himedia, Mumbai, India) and 10% ADC (albumin–dextrose–catalase, Becton Dickinson, Sparks, MD, USA), with shaking at 37ºC until mid-log phase or on Middlebrook 7H10 agar (Difco Laboratories, Detroit, USA) supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase, Becton Dickinson, Sparks, MD, USA) and 0.2% (v/v) glycerol in the 5% CO_2 incubator. *M. tuberculosis* strain H37Rv was the parent strain for all the mutants generated for the study.

RIF, INH, reserpine and piperine were from Sigma Aldrich (St. Louis, MO, USA). RIF was dissolved in dimethyl sulphoxide (DMSO, Rankem, India) to prepare the stock solution of 10 mg/ml. Reserpine and piperine were also dissolved in DMSO (Rankem, India) to prepare the stock solution of 1 mg/ml. INH was dissolved in water to make a stock solution of 10 mg/ml and was filter sterilized with the help of 0.22 µm syringe driven disposable filters (Millipore, USA).

Generation of drug resistant mutants: Spontaneous resistant mutants as well as serially passaged mutants against RIF or INH were generated. Spontaneous mutants were generated by spreading the parent strain (*M. tuberculosis* H37Rv) on Middle brook 7H10 agar plates containing the critical concentration of respective drugs. Briefly, *M. tuberculosis* H37Rv was cultured in Middle brook 7H9 broth for 10 – 14 days in a shaking incubator at 37ºC. More than 10^8 CFU of the cell suspension was then spread on 7H10 agar plates containing either RIF (2µg/ml) or INH (1µg/ml). The plates were incubated at 37ºC for 2 to 3 weeks. Isolated colonies obtained after incubation were picked and streaked on drug containing media to confirm the resistant phenotype. Mutants were also obtained by serial passage on different concentrations of drug starting from the drug's sub-inhibitory concentration i.e. ½MIC. For both RIF and INH the concentrations used were 0.06µg/ml to 1µg/ml.

Determination of Minimum Inhibitory concentration (MIC) and synergy testing: The two-fold serial broth microdilution method was used to test the susceptibilities of the *M. tuberculosis* H37Rv, *M. tuberculosis* RIF^r and *M. tuberculosis* INH^r in accordance with the standards of Clinical and Laboratory Standards Institute[28]. Serial dilutions of RIF and INH ranged from 512µg/ml to 0.06µg/ml for MIC determination.

The combination studies of RIF and INH were also performed on the above mutants. The MICs of RIF and INH were determined in Middlebrook 7H9 broth supplemented with 10% ADC in the presence of efflux pump inhibitors (EPIs) like reserpine and piperine by the broth chequer board method in microtitre plates[29]. RIF was tested in the range 512 µg/ml to 2 µg/ml and INH 128 µg/ml to 1 µg/ml in combination with EPIs at 25µg/ml. The final bacterial inoculum of 5×10^5cfu/mL was added to each well. The plates were incubated at 37ºC in 5% CO_2 for 3–4 weeks[30]. The plates were read visually, and the minimum concentration of the drug showing no turbidity was recorded as the MIC.

Quantitative real time RT-PCR (qRT-PCR): RNA isolation: In order to analyse the transcriptional profile of 20 putative efflux genes, *M. tuberculosis* H37Rv, *M. tuberculosis* RIF^r and *M. tuberculosis* INH^r were grown to mid-log phase in Middlebrook 7H9 broth supplemented with 10% ADC in the presence of sub inhibitory concentration (1/4 the MIC) of respective drugs (RIF and INH). *M. tuberculosis* H37Rv was included as a control.

Total mycobacterial RNA was extracted using TRIZOL® (Invitrogen, CA, USA) reagent according to manufacturer's instructions. Quality assessment and quantification were done by examination on the denaturing formaldehyde (1.2%) agarose gel and measuring the A_{260}/A_{280} ratio using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA). Sample showing clear bands of 23S and 16S bacterial rRNA subunits indicated little degradation of RNA and A_{260}/A_{280} ratio between 1.8 and 2.1 were processed further.

cDNA preparation: Reverse transcription was carried out by QuantiTech® Reverse Transcription kit (Qiagen, Germany) according to manufacturer's instructions. Total RNA (1µg) was treated with gDNA wipeout buffer (1×) at 42ºC for 2 min to remove genomic DNA contamination. The RT reaction mixture contained treated RNA, 1µl RT primer mix, 1× Quantiscript RT buffer, and 1µl reverse transcriptase in a total volume of 20µl. The reaction was incubated at 42ºC for 15 min. and then at 95ºC for 5 min. and then cooled to 4ºC.

Real-Time PCR: Quantitative real time PCR was performed with SYBR green master mix (Roche Applied Science, Germany) in LightCycler® 480 Real-Time PCR System (Roche Applied Science, Germany). The 16S rRNA gene was used as housekeeping gene. The reaction volume of 20µl was prepared containing 10µl of 2× LightCycler® 480 DNA SYBR Green I Master, 0.5µM of each gene specific primers and 1µl of cDNA as template. After the initial incubation at 95ºC for 10 min to activate the enzyme, 45 cycles of amplification and quantification was performed as per the following program: denaturation at 95ºC for 10 sec, annealing (as per the primer pair) at 57ºC/58ºC/59ºC/60ºC for 20 sec, extension at 72ºC for 20 sec with a single fluorescence measurement. Reaction was cooled at 40ºC for 30 sec. Each reaction was performed in triplicates. A no-template control was always included with each run. Primers used are listed in table 1. The crossing point (C_P) of each reaction was analysed by the method of second derivative maximum algorithm. C_P was defined as cycle number at detection threshold.

Relative Quantification Analysis: The expression level of efflux pump gene mRNA in each sample was analysed by Light Cycler Relative Quantification Software (Roche Diagnostics) as described earlier[31]. Briefly, the quantity of a target and a reference gene is a function of the PCR efficiency and the sample C_P.

No standard curve is required in this case. C_P value is more reliably proportional to the initial template concentration. Differences in PCR efficiency results from different primers and can be corrected by the software. Results are expressed as the target/reference ratio of each sample normalised by the target/reference ratio of the calibrator. The calibrator is included in each run and its ratio is set to a value of 1. This normalisation provides a constant calibrator point between PCR runs.

Data of real-time PCR, including calibrator and samples, were imported into the Relative Quantification Software and analysed with the Fit Coefficients File. Finally the normalised ratios were calculated. These ratios directly reflect the expression level of efflux pump mRNA.

Analysis of upstream region of the efflux pump genes: To detect mutations in the promoter region which may be responsible for over expression of the efflux pumps, we amplified approximately 350-450 bp amplicon covering ~ 300 bp upstream region from the probable Transcriptional Start Site (TSS) using specific primers and PCR conditions as described in the Table-2. Subsequent sequencing of the PCR products was performed in the automated ABI 3730xl DNA Analyzer (Life Technologies, Applied Biosystems, USA) and analysed using Chromas version 2.33 (Technelysium Pty Ltd, Australia; Chromous Biotech Pvt. Ltd., Bangalore). The nucleotide sequences of the mutants were compared to the wild type strain *M. tuberculosis* H37Rv with the help of the nucleotide BLAST alignment tool.

Molecular modelling and docking analysis: To understand the binding interactions between the drug molecule and efflux pump in-silico analysis was performed. An efflux pump protein (Rv1410c) was modelled for analysis. Protein sequence of Rv1410c was obtained from Tubercu List web server (http://genolist.pasteur.fr/TubercuList/) and homology search was done using BLASTp[32]. The database chosen for BLASTp was Protein Data Bank (PDB) which resulted in the identification of *Escherichiacoli* YajR MFS transporter (PDB code: 3WDO)[33] sharing 37% sequence identity with Rv1410c. Modelling of protein was carried out using MODELLER 9.12[34] with 3WDO as template. The theoretical model was evaluated for potential structural errors using ProSA web server[35] and SAVES (The Structure Analysis and Verification Server v4) web server (http://nihserver.mbi.ucla.edu/SAVES/). Highly conserved residues were identified using ConSurf[36]. QSiteFinder[37] was employed to predict the possible ligand binding sites of the model.

Docking of RIF with the modelled Rv1410c was performed using AutoDock4.2[38]. The 3D structures of RIF (DB01045) and tetracycline (DB00759) were downloaded from DrugBank database (www.drugbank.ca/drugs/) in mol format and converted to .pdb format using Discovery Studio 3.5 Visualizer (Accelrys). Ligand and receptor were prepared in AutoDockTools 1.5.4 prior to docking as per the instructions. Blind Docking parameters[39] were followed to dock the flexible ligand to the rigid receptor with minor modification. Briefly, for the whole protein target, grid maps with 0.5Å spacing was generated.

The Lamarckian genetic algorithm and the pseudo-Soils and Wets methods were applied for minimization using default parameters. The number of generations were set to 10 million in all runs. The number of runs were 50 with 25×10^6 energy evaluations. The population in the genetic algorithm was kept 250. A re-docking to increase precision of ranking was also performed with another 50 runs at the sites of high binding energy with other parameters same as above. Blind docking of tetracycline was also done to validate the binding site identified after docking with RIF. Attractive forces (H-bond and hydrophobic interactions) between the ligand and protein were analysed using LigPlot v1.4.5[40]. Ligplot algorithm converts the 3D protein-ligand complexes generated during docking to 2D diagrams.

Table-1: List of gene specific primers used in quantitative real time RT-PCR.

Gene		Primer sequence (5'→3')	Amplicon size (bp)	Annealing Temperature (Tm)
16S RNA	FP: RP:	GTCAAGTCATCATGCCCCTT CACCTTCGACAGCTCCCTCC	282	58
Rv2936/*drrA*	FP: RP:	CGAGCAATTCAGCCTCGTA ATCAGGATGATGCGGTCACT	264	59
Rv2937/*drrB*	FP: RP:	GCCAGCAACTTAGGGCAATA GATCGCGATAACCAGTAGGC	291	59
Rv2938/*drrC*	FP: RP:	GCCTGATCTCTCGAATCCTG CAAACCCGTGGAGAAGAAGA	257	59
Rv2846c/*efpA*	FP: RP:	CTGGATGTCAGGCATTACCA GACGAACGGGATGAAACCTA	259	58
Rv3065/*mmr*	FP: RP:	ATACCTCTTGTGCGCGATCT AGAAACAGTACGGCGACCAG	225	59
Rv0783c/*emrB*	FP: RP:	GGCGTTGGATTTTCTTGGTC GCGGATGTTCTGTGCGGTAC	276	60
Rv0849	FP: RP:	CGCTGGTTGGATTAGTATTGC CGCCGCAATCAAGAGATATT	299	58
Rv1145/*mmpL13a*	FP: RP:	CGTTGGTTTACAACGTGACC ATCACCAGCAGGTCGTCTTT	250	58
Rv1146/*mmpL13b*	FP: RP:	CCTCCGATATCCAGCTCAAG TCCTGGAAGATCCACACCA	265	59
Rv1250	FP: RP:	TGATCCCACTGGAGGTCTTC CTCGCATAAGAGCCATGTCA	287	59
Rv1258c	FP: RP:	CCTCAACCTGGCCTTTATTG GACGGTCAGGTCAATCATCC	259	58
Rv1410c/P55	FP: RP:	TGGTTGATGTCGAGCTGTTC CCAGTGGGAAATAAGCCAGT	200	57
Rv1634	FP: RP:	TTGATGGCCATGTTGGTG CGACATGGTCAGGTAAATCC	300	57
Rv1819c/*bacA*	FP: RP:	ATATCTTCGGCGTGTCCATC CGCTGTAGCTGGGTACCTTC	226	58
Rv2136c	FP: RP:	GGTATGTCATCATCGGCACA GTTTGGGCAATACCAACCAC	211	58
Rv2209	FP: RP:	CGCAGGTCTGGTTTGCTC ACCAATCCAATGCTGGAAAG	267	59
Rv2333c/*stp*	FP: RP:	GTGCTACGTCGAGGAGTCGT CCATAGACGGCGAAGAACAC	294	59
Rv2994	FP: RP:	CGATCCTGTGGCGGATAC TCATATCTCGAGCCCTCGTT	286	59
Rv1877	FP: RP:	CTGTTTGGCAGCCCAGTATT CTGCGACATCAGCAGGAAC	273	59
Rv2459	FP: RP:	GTGGGACAGCTGACGCTTAT ATCAGAGTCCCGAAAAAGCA	257	59

Table-2: Primers used for DNA sequencing.

Gene name		Primer sequence (5'→3')	Position[a]	Amplicon (bp)
Rv2936/*drrA*	FP: RP:	CCGACATTCACTTCCGGTAT AATCTTGCCCTTGCCGTAG	-366 to -347 +66 to +48	432
Rv2937/*drrB*	FP: RP:	CACCGCGAATGAACTCAAG CCGATAGAGCACCCACATCT	-327 to -309 +111 to +92	438
Rv2938/*drrC*	FP: RP:	TCGGTTTTTGCCTACTGGTT GTTGGTCTGCAAAAGGGTCT	-358 to -339 +105 to +86	463
Rv3065/*mmr*	FP: RP:	GGTAAACAGCCCGGTTGC TGCCATAACCCACTAGACAGC	-358 to -341 +121 to +101	479
Rv0783c/*emrB*	FP: RP:	CAGGGTGCTCTCACCTATCC ACCCCACATCGAGCCTATC	-230 to -211 +121 to +103	351
Rv0849	FP: RP:	CAATGTCGATTACCGTGCAT GATGTTGATGCCGAACTGGT	-363 to -344 +78 to +59	441
Rv1145/*mmpL13a*	FP: RP:	GGTGCAAGTCTTCGAGCTG ATGAAGACGAAGACCGCAAA	-367 to -349 +74 to +54	441
Rv1258c	FP: RP:	ACCTCCTCACGCCAACTG AACACCAGCCACGGAAAC	-300 to -282 +103 to +86	404
Rv1410c/P55	FP: RP:	CAACGTGCTGGCGAATTT CCAACGCTGTTCATGATGTC	- 288 to -271 +113 to +93	401
Rv1819c/*bacA*	FP: RP:	TCATCCCGTTGGTGATCC ATATCGCGAGCACACAGATG	-338 to -321 +112 to +93	450
Rv2209	FP: RP:	ACCGATAGCGGTTCTGGTAG CAGATGACCGGCAGTACCAC	-314 to -295 +131 to +112	445
Rv1877	FP: RP:	CATCGGTTCGTTGCGTATC CACAAGTGCGGTGAAAATAATG	-346 to -328 +102 to +80	448

Table-3: Antimicrobial activity of drug alone and in the presence of efflux pump inhibitors against *M. tuberculosis* mutant.

M. tuberculosis strains	MIC (µg/ml)					
	Rifampicin			Isoniazid		
	alone	+ reserpine	+ piperine	alone	+ reserpine	+ piperine
H37Rv	0.12	ND	ND	0.25	ND	ND
RIF^r	256	128	64	0.25	ND	ND
INH^r	0.06	ND	ND	128	64	64

The concentration of reserpine and piperine was 25µg/ml. ND: Not determined.

Results and Discussion

Selection of drug resistant mutants of *M. tuberculosis* H37Rv: The pan sensitive strain, *M. tuberculosis* H37Rv was exposed to the critical concentration of RIF (2 µg/ml) and INH (1µg/ml) to generate RIF-resistant and INH-resistant mutants respectively.

Twelve randomly picked RIF-resistant were further analyzed and found to be highly resistant (MICs ≥ 128 µg/ml) towards RIF. No cross-resistance to other drugs was detected in the mutants. Likewise, INH-resistant 6 colonies were randomly picked and their resistant profiles showed an elevated INH MIC of 128µg/ml. Cross-resistance to RIF and other drugs was also found to be absent.

Effect of Efflux Pump Inhibitors (EPIs) on drug susceptibility: To test for the involvement of efflux on the increased resistance to drugs, the drug's MIC were determined in the presence and absence of EPIs. Reserpine a well known EPI along with piperine which was also demonstrated to be an EPI[41] was used. Out of all the 20 RIF resistant mutants, only one mutant (RIF^r) exhibited 2-fold reduction in RIF MIC with reserpine and 4-fold reduction in RIF MIC with piperine and among the 6 INH resistant mutant only one mutant (INH^r) was found to have 2-fold reduction in INH MIC with reserpine as well as piperine as indicated in Table-3. Hence for further studies, both RIF^r and INH^r were selected.

In this study an attempt was made to understand the role of efflux pumps of *Mycobacterium tuberculosis* in resistance to

RIF and INH. The RIF^r and INH^r isolates used for transcriptional analysis of efflux pump genes exhibited a high level of MIC i.e. 256µg/ml and 128µg/ml respectively. In presence of EPIs, a two- to four- fold reduction was observed for RIF^r isolate and a two-fold reduction for INH^r isolate. A high level of MIC for both the isolates suggests occurrence of stable classical chromosomal mutations, nevertheless the reduction of MIC in presence of inhibitors of efflux pumps strongly indicates the presence of active efflux pumps. To confirm this hypothesis quantitative real time analysis was performed for 20 probable efflux pumps genes.

Expression analysis of genes coding for efflux pumps in response to RIF and INH: The isolates RIF^r and INH^r isolates to investigate the transcription level of 20 efflux pump genes when grown in the presence of sub-inhibitory concentrations of RIF (64µg/ml) and INH (32µg/ml) respectively. Only those genes showing more than 2-fold over expression following induction were considered significant. Twelve efflux pump genes were found to be over expressed during stress produced individually by the drugs (Figure-1). RIF stress induced altogether 10 efflux pump genes, Rv1877, Rv2209, Rv1258c, Rv1410c, Rv1819c, Rv1145, Rv2936, Rv2937, Rv0783 and Rv0849. Low level induction of 2-fold to 4.5-fold was observed for Rv1877, Rv2209, Rv1258c, Rv1819c and Rv2937 whereas high level induction of 7-fold to 22-fold was recorded for Rv1410c, Rv1145, Rv2936, Rv0783 and Rv0849. INH stress up regulated the expression of 4 efflux pump genes, Rv3065, Rv1258c, Rv1819c and Rv2938. While Rv1258c was highly induced (upto 15-fold), Rv3065, Rv1819c and Rv2938 were induced moderately (2-fold to 3.8-fold). Two efflux pumps genes, Rv1258c and Rv1819c were induced under the pressure of RIF and INH both. Although the induction of Rv1819c did not differed much when under either RIF (2-fold) or INH (3-fold) stress, the fold induction of Rv1258c was 2-fold under RIF and upto 15-fold under INH (Figure-1). The efflux pump genes reported in this study belonged to four different transporter families found in mycobacteria, namely, the ATP-binding cassettes (ABC), the major facilitator super family (MFS), the small multidrug resistance (SMR) family and the resistance-nodulation-division (RND) super family.

Characterization of mutations in the promoter regions of the mutants: The analysis of the probable promoter region of the over expressed efflux pump genes was done to determine mutation(s) that may have played a role in inducing efflux pump genes. Sequencing of all the samples (Ten for RIF^r and 4 for INH^r) and subsequent BLAST of the sequence indicated no change in nucleotides whatsoever (data not shown).

Transcriptional analysis was undertaken for both the resistant isolates when grown under the pressure of sub-inhibitory concentration of drug. The RT-PCR analysis revealed that the resistance to RIF and INH could be a reflection of over expression of efflux pump genes under drug pressure. INH was able to induce four efflux pump genes, viz. Rv1258c, Rv1819c, Rv2938 and Rv3065. While Rv1258c showed high level expression in consistency with an earlier findings[42] all the other genes were moderately induced. The induction of Rv1819c, is in agreement with the finding of Jiang et al. and Gupta et al. who reported its induction in INH-treated Mtb cultures[43,44]. Similar observation for Rv3065 was reported by Machado et al. and Gupta et al. although Rodrigues et al. noted that it does not have a direct effect on the efflux of INH[44-46]. Rv2938 (drrC) was reported to be one of the most over expressed ABC transporter gene among 25-31% of MDR isolates under RIF/INH stress[47]. During RIF stress, a total of 10 efflux pump genes were induced. Low level induction of Rv1258c and Rv1819c was observed even here. Jiang et al. and Gupta et al. reported Rv1258c induction in presence of RIF and INH both[43,48]. Gupta et al. also found low level induction of Rv1819c in isolates of Mtb when grown in presence of RIF[44].

Our finding extends further evidences in support of these two efflux pumps involvement in multidrug mechanism. A member of the RND family, Rv1145 was found to be highly induced. Rv1877 and Rv2937 (drrB) were also found to be slightly induced. In presence of RIF we found increased induction of Rv0783 (upto 7-fold) and a very high induction of Rv2936 (drrA) (upto 22-fold). Pang et al. reported Rv0783 and Rv2936 were induced upto 5-fold and 7-fold respectively in a rifampin monoresistant mutant in absence of drug[49]. Rv1410c encoded protein is considered as a membrane protein implicated in low level aminoglycoside, tetracycline[50], INH[45] and RIF[43] efflux in mycobacteria. Our studies showed that it was highly induced only in presence of RIF. The induction of other two genes mentioned below during RIF stress, however, are novel. While Zang et al. found Rv0849 induction in presence of only INH[42], and Gupta et al. reported Rv2209 induction only under ofloxacin stress[44], we report both of their induction under RIF pressure. Since mutations within promoter region of the transporter gene could be a cause of efflux pump overexpression[52] we sequenced the respective promoter regions of all the over expressed efflux pumps genes. Absence of mutations indicated some other mechanisms at work. Other mechanism includes mutations in the local repressor gene, mutations in a global regulatory gene and insertion elements upstream of the transporter gene[53].

Molecular modelling: In order to determine the active site and residues involved in efflux mechanism the efflux pump Rv1410c (a MFS transporter) was selected for 3D homology modelling. A theoretical 3D model can provide valuable insight into the structural and mechanistic studies. The model was generated by MODELLER 9.12 using the crystal structure of a MFS transporter[33] for which the sequence identity was 37%. As shown in Figure-2, the generated model consisted of 12 transmembranes (TMs) helix that are organized into two discretely folded domains, the N and C domains, each containing six consecutive TMs. This unique arrangement, called MFS fold, is a common feature of MFS transporters[54]. Both the N and C termini are located towards the cytoplasmic

side of the membrane. TMs 1, 4, 7, and 10 are positioned in the center of the model, TMs 2, 5, 8, and 11 are on the sides of the structure while TMs 3, 6, 9, and 12 are placed outside of TMs 1, 4, 7, and 10. Connecting segments between consecutive TMs, particularly towards cytoplasmic side play important role[55] and the conserved sequence (DRXXRR) common to all MFS is also present between TMs 2 and 3 in our model. The linker connecting the two domains is 31 residues long (Val180 to Glu206) and exists in the form of unstructured α-helix[55]. Towards the cytoplasmic side helix and sheets domains are present, this unique structural feature although absent in majority of MFS transporters, is also present in the crystal structure of another MFS transporter XylE[55].

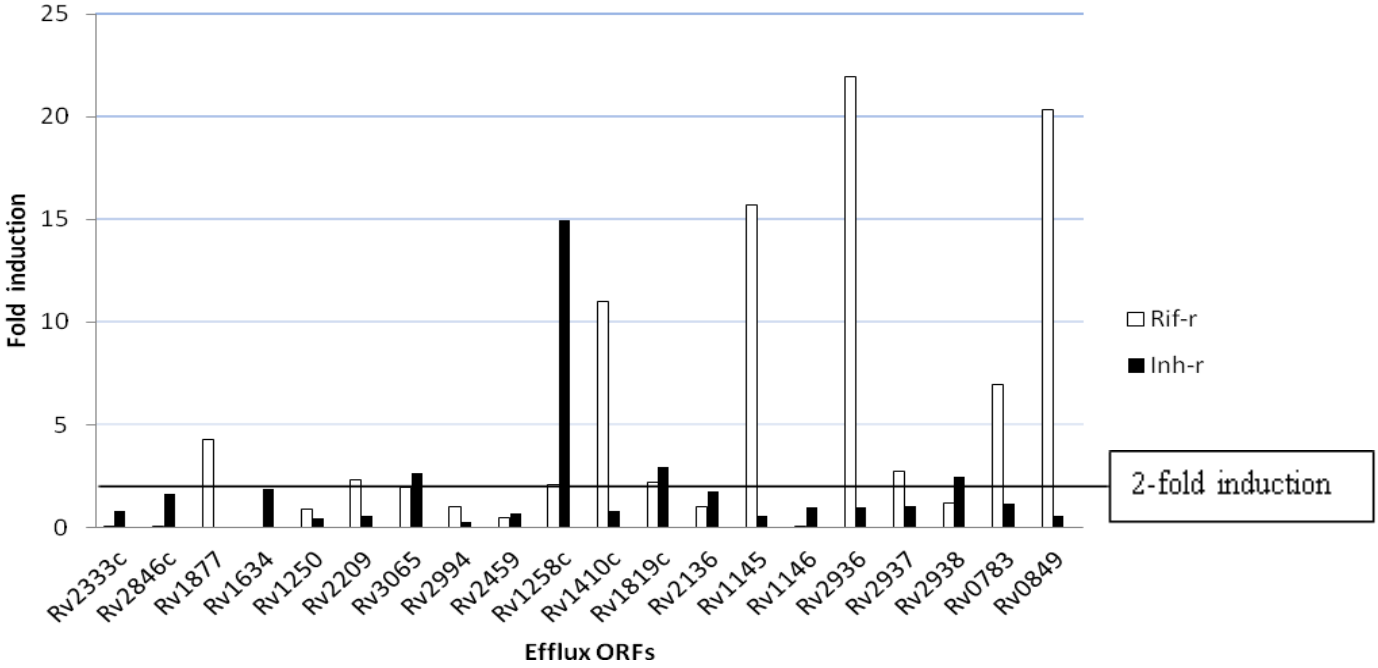


Figure-1: Quantitative real time PCR analysis. Relative expression levels of probable 20 efflux pump genes in the rifampin resistant and isoniazid resistant mutants. qRT-PCR analysis was performed in triplicates. More than 2-fold induction was considered significant.

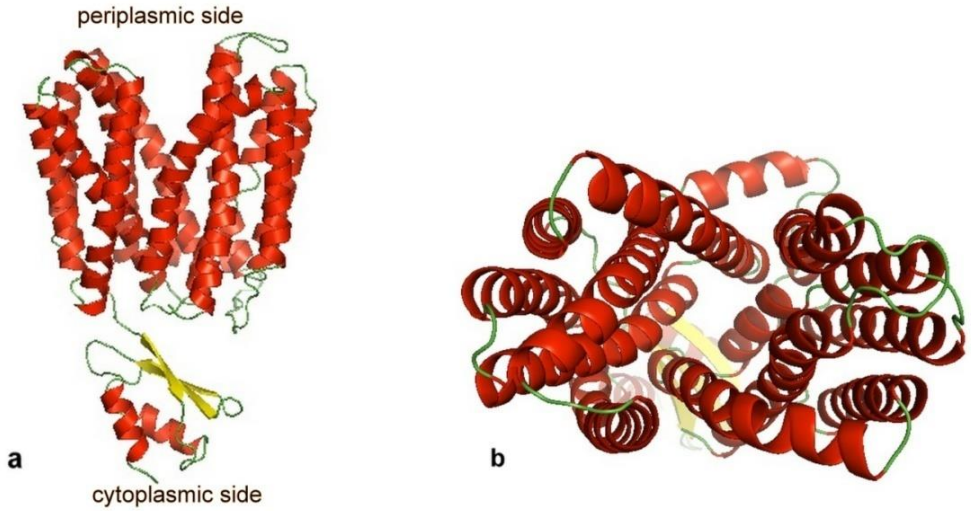


Figure-2: Final model of Rv1410c generated using Modeller 9.12. (a) The side view of the model positioned so that the periplasmic side is on top and cytoplasmic side on the bottom. The 12 transmembrane helix are also illustrated. (b) The cross-section view from the periplasmic side illustrating the common structural fold known as 'MFS fold'. All structures are prepared using Pymol.

The quality of the model was subjected to various checks through SAVS web server. Ramachandran plot (Figure-3) assessed the stereochemical quality and showed that 95.4% (371/450) of the residues lie in the most favoured regions, 3.9% (15/450) were in the allowed regions with only 0.66% (3/450) appeared in the outlier regions. Over 90% residues in the most favoured regions are expected for a good quality model. Verify3D analyzes the compatibility of an atomic model (3D) with its own amino acid sequence (1D).

The analysis plot showed that at least 80% of the amino acids have scored = 0.2 in the 3D1D profile giving Pass as result. Errat assess the statistics of non-bonded interactions between different atom types. Its output plot is represented in Figure-4 with an overall quality factor of 96.59% indicating a good high resolution model. To recognize the errors in the model ProSA-web was employed. Figure 5 displays the overall model quality with the Z-score of -6.84 which is well in the range of native conformations.

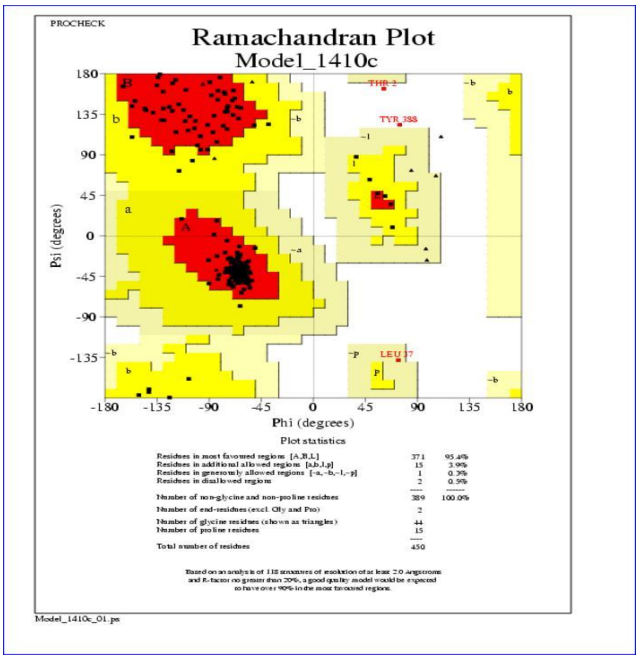
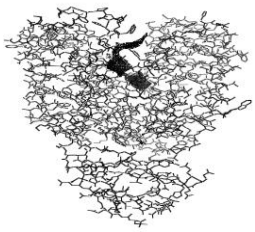


Figure-3: Quality check of modelled Rv1410c by Ramachandran Plot. Regions are nomenclature as: most favoured regions (red colour), additional allowed regions (yellow colour) and generously allowed region (brown colour).

ConSurf server was used for estimating the evolutionary conservation of amino acid positions in the model based on the phylogenetic relations between homologous sequences. Amino acids evolutionary conservation implies their structural and functional importance. As displayed in Figure-6 the amino acids are given color coded grade from highly conserved (magenta, grade 9) to average (white, grade 5) to highly variable (blue, grade 1). For our model, 107 amino acid obtained grade 9. To predict the ligand binding site, Q-SiteFinder was employed. It ranks the energetically most favourable cluster formed after calculating total interacting energies of the favourable probes with protein. The result is summarized in Table-4. The most favourable binding sites contain amino acids having conservation score in the range of 7 to 9 except one residue (grade 6). These residues can be of functional importance. Docking analysis was undertaken to determine whether the conserved amino acids as depicted by ConSurf and/or the residues involved in the most favourable binding site as reported by Q-SiteFinder are involved in interaction with the drug RIF and tetracycline. Using the Lamarckian algorithm of the AutoDock4 program blind docking was done on the theoretical model of Rv1410c with RIF as described in the methods. Re-docking was done to increase precision which yielded the best conformation with the lowest binding energy of -10.05 kcal/mol. The docking score between the ligand and the receptor represents energy terms such as electrostatic, van der Waals, and the energy of salvation. Based on docking simulations results, best docked conformation's hydrophobic and H-bond interactions are illustrated using LigPlot+ diagram in Figure-7. Figure-7a shows key binding and catalytic residues involved, Pro28, Phe143, Ala144, Met147, Val148, Phe227, Pro231, Ala241, His244, Trp245, and Tyr248. All the above residues were involved in hydrophobic interaction except His244. The imidazole side chain of His244 residue formed a H-bond formation with the RIF of 2.45 Å length. Since tetracycline is also a substrate for Rv1410c[50], it was also docked to the model as described in the methods. Residues involved in hydrophobic interactions are Val148, Met147, Leu249, His244, Ala 241, Phe143, Ala144, Tyr248, and Met252. An H-bond of 3.06 Å with Trp245 is also found (Figure-7b).

Table-4: Q-SiteFinder analysis of the model of Rv1410c. The energetically most favoured site with residues involved in interactions are shown. Structure prepared using PyMol.

Binding Site 1	Residues involved
	Phe16, Arg19, Met20, Met23, Phe24, Gly53, Gln56, Ala57, Gln60, Ala112, Gly136, Val137, Phe139, Gly140, Phe143, Ala144, Met147, Val148, His220, Leu223, Met224, Phe227, Trp245, Tyr248, Leu249, Met252, Phe256, Phe308, Phe311, Asn312, Glu315, Ser339, Thr340, Gln342, Phe343, Val346

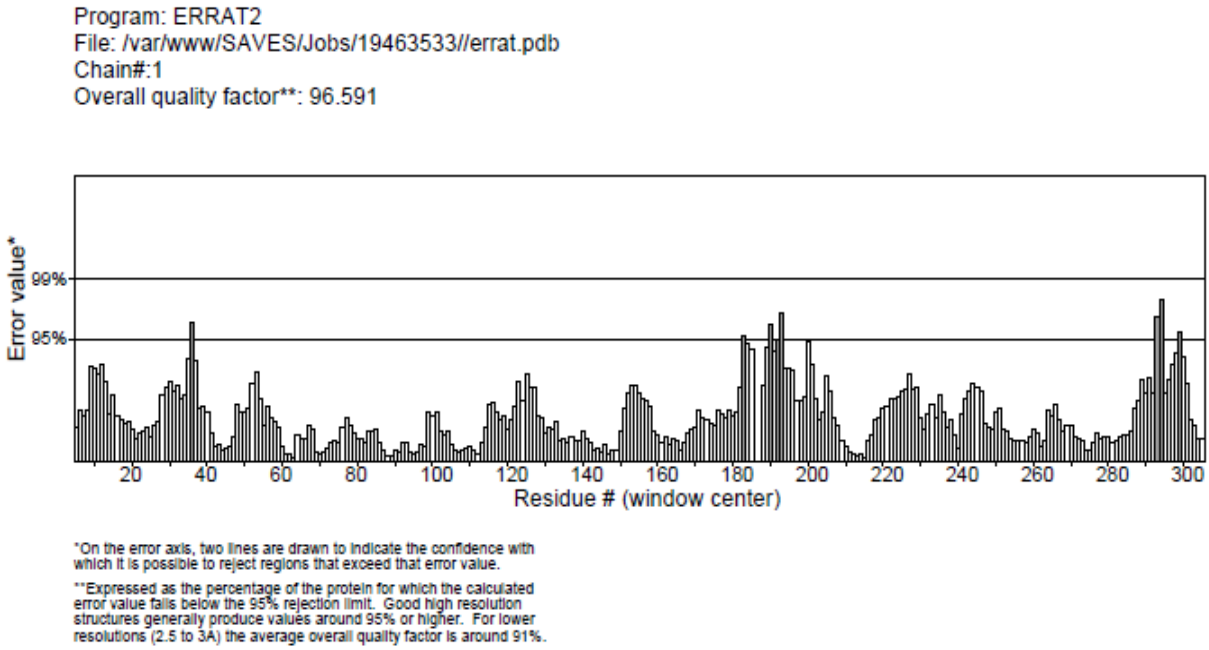


Figure-4: Stereochemical property validation result through Errat Program.

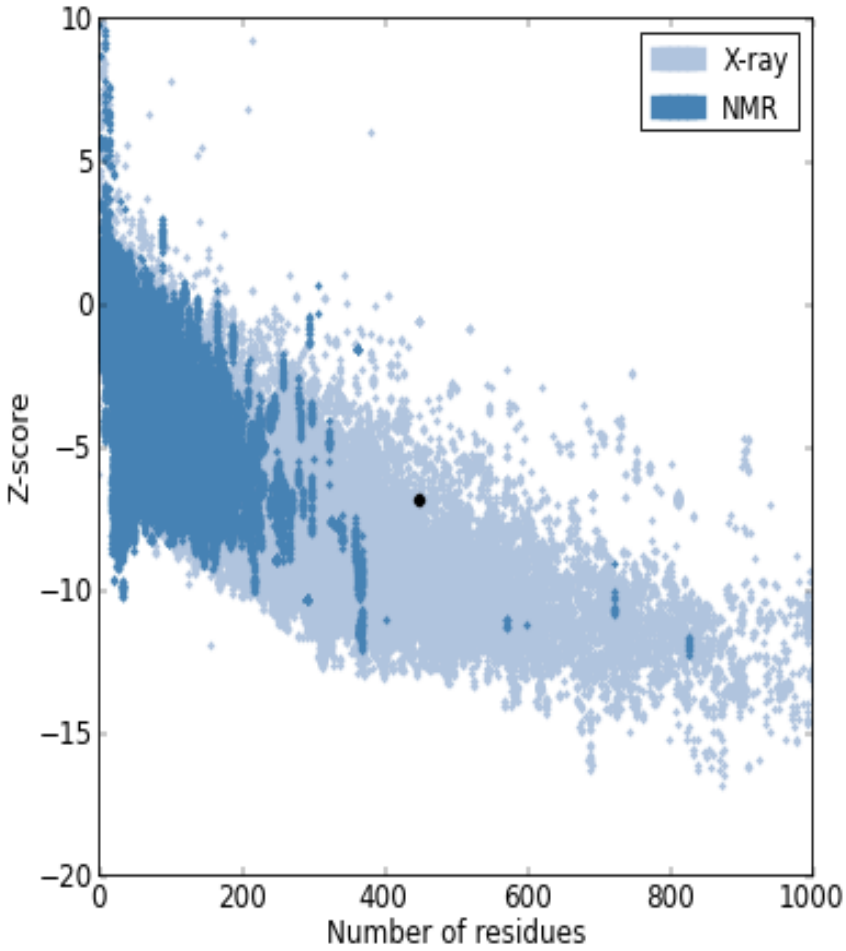


Figure-5: Investigation of the model using ProSA-web server. It indicates that protein structure has features characteristics for native structures with the Z-score of -6.84, represented as a black dot.

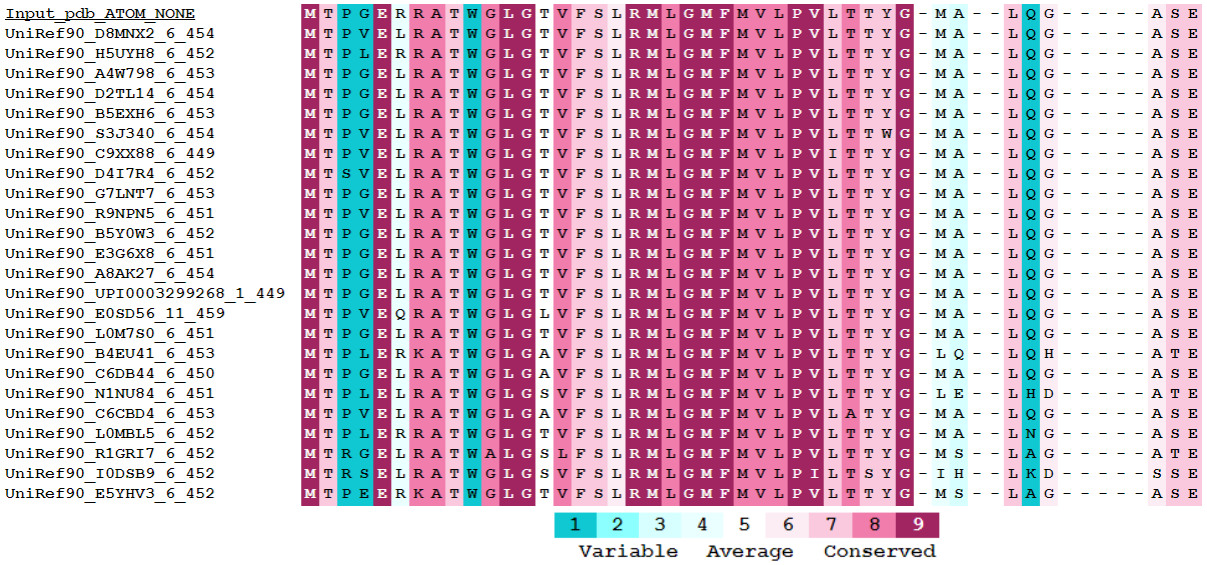


Figure-6: ConSurf server analysis. The colour coded amino acid conservation score of few amino acids of the model of Rv1410c.

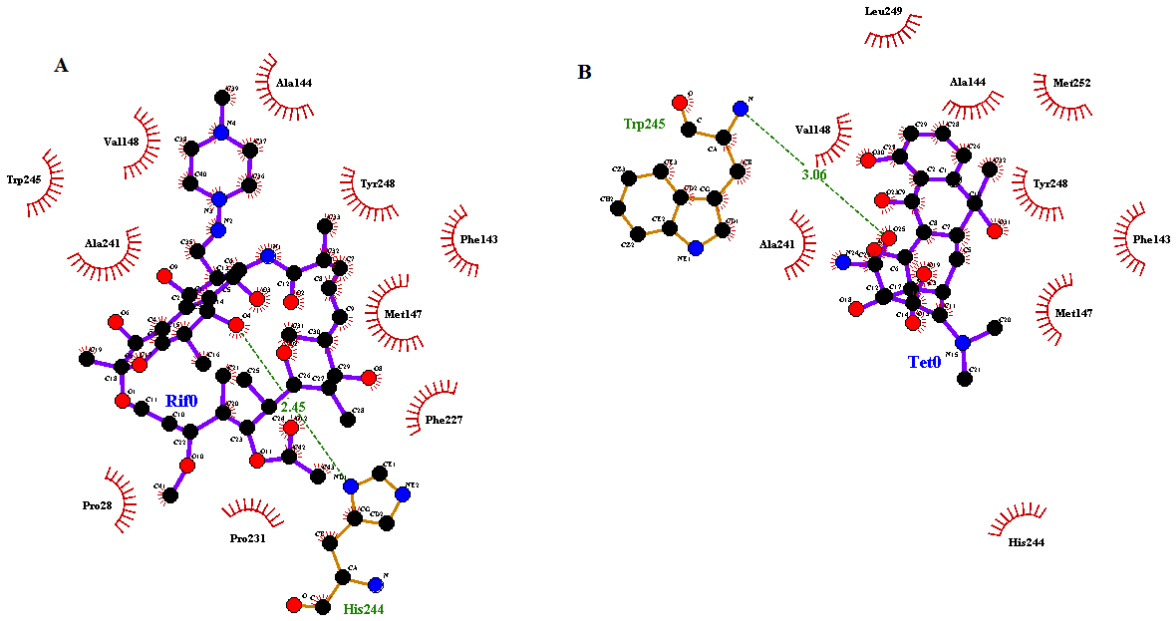


Figure-7: Ligplot diagrams illustrating protein-ligand interactions during docking. (A) Rifampin, (B) Tetracycline. Ligands are represented in purple. C, N, and O atoms are represented in black, blue and red respectively. Hydrophobic interactions and H-bond are represented by red spokes radiating towards the ligand atoms they contact and green lines indicating the bond length respectively.

In silico approach gave valuable insight into the binding mechanisms of effux pump proteins to the antimycobacterial drugs. A member of MFS family was selected (Rv1410c) as they represent one of the ancient and largest families of secondary transporters present in all kingdoms of life[56-58] . The efflux pumps belonging to them are also well known to contribute to drug resistance in several clinically important bacterial pathogens as reported by others[59] as well as in this study. The template (3WDO) chosen shared 37% homology with the aminoacid sequence of Rv1410c. Although the other high resolution MFS transporter structures available in PDB database share low sequence similarity, yet they share a common conserved structural feature known as MFS fold[54]. Hence a successful model even with low sequence identity can be predicted as was also observed for solute carrier transporters[60]. The model of Rv1410c contains all the general and unique features of a typical MFS transporter protein[54]. Evaluation and validation of the model through various web based server indicated the overall quality of the model. Consurf, Q-SiteFinder and blind docking analysis were undertaken to

successfully predict the binding site/residues of the model. Consurf analysis provides evolutionary conserved residues that may be of functional importance and grades them with the conservation score of 9 (highly conserved) to 1 (most variable) while Q-SiteFinder detects and characterizes functional sites on protein also known as ligand binding sites. Interestingly, these two analysis complemented each other in the sense that all the residues (36 in no.) predicted by Q-SiteFinder lie in the conservation grade range of 9 to 7 except Val137 (grade-6) signifying the functional conservation of the residues involved in the most possible binding pocket for ligand binding. For efficient docking without prior knowledge of binding site blind docking is an effective tool[39]. In general, the higher ability of ligands to form hydrophobic interactions with hydrophobic amino acids of the binding site, the higher the binding affinity. RIF and tetracycline are surrounded by hydrophobic amino acids, therefore are harder to remove under physiological conditions. Presence of H-bond further stabilises the complex in both the case. These residues may facilitate the binding of drug and can be key amino acids in efflux related drug resistance.

Conclusion

This study highlights the involvement of efflux pumps as a mechanism of drug resistance development in Mycobacterium tuberculosis. Various genes over expressed need to be characterized further in terms of time-kill kinetics and in-vivo studies also the use of efflux pump inhibitors need to be assessed as an effective anti-tb regime against MDR TB.

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References

1. World Health Organization. (2023). *Global tuberculosis report 2023*. World health organization.

2. Datta, D., Jamwal, S., Jyoti, N., Patnaik, S., & Kumar, D. (2024). Actionable mechanisms of drug tolerance and resistance in Mycobacterium tuberculosis. *The FEBS journal*, 291(20), 4433-4452.

3. Udwadia Z.F., Amale, R. A., Ajbani, K. K., & Rodrigues, C. (2012). Totally drug-resistant tuberculosis in India. *Clinical Infectious Diseases*, 54(4), 579–581.

4. Nikaido, H. (2001). Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Seminar in Cell and Developmental Biology,* 12, 215–223

5. Heym, B., Alzari, P.M., Honore, N. & Cole, S.T. (1995). Missense mutations in the catalase-peroxidase gene, *katG*, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. *Molecular Microbiology,* 15, 235–245.

6. Heym, B., Saint-Joanis, B. & Cole, S.T. (1999). The molecular basis of isoniazid resistance in *Mycobacterium tuberculosis*. *Tubercle and Lung Disease,* 79, 267–271.

7. Ramaswamy, S. V., Reich, R., Dou, S. J., Jasperse, L., Pan, X., Wanger, A., ... & Graviss, E. A. (2003). Single nucleotide polymorphisms in genes associated with isoniazid resistance in Mycobacterium tuberculosis. *Antimicrobial agents and chemotherapy*, 47(4), 1241-1250.

8. Baulard, A. R., Betts, J.C., Engohang-Ndong, J., Quan, S., McAdam, R.A., Brennan, P.J., Locht, C. and Besra, G.S. (2000). Activation of the pro-drug ethionamide is regulated in mycobacteria. *Journal of Biological Chemistry.* 275, 28326–28331.

9. Morlock, G.P., Metchock, B., Sikes, D., Crawford, J.T. and Cooksey, R.C. (2003). *Eth A*, *inh A*, and *kat G*loci of ethionamide-resistant clinical *Mycobacterium tuberculosis* isolates. *Antimicrobial agents and Chemotherapy* 47, 3799–3805.

10. Zainuddin, Z.F. and Dale, J.W. (1990). Does *Mycobacterium tuberculosis* have plasmids? *Tubercle* 71:43–49.

11. Telenti, A. (1997). Genetics of drug resistance in tuberculosis. *Clinics in Chest Medicine,* 18, 55–64.

12. Mitchison, D. A. (1979). Basic mechanisms of chemotherapy. *Chest*, 76(6), 771-776.

13. Musser J. M. (1995). Antimicrobial agent resistance in mycobacteria: molecular genetics insights. *Clinical Microbiology Reviews*, 8, 496–514.

14. Hazbon, M.H., Brimacombe, M., Bobadilla Del Valle, M., Cavatore, M., Guerrero, M.I., Varma-Basil, M., Billman-Jacobe, H., Lavender, C., Fyfe, J., García-García, L. & León, C.I., (2006). Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrobial agents and Chemotherapy,* 50, 2640–2649.

15. Basso L.A., Zheng R., Musser J.M., Jacobs Jr, W.R., & Blanchard J.S. (1998). Mechanisms of isoniazid resistance in *Mycobacterium tuberculosis:* enzymatic characterization of enoyl reductase mutants identified in isoniazid-resistant clinical isolates. *Journal of Infectious Diseases,* 178, 769–775.

16. Miesel L., Weisbrod T.R., Marcinkeviciene J.A., Bittman, R., & Jacobs, W.R.Jr. (1998). NADH dehydrogenase defects confer isoniazid resistance and conditional lethality in *Mycobacterium smegmatis*. *Journal of Bacteriology*, 180, 2459–67.

17. Larsen, M.H., Vilchèze, C., Kremer, L., Besra, G.S., Parsons, L., Salfinger, M., Heifets, L., Hazbon, M.H., Alland, D., Sacchettini, J.C. & Jacobs, W.R. Jr. (2002). Over expression of inhA, but not kasA, confers resistance to isoniazid and ethionamide in *Mycobacterium smegmatis*,

M. bovis BCG and *M. tuberculosis*. *Molecular Microbiology,* 46, 453–466.

18. Dalla Costa, E.R., Ribeiro, M.O., Silva, M.S., Arnold, L.S., Rostirolla, D.C., Cafrune, P.I., Espinoza, R.C., Palaci, M., Telles, M.A., Ritacco, V., Suffys, P.N., Lopes, M.L., Campelo, C.L., Miranda, S.S., Kremer, K., da Silva, P.E., Fonseca, Lde S., Ho, J.L., Kritski, A.L. & Rossetti, M.L. (2009). Correlations of mutations in katG, oxyR-ahpC and inhA genes and in vitro susceptibility in *Mycobacterium tuberculosis* clinical strains segregated by spoligotype families from tuberculosis prevalent countries in South America. *BMC Microbiology,* 9, 39.

19. Ando H., Kitao T., Miyoshi-Akiyama T., Kato, S., Mori, T., &Kirikae, T. (2011). Downregulation of *katG* expression is associated with isoniazid resistance in *Mycobacterium tuberculosis*. *Molecular Microbiology,* 79: 1615–28.

20. Louw, G.E., Warren, R.M., Gey van Pittius, N.C., McEvoy, C.R.E., Van Helden, P.D. & Victor, T.C. (2009). A Balancing Act: Efflux/Influx in Mycobacterial Drug Resistance. *Antimicrobial agents and Chemotherapy*, 53(8), 3181-3189.

21. Dhamdhere, G. & Zgurskaya, H.I. (2010). Metabolic shutdown in *Escherichia coli* cells lacking the outer membrane channel TolC. *Molecular Microbiology,* 77, 743–754.

22. Lau, S.Y. & Zgurskaya, H.I. (2005). Cell division defects in *Escherichia coli* deficient in the multidrug efflux transporter AcrEF-TolC. *Journal of Bacteriology*, 187, 7815–7825.

23. Krulwich, T.A., Lewinson. O., Padan, E., & Bibi, E. (2005). Do physiological roles foster persistence of drug/multidrug-efflux transporters? A case study. *Nature Reviews Microbiology,* 3, 566 –572.

24. De Rossi, E., Aínsa, J. A, & Riccardi, G. (2006). Role of mycobacterial efflux transporters in drug resistance: an unresolved question. *FEMS Microbiology Reviews,* 30, 36–52.

25. Pasipanodya, J.G. & Gumbo, T. (2011). A new evolutionary and pharmacokinetic-pharmacodynamic scenario for rapid emergence of resistance to single and multiple anti-tuberculosis drugs. *Current Opinion in Pharmacology,* 11(5), 457–463.

26. Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., Edelstein, P. H., Cosma, C. L., & Ramakrishnan, L. (2011). Drug Tolerance in Replicating Mycobacteria Mediated by a Macrophage-Induced Efflux Mechanism. *Cell,* 145, 39–53.

27. Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E. III, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K. & Barrell, B.G. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature,* 393, 537–544.

28. MA, W. (2006). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. *Clsi (Nccls)*, 26, M7-A7.

29. Eliopoulos, G.M. & Wennersten, C.B. (2002). Antimicrobial activity of quinupristin–dalfopristin combined with other antibiotics against vancomycin-resistant enterococci. *Antimicrobial agents and Chemotherapy*, 46, 1319–24.

30. Alvireaz-Freits, E.J., Carter, J.L. & Cynamon, M.H. (2002) In vitro and in vivo activity of gatifloxacin against Mycobacterium tuberculosis. *Antimicrobial agents and Chemotherapy,* 46, 1022–5.

31. Sanger, G., & Goldstein, C. (2001). Principles, workflows and advantages of the new Light Cycler Relative Quantification Softwar. *Biochemical,* 3, 15-17

32. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology*, 215(3), 403-410.

33. Jiang, D., Zhao, Y., Wang, X., Fan, J., Heng, J., Liu, X., Feng, W., Kang, X., Huang, B., Liu, J., & Zhang, X.C. (2013). Structure of the YajR transporter suggests a transport mechanism based on the conserved motif A. *Proceedings of the National Academy of Sciences of the United States of America,* 110, 14664-14669.

34. Sali, A., & Blundell, T.L. (1993). Comparative protein modelling by satisfaction of spatial restraints. *Journal of Molecular Biology,* (3), 779–815

35. Wiederstein M. & Sippl M.J. (2007). ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Research*, 35, W407–W410.

36. Glaser, F., Pupko, T., Paz, I., Bell, R.E., Bechor, D., Martz, E. & Ben-Tal, N. (2003). ConSurf: Identification of Functional Regions in Proteins by Surface-Mapping of Phylogenetic Information. *Bioinformatics,* 19, 163-164.

37. Laurie, A.T. & Jackson, R.M. (2005). Q-SiteFinder: an energy-based method for the prediction of protein-ligand binding sites. *Bioinformatics,* 21, 1908-1916

38. Morris, G.M., Huey, R., Lindstrom, W., Sanner, M.F., Belew, R.K., Goodsell, D.S. & Olson, A. J. (2009). Autodock4 and AutoDockTools4: automated docking with selective receptor flexibility. *Journal of Computational Chemistry*, 16, 2785-91

39. Hete´nyi, C. & van der Spoel, D. (2002). Efficient docking of peptides to proteins without prior knowledge of the binding site. *Protein Science,* 11, 1729–1737.

40. Wallace, A.C., Laskowski, R.A. & Thornton, J.M. (1995). LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Engineering, Design and Selection,* 8, 127–134

41. Khan, I.A., Mirza, Z.M., Kumar, A., Verma, V., & Qazi, G.N. (2006). Piperine, a phytochemical potentiator of ciprofloxacin against Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy,* 50, 810-812

42. Zhang, J.R., Li, G.L., Zhao, X.Q., Wan, K.L. &Lü, J.X. (2013). A primary investigation on the isoniazid-induced alterations in efflux gene expression among the isoniazid resistant *Mycobacteriumtuberculosis* clinical isolates. *Zhonghua Liu Xing Bing Xue Za Zhi*, 34(4), 379-84

43. Jiang, X., Zhang, W., Zhang, Y., Gao, F., Lu, C., Zhang, X., & Wang, H. (2008). Assessment of efflux pump gene expression in a clinical isolate *Mycobacteriumtuberculosis* by real-time reverse transcription PCR. *Microbial drug resistance*, 14(1), 7-11.

44. Gupta, A.K., Katoch, V.M., Chauhan, D.S., Sharma, R., Singh, M., Venkatesan, K. & Sharma, V.D. (2010). Microarray analysis of efflux pump genes in multidrug-resistant *Mycobacteriumtuberculosis* during stress induced by common anti-tuberculous drugs. *Microbial drug resistance,* 16(1), 21-8.

45. Machado, D., Couto, I., Perdigão, J., Rodrigues, L., Portugal, I., Baptista, P., Veigas, B., Amaral, L., & Viveiros, M. (2012). Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. *PLOSone,* 7(4), e34538

46. Rodrigues, L., Villellas, C., Bailo, R., Viveiros, M. &Aínsa, J.A. (2013). Role of the Mmr efflux pump in drug resistance in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy,* 57(2), 751-7.

47. Ghajavand, H., Kargarpour Kamakoli, M., Khanipour, S., Pourazar Dizaji, S., Masoumi, M., Rahimi Jamnani, F., ... & Vaziri, F. (2019). Scrutinizing the drug resistance mechanism of multi-and extensively-drug resistant Mycobacterium tuberculosis: mutations versus efflux pumps. *Antimicrobial Resistance & Infection Control*, 8(1), 70.

48. Gupta, A.K., Chauhan, D.S., Srivastava, K., Das, R., Batra, S., Mittal, M., Goswami, P., Singhal, N., Sharma, V.D., Venkatesan, K., Hasnain, S.E. & Katoch, V.M. (2006). Estimation of efflux mediated multi-drug resistance and its correlation with expression levels of two major efflux pumps in mycobacteria. *Journal of Communicable Diseases*, 38(3), 246-54.

49. Pang, Y., Lu, J., Wang, Y., Song, Y., Wang, S., & Zhao, Y. (2013). Study of the Rifampin monoresistance mechanism in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*, 57, 893-900

50. Silva, P.E., Bigi, F., Santangelo, M.P., Romano, M.I., Martín, C., Cataldi, A. & Aínsa, J.A. (2001). Characterization of P55, a multidrug efflux pump in *Mycobacteriumbovis* and *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy,* 45(3), 800-4.

51. Rodrigues, L., Machado, D., Couto, I., Amaral, L. & Viveiros, M. (2012). Contribution of efflux activity to isoniazid resistance in the *Mycobacterium tuberculosis* complex. *Infection, Genetics and Evolution,* (4), 695-700.

52. Kaatz, G.W., Thyagarajan, R.V. & Seo, S.M. (2005). Effect of promoter region mutations and *mgrA*overexpression on transcription of *norA*, which encodes a *Staphylococcus aureus* multidrug efflux transporter. *Antimicrobial Agents and Chemotherapy*, 49, 161–169.

53. Piddock, L.J.V. (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clinical microbiology reviews*, 19(2), 382-402.

54. Yan, N. (2013). Structural advances for the major facilitator superfamily (MFS) transporters. *Trends in Biochemical Sciences,* 38(3), 151-159

55. Sun, L., Zeng, X., Yan, C., Sun, X., Gong, X., Rao, Y., & Yan, N. (2012). Crystal structure of a bacterial homologue of glucose transporters GLUT1–4. *Nature*, 490(7420), 361-366.

56. Pao, S.S., Paulsen, I.T. & Saier, M.H. Jr. (1998). Major facilitator superfamily. *Microbiology and Molecular Biology Reviews,* 62, 1–34

57. Marger, M.D. & Saier, M.H., Jr (1993). A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends in Biochemical Sciences,* 18, 13–20

58. Reddy, V. S., Shlykov, M. A., Castillo, R., Sun, E. I., & Saier Jr, M. H. (2012). The major facilitator superfamily (MFS) revisited. *The FEBS journal*, *279*(11), 2022-2035.

59. Saidijam, M., Benedetti, G., Ren, Q., Xu, Z., Hoyle, C.J., Palmer, S.L., Ward, A., Bettaney, K.E., Szakonyi, G., Meuller, J., Morrison, S., Pos, M.K., Butaye, P., Walravens, K., Langton, K., Herbert, R.B., Skurray, R.A., Paulsen, I.T., O'reilly, J., Rutherford, N.G., Brown, M.H., Bill, R.M. & Henderson, P.J.. (2006). Microbial drug efflux proteins of the major facilitator superfamily. *Current Drug Targets,* 7, 793–811.

60. Schlessinger, A., Khuri, N., Giacomini, K. M., & Sali, A. (2013). Molecular Modeling and Ligand Docking for Solute Carrier (SLC) Transporters. *Current Topics in Medicinal Chemistry*, 13, 843-856.