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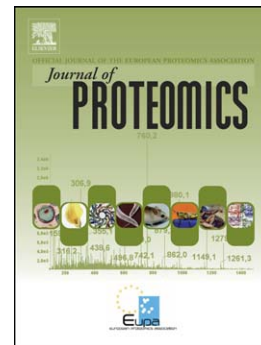
Proteomic analysis of ofloxacin-mono resistant *Mycobacterium tuberculosis* isolates

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**RESEARCH ARTICLE**

Title: Proteomic analysis of ofloxacin-mono resistant *Mycobacterium tuberculosis* isolates

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**ABSTRACT**

Drug resistance particularly, multi drug resistance tuberculosis (MDR-TB) has emerged as a major problem in the chemotherapy of tuberculosis. Ofloxacin (OFX) has been used as second-line drug against MDR-TB. The principal target of the OFX is DNA gyrase encoded by *gyrA* and *gyrB* genes. Many explanations have been proposed for drug resistance to OFX but still some mechanisms are unknown. As proteins manifest most of the biological processes, these are attractive targets for developing drugs and diagnostics/therapeutics. We examined the OFX resistant *M.tuberculosis* isolates by proteomic approach (2DE-MALDI-TOF-MS) and bioinformatic tools under OFX induced conditions. Our study showed fourteen proteins (Rv0685, Rv0363c, Rv2744c, Rv3803c, Rv2534c, Rv2140c, Rv1475c, Rv0440, Rv2245, Rv1436, Rv3551, Rv0148, Rv2882c and Rv0733) with increased intensities in OFX resistant and OFX induced as compared to susceptible isolates. Bioinformatic analysis of hypothetical proteins (Rv2744c, Rv2140c, Rv3551 and Rv0148) revealed the presence of conserved motifs and domains. Molecular docking showed proper interaction of OFX with residues of conserved motifs. These proteins might be involved in the OFX modulation/neutralization and act as novel resistance mechanisms as well as potential for diagnostics and drug targets against OFX resistance.

**Biological Significance**

In this study we employed two dimensional gel electrophoresis coupled with mass spectrometry and bioinformatics tools. To the best of our knowledge it is the first ever report on proteomics of ofloxacin (OFX) resistant *M. tuberculosis* isolates and their induction with OFX. Out of fourteen proteins (increased intensities) four proteins (Rv2744c, Rv2140c, Rv3551 and Rv0148) were with unknown functions. Molecular docking revealed that conserved motifs of hypothetical proteins interact with OFX. We hypothesized that these

proteins might be neutralizing the effect of OFX, explored novel resistance mechanism and in survival of mycobacteria in the presence of OFX. These findings need further exploitation to develop newer therapeutic agents against these targets.

## Introduction

*Mycobacterium tuberculosis* is the etiological agent of tuberculosis (TB), a potentially fatal illness which results in approximately 2 million deaths worldwide annually. Global emergence of drug resistance is a dangerous alarm. The increase in the incidence of MDR-TB and emergence of XDR-TB presents tremendous challenges to the global efforts to battle TB. Globally, 3.5% of new and 20.5% of previously treated TB cases had MDR-TB. In 2013, an estimated 4.8 lakhs people developed MDR-TB in the world [1]. Ofloxacin (OFX) is one of the most widely available fluoroquinolones (FQs) which is routinely administered to patients with MDR-TB in developing countries, because it is less expensive. The principle target of FQs is DNA gyrase encoded by *gyrA* and *gyrB* genes. FQ resistance in *M.tuberculosis* is mainly due to the acquisition of point mutations within the quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB*. But only 42-85% of resistant *M.tuberculosis* isolates have mutation in *gyrA* QRDR and till date no isolates have been associated with *gyrB* QRDR mutation [2]. The other mechanisms of fluoroquinolones resistance are alteration in the drug target, permeability barrier and efflux of the drug from

the cell [3]. However, exact mechanisms of resistance to FQs are not fully known. It is also possible that fluoroquinolones have some other mechanisms of action and development of resistance. Two-dimensional gel electrophoresis (2-DE) coupled with MALDI-TOF mass spectrometry (MS) and bioinformatics is a powerful and efficient tool for rapid identification of proteins. Comparative proteomic studies addressing whole cell proteins with first and second line drugs resistant isolates have been reported [4-5]. In spite of advancement in mycobacterial research, the knowledge about genes and their corresponding functional proteins under drug pressure with susceptible and resistant isolates with FQs has remained unaddressed. Therefore, the present study was designed to investigate the proteome analysis of OFX mono-resistant *M.tuberculosis* isolates exposed to sub-MIC concentrations of OFX using proteomic and bioinformatic approaches. Such study under induced conditions might help in further understanding the mechanisms of drug resistance and in modifying the OFX dosage for resistant isolates and developing new agents based on inducible protein profile of the OFX mono-resistant *M.tuberculosis* clinical isolate under drug pressure.

## **Materials & methods**

### ***M.tuberculosis* isolates & drug susceptibility testing**

Three total susceptible (rifampicin, isoniazid, ethambutol, pyrazinamide, streptomycin, kanamycin, amikacin and ofloxacin) and three OFX mono-resistant (susceptible to other first line and second line drugs) *M.tuberculosis* isolates were obtained from the Mycobacterial Repository Centre of the Institute. Our Institute is the NRL (National Reference Laboratory) and these isolates are clinical isolates. These are not deposited in public strain collection centres. Susceptibility testing for all the drugs was performed by LJ proportion [6] and REMA methods [7-8]. Cultures were grown in Sauton's liquid medium at 37°C and harvested in late log phase (4 weeks). Sauton's medium was preferred for scale up

of culture over MB7H9 as it is less expensive, do not require any enrichment like OADC. Further albumin in OADC (MB7H9) might interfere with proteomic studies.

### **Culture and drug induction**

Susceptible and OFX mono-resistant isolates were grown in Sauton's liquid medium at 37 °C for four weeks. After four weeks each culture of OFX mono-resistant isolates were induced with OFX (sub-MIC-2µg/ml) for 36 hrs and one flask of each (OFX mono-resistant) was not induced with OFX. As the generation time of *M.tuberculosis* is 24 to 36 hrs, sufficient time was given so that all proteins of mycobacteria are expressed.

### **Mycobacterial cell lysate preparation and protein precipitation**

Mycobacterial cell lysate was prepared according to previously published protocol [9]. Cells after washing with normal saline were suspended in sonication buffer and broken by intermittent sonication at 4 °C for 20 min. The homogenate was centrifuged and supernatant collected. Cell lysate proteins were precipitated using published protocol [10] and protein concentrations were estimated by Bradford method [11]. Protein extractions were performed on three identical cultures and processed samples to provide biological and technical replicates.

### **Two-dimensional gel electrophoresis**

Isoelectric focusing (IEF) was carried out using the method of "in gel rehydration" with slight modifications [4, 12]. Proteins were separated in second dimension on 12% SDS-polyacrylamide gels in a vertical electrophoretic unit PROTEAN Plus Dodeca Cell (BIO-RAD, Hercules, CA, USA) at a constant voltage of 250 V for 5-6 h and gels were stained with Coomassie Brilliant Blue R250 to visualize proteins. Gels were analyzed using PDQuest

software version 8.0.0 (BIO-RAD, Hercules, CA, USA). System picks up the spots with differential intensity of significant levels by using inbuilt Student's t-test for the statistical analysis by PDQuest software. Beside PDQuest analysis we also carried out manual analysis to rule out any artifacts. Protein spots which showed increased intensities with more than 1.5 folds were selected for identification.

### **In-gel digestion of protein and Mass spectrometric analysis**

Method of Shevchenko et al [13] was followed with slight modifications. Protein spots of interest were excised from the 2D gels and digested by trypsin. Digested samples were desalted and concentrated on C-18 ZipTips (Millipore, Billerica, MA, USA) using the manufacturer's protocol. Mass spectra of digested proteins were acquired using Autoflex II TOF/TOF 50 (Bruker Daltonik GmbH, Leipzig, Germany) in positive reflectron mode, in the detection range of 500-3000 m/z. External calibration to a spectrum, acquired for a mixture of peptides with masses ranging from 1046 to 2465 Da, was done prior to acquisition. Proteolytic masses obtained were then processed through Flex Analysis v.2.4 programme for peak detection of proteins. Peak detection in MALDI spectra and submission of peak lists to the Peptide mass fingerprint (PMF) Mascot server were done using the Mascot Wizard program (Matrix Science, U.K). Peptide mass tolerance was set in range of 50-100 ppm (to acquire the best MASCOT score) with carbamidomethyl-cystein set as fixed modification, oxidation of methionine as variable modification and 0 or 1 missed cleavage site allowed.

### **Bioinformatic analysis**

Protein sequences of identified proteins with unknown function were retrieved from Tuberculist server <http://genolist.pasteur.fr/TubercuList/BLAST> [14] runs were performed at the NCBI server <http://blast.ncbi.nlm.nih.gov/> using the default threshold E-value of 10 and inclusion threshold value of 0.005. Motif and domain searches were made on EBI server

<http://www.ebi.ac.uk/Tools/InterProScan/> employing InterProScan. Orthologs from other species of mycobacteria and human were obtained from KEGG <http://www.genome.jp/kegg/> by single-directional besthit option (SBH) and the same server was employed for multiple sequence alignments (CLUSTALW) [15], using a set of five organisms: mbo (*Mycobacterium bovis*), maf (*Mycobacterium africanum*), mav (*Mycobacterium avium*), mle (*Mycobacterium leprae*) and hsa (*Homo sapiens*). Sequences of H<sub>37</sub>Rv were submitted for 3-dimensional structure predictions at I-TASSER server <http://zhang.bioinformatics.ku.edu/I-TASSER/>. Structures were selected on the basis of RMSD values and agreement with Ramachandran Plot using VMD software (University of Illinois). Selected structures were docked with OFX (structure obtained from <http://www.drugbank.ca>) using Patch Dock server [16] <http://bioinfo3d.cs.tau.ac.il/PatchDock/> and results were refined using FireDock server [17-18] <http://bioinfo3d.cs.tau.ac.il/FireDock/>. Interacting amino acid side chains, drug molecule orientation and docking feasibility was based on Fire Dock scores and visualizations with VMD software.

## Results

The goal of this study was to identify proteins of increased intensities in the OFX mono-resistant *M.tuberculosis* isolates grown in presence and absence of drug OFX. The MIC of OFX for resistant isolates was found to be 4µg/ml by REMA plate method. We used a combination of two-dimensional electrophoresis (2-DE) and matrix-assisted-laser-desorption/ionization-time of flight-mass spectrometry (MALDI-TOF/MS) to identify increased intensities proteins of *M.tuberculosis* isolates. Fig. 1(A), 1(B) and 1(C) shows the 2-DE profile of *M. tuberculosis* total susceptible, OFX mono-resistant isolates grown without drug and with drug respectively. 2-DE gel profile of OFX mono-resistant isolates whether uninduced or induced with drug exhibited many proteins with increased intensities.



Interestingly, both constitutive and inducible proteins showed increased intensities. The susceptible isolates were included to get a preliminary idea on the pattern of constitutive changes in the resistant isolates. Intensities of six proteins were found to be increased in OFX mono-resistant isolates on comparing with total susceptible isolates. When 2-DE profile of OFX mono-resistant isolates (absence of drug) was compared with OFX mono-resistant isolates grown in presence of drug, fourteen protein spots were identified. Six proteins were same as upregulated in OFX mono-resistant isolates and eight new proteins were found to be upregulated in induced cultures. The levels of difference in protein spots intensity (cut limit  $\geq 1.5$  fold changes) have been represented as densitometric ratio in Table-1. These protein spots were identified by MALDI-TOF mass spectrometry (Table 1). The identified proteins are elongation factor Tu (Rv0685), fructose-bisphosphate aldolase (Rv0363c), 35 kDa protein (Rv2744c), MPT51/MPB51 antigen (Rv3803c) elongation factor P (Rv2534c), conserved hypothetical protein TB18.6 (Rv2140c), aconitate hydratase (Rv1475c), 60 kDa chaperonin 2 (Rv0440), 3-oxoacyl-[acyl-carrier-protein] synthase 1 (Rv2245), glyceraldehyde-3-phosphate dehydrogenase (Rv1436), putative CoA-transferase subunit alpha (Rv3551), hypothetical protein (Rv0148), ribosome-recycling factor (Rv2882c), adenylate kinase (Rv0733). Out of fourteen, Rv0685, Rv2534c and Rv2882c belonged to information pathways, Rv0363c, Rv1475c, Rv1436, Rv3551, Rv0148 and Rv0733 to intermediary metabolism and respiration, Rv3803c and Rv2245 to lipid metabolism, Rv0440 to virulence, detoxification and adaptation, Rv2744c and Rv2140c to conserved hypothetical category.

### **Bioinformatic Analysis**

Identified proteins (hypothetical and others) were bioinformatically characterized by using different servers and softwares such as BLASTP, InterProScan, Multiple Sequence Alignment and Molecular Docking. These are Rv2744c, Rv2140c, Rv3551, Rv0148, Rv2534c, Rv0685, Rv3803c, Rv2882c, Rv2245, Rv1436, Rv0363c and Rv0440.

## BLASTP

BLASTP analysis was performed for hypothetical proteins only. The conserved alanine rich hypothetical protein Rv2744c protein exhibited significant homology with hypothetical and phage shock protein A (PspA) of all *M. tuberculosis* complex and NTMs but *M. leprae* exhibit homology with cell division protein. Rv2140c exhibited significant homology with conserved hypothetical protein of *M. bovis*, *M. bovis* BCG, *M. avium*, *M. africanum*, *M. leprae* and some species of mycobacterium showed homology with phospholipid-binding protein. Other hypothetical protein Rv3551 exhibited significant homology with CoA-transferase subunit alpha of *Mycobacterium* species as well as other bacteria. Hypothetical protein Rv0148 was found conserved (100 % identical) in species of mycobacteria (*M. tuberculosis* complex and NTM), belongs to the short-chain dehydrogenases/reductases (SDR) family and thought to be involved in cellular metabolism. Dendrogram for Rv2140c, Rv2744c, Rv3551 and Rv0148 are shown in Fig.2 A, B, C and D, respectively.

## Phylogenetic analysis

Orthologs of hypothetical proteins from other species of mycobacteria and human were obtained from KEGG (<http://www.genome.jp/kegg/>) by single-directional best-hit option (SBH) and same server was employed for multiple sequence alignments (CLUSTALW). Multiple sequence alignment of *mtu* (*M. tuberculosis*) proteins was performed for the set of five organisms, *mbo* (*M. bovis*), *maf* (*M. africanum*), *mav* (*M. avium*), *mle* (*M. leprae*) and *hsa* (*Homo sapiens*) {Table-2}. Results show that all proteins exhibited 100% homology to their corresponding proteins in *M. bovis* and *M. africanum* which are members of tuberculosis complex. Rv2744c exhibited 88% homology with *M. avium*, 90.40% homology with PspA of *Mycobacterium liflandii* and 24.6% homology

with myosin heavy chain of human and 26.6% homology with cell division protein of *M. leprae*. Rv2140c showed 83% with *M. avium*, 86.3% with *M. leprae* and 26.4% homology with peroxisome proliferator-activated receptor  $\alpha$  of human. Rv3551 showed 87.2% with *M. avium*, 25.2% homology with serine protease of *M. leprae* and 24.8% homology with 3-oxoacid CoA transferase 2. Rv0148 showed 87.6% homology with *M. avium*, but 50% & 33.5% homology with human and *M. lepre* protein.

#### **InterProScan analysis:**

Motif and domain searches were made on EBI server (<http://www.ebi.ac.uk/Tools/InterProScan/>) employing InterProScan for proteins which were docked with OFX. InterProScan analysis of Rv2744c showed the presence of PspA domain with amino acid residues from 3-242 (PF04012). Rv2140c showed motif (#PF01161) from residues 17-175 characteristic motif of Phosphatidylethanolamine-binding protein and Raf kinase inhibitor-like protein. Rv3551 showed motif (#PF01144) from residues 6-233 characteristic motif which provides a signature for coenzyme A transferase. Rv0148 showed motifs (#PF00106) from residues 8-183 characteristic motif which provides a signature for short chain dehydrogenase. Rv2534c (Elongation factor P) showed conserved OB domain (#PF01132) from residues 67-121 and C-terminal domain (#PF09285) from residues 129-184. Rv0685 (Elongation factor Tu) possess EF-Tu domain 2(#PF03144) and C-terminal domain (#PF03143) from residues 226-296 and 301-395 respectively. Rv3803c (MPT51 antigen) showed conserved Alpha/Beta hydrolase fold from residues 35-297. Rv2882c possess conserved Ribosome recycling factor (RRF) domain (#PF01765) from residues 21-183. Rv2245 showed conserved N-terminal and C-terminal domain of Beta-ketoacyl synthase

from residues 13-253 and 262-374 respectively. Rv1436 possess conserved NAD (P) binding domain (#PF00044) from residues 3-158. Rv0363c showed conserved domain Fructose-bisphosphate aldolase class-II (#PF01116) from residues 9-340. Rv0440 showed conserved GroEL like equatorial domain (G3DSA:1.10.560.10) from residues 3-186 and 367-515.

### **3D modelling and docking**

Molecular docking analysis of selected 3D models (showing less than 2% discrepancy from Ramachandran plot) of identified proteins with unknown function and other proteins were performed to detect their binding with OFX. Parameters used for selection of 3D models and molecular docking are represented in Table 3. Docking of Rv2744c with OFX shows the interaction with the conserved domain of phage shock protein A (PspA). Rv2140c interacting residues are in close vicinity with Usp domain. Rv3551 with OFX shows the interaction with the conserved motif of coenzyme A transferase. Rv0148 showed the interaction of OFX with the residues of characteristic motif which provides a signature for short chain dehydrogenase domain (Fig. 3). OFX binds to conserved Nucleic acid binding OB and C-terminal domain of Rv2534c. Rv0685 showed the interaction of OFX with the residues of EF-Tu domain- 2 and C-terminal domain. Rv3803c and Rv2882c showed binding of OFX to conserved Alpha/Beta hydrolase fold and domain of the ribosome recycling factor (RRF) respectively. In Rv2245 OFX interacts with conserved N and C-terminal domain of Beta ketoacyl synthase. Rv1436 and Rv0363c OFX binds to conserved NAD (P) binding domain and Fructose bisphosphate aldolase class II respectively. Rv0440 showed the interaction of OFX with the residues of conserved GroEL like equatorial domain.

### **Discussion:**

In this study, we have analyzed and compared the protein profiles of OFX mono-resistant *M.tuberculosis* isolates (uninduced and induced with drug OFX) by 2DE-MALDI-TOF/MS and bioinformatic tools. The identified proteins showed increased intensities in the resistant isolates and might be contributing in conferring resistance or serve as potential diagnostic markers/ therapeutic targets. Six proteins were found with increased intensities in resistant as compared to total susceptible isolates. Besides this, eight new proteins were found to have increased intensities in induced as compared to uninduced resistant isolates. Of the fourteen proteins, eight belonged to metabolic pathways and six belonged to information pathways, conserved hypothetical and virulence/detoxification/adaptation categories.

Spot 1 (Rv0685), an elongation factor-Tu, is a conserved protein involved in the elongation phase of translation and posttranslational modifications. It also has RNA chaperone activities, ensuring that tmRNA adopts an optimal conformation during aminoacylation [19]. Ef-Tu phosphorylation is implicated in acclimation to the stress conditions encountered during the course of infection. Sajid et al [20] reported that phosphorylation of Ef-Tu by Protein Kinase B reduced its interaction with GTP, suggesting reduction in protein synthesis. Spot 2 is fructose 1,6-bisphosphate aldolase/FBA (Rv0363c) which catalyzes reversible aldol condensation of dihydroxy acetone phosphate (DHAP) with G3P to form fructose 1, 6-bisphosphate (FBP). It is reported to be over expressed in *M.tuberculosis* under low oxygen tension [21-23] and in latent TB [24]. It is crucial to supply these precursor molecules into various biochemical pathways; essential for the survival of *M.tuberculosis* under hypoxic environment and might be promising new target. Spot 3 is a hypothetical protein (Rv2744c/35 kDa antigen), homologous to phage shock protein A (PspA) of *E.coli* and involved in maintaining the proton motive force [25]. It was found to be over expressed upon exposure to vancomycin and cell wall damaging antibiotics, suggesting their role in resistance to cell envelope stress [26]. PepD functions to proteolytically regulate Rv2744c

levels to help maintain cell wall/cell envelope homeostasis in *M.tuberculosis*. Spot 4 (MPT51/MPB51/Rv3803c) is involved in cell wall mycoloylation. It has mycolyltransferase enzyme activity required for the biogenesis of cord factor which is necessary for maintaining the cell wall integrity [27-28]. Ag85 complex was recently reported to be an immunodominant marker for TB [29].

Spot 5, an elongation factor P/EF-P (Rv2534c), is a soluble protein that binds to the ribosome and stimulates peptide bond formation [30-32]. It is down regulated in *M.tuberculosis* grown under nutrient starvation [33]. However, we found the over expression of this protein under OFX stress which might be to enhance the protein synthesis. Spot 6 is identified as hypothetical protein (Rv2140c) of unknown function and might be playing some role in imparting resistance. Spot 7 (Rv1475c) which encodes aconitase catalyzes the reversible isomerization of citrate and isocitrate via cis-aconitate. Mycobacterial Acn participates in energy metabolism, serves as an iron-responsive element (IRE) binding protein and upregulates under high iron concentration and down regulates during nutrient starvation [34-35]. Spot 8 (Rv0440), a 60 kDa Chaperonin-2 prevents misfolding and promotes the refolding of unfolded/misfolded polypeptides generated under stress conditions. Duplicate copies of this gene suggest its importance as an energy independent chaperonin in slow metabolism efficient genome of *M.tuberculosis* [36]. Spot 9 (Rv2245) which encodes beta-ketoacyl-ACP synthase/KasA is an essential enzyme of fatty acid biosynthesis pathway in *M.tuberculosis*. Lack of KasA encoding gene has been shown to induce cell lysis of mycobacteria [37-38] and might be a promising antimycobacterial drug target.

Spot 10 is glyceraldehyde 3-phosphate dehydrogenase/GAPDH (Rv1436), an essential glycolytic enzyme, which catalyzes oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate. Florczyk et al [39] showed that GAPDH of mycobacteria is upregulated in standing culture. It may be necessary to satisfy the energy

demands of these mycobacteria during adjustment to growth under reduced oxygen tension. Boradia et al [40] reported the over-expression of GAPDH, correlated with a corresponding increase in transferrin binding and iron uptake in mycobacteria. Spot 11 is probable CoA-transferase alpha subunit (Rv3551). CoA-transferases transfer the CoA moiety from a donor (e.g. acetyl CoA) to an acceptor, (R)-2-hydroxyglutarate forming acetate [41]. In *M.tuberculosis*, its function is unknown. Spot 12 is probable short-chain dehydrogenases/reductases (Rv0148) and possess two binding domains-NAD and substrate. As the gene has been retained in the genome through selective advantage, it might play a key role in pathogenesis and immunomodulation [42]. Spot 13 (Rv2882c) is identified as ribosome recycling factor (RRF) and responsible for the release of ribosomes from mRNA at the termination of protein biosynthesis. Protein synthesis is dramatically reduced upon loss of RRF *in vivo* [43-44] and in the absence of RRF *in vitro* [45]. RRF might be of interest in developing sub cellular vaccines. It is expected to increase the efficiency of translation under stress conditions, necessary for the survival of bacilli. Spot 14 (Rv0733) encodes adenylate kinase (Adk), a ubiquitous enzyme that contributes to the homeostasis of adenine nucleotides in eukaryotic and prokaryotic cells. Adk secreted by *M.tuberculosis* is a cytotoxic factor and promote apoptosis, may be important for the initiation of infection, bacterial survival, and escape from the host immune response [46].

In the present study we observed that on performing molecular docking, OFX interacted with residues of conserved domain of proteins which might alter the functions. It is predicted that these proteins might be exhibiting increased intensities to neutralize/compensate the effect of OFX which might lead to OFX resistance.

## Conclusions

To conclude, we have identified proteins from OFX mono-resistant *M.tuberculosis* isolates uninduced and induced with OFX by 2DE-MALDI-TOF/MS and bioinformatic tools.

Fourteen protein spots showed consistently increased intensities in OFX resistant isolates induced with drug. We expect that these proteins might be contributing in conferring resistant phenotype. Bioinformatic analysis of the four proteins with unknown functions (Rv2744c, Rv2140c, Rv3551 and Rv0148) revealed the presence of conserved motifs and domains. Molecular docking showed proper interaction of OFX with these proteins and suggested that increased intensities of these molecules might be to inhibit/modulate/compensate the effect of the drug. Another major finding of the study was the overexpression of various enzymes (fructose-bisphosphate aldolase, putative CoA-transferase subunit alpha, glyceraldehyde-3-phosphate dehydrogenase, KasA, adenylate kinase and aconitate hydratase) and EF-Tu, EF-P and ribosome recycling factor involved in major metabolic processes and translation machinery, that could be crucial for the initial survival of the cells under prolonged adverse conditions. These preliminary findings need further validation and subsequent exploitation for the development of novel diagnostic markers or newer therapeutic agents targeting directly to gene/protein responsible for drug resistance and this could open new windows of hope for mankind to fight against the deadly disease of tuberculosis, especially multidrug resistant.

**Conflict of interest:** There is no conflict of interest among the authors.

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**Figure captions**

**Fig.1:** Composite images of 2D gel profiles M.tuberculosis isolates generated by PDQuest software: (A) Susceptible (B) Ofloxacin mono-resistant (C) Ofloxacin mono-resistant Induced with ofloxacin. Spots indicated by arrow were excised and analyzed by MS.

**Fig.2:** Phylogenetic tree of hypothetical proteins: A-2140C, B-Rv2744c, C-Rv3551 and D-Rv0148.

**Fig. 3:** 3D model of hypothetical proteins showing docking with OFX: A, B, C and D shows molecular docking of Rv2744c, Rv2140c, Rv0148 and Rv3551 with drug molecule OFX (blue, green and red color) respectively, yellow color shows interacting residues of conserved domain which create the cavity for drug. E, F, G, H, I, J, K and L shows OFX (blue color) docking with Rv2534c, Rv0685, Rv3803c, Rv2882c, Rv2245, Rv1436, Rv0363c and Rv0440 respectively, Yellow color shows interacting residues of conserved domains.

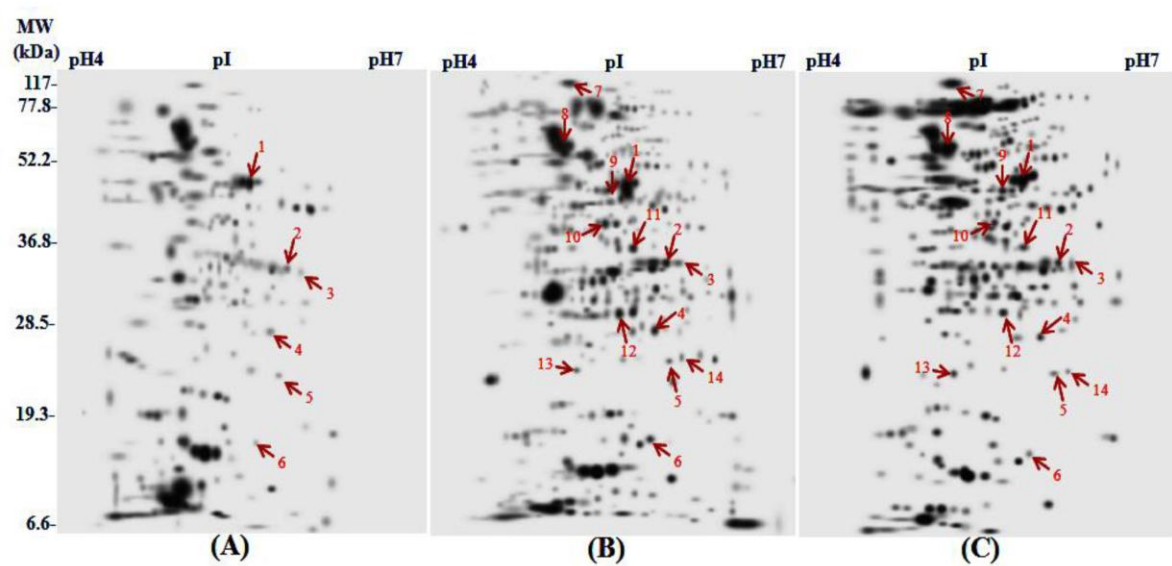


Figure 1

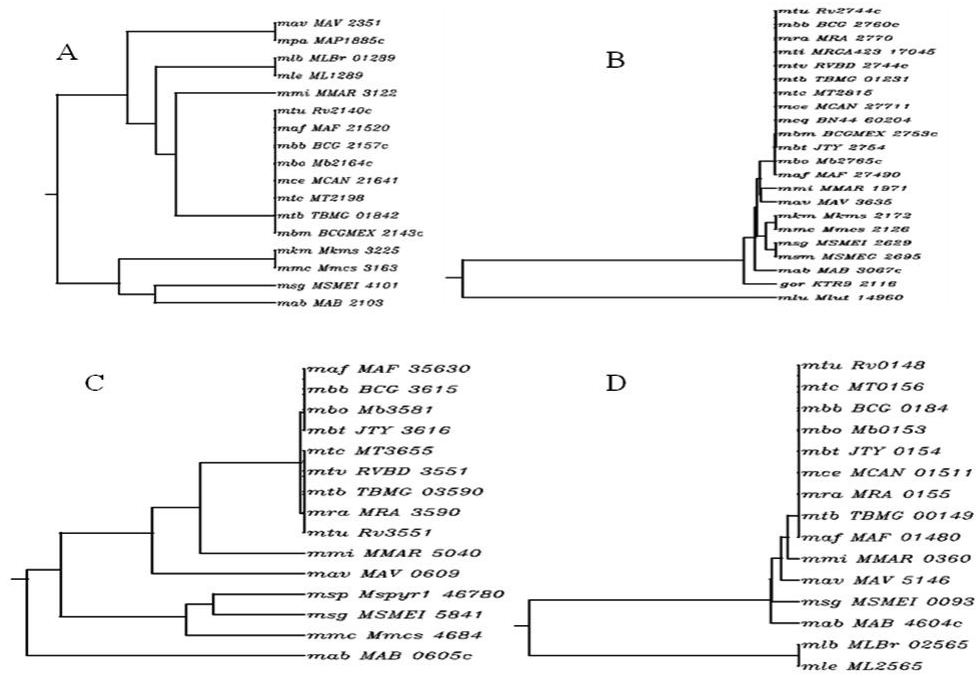


Figure 2



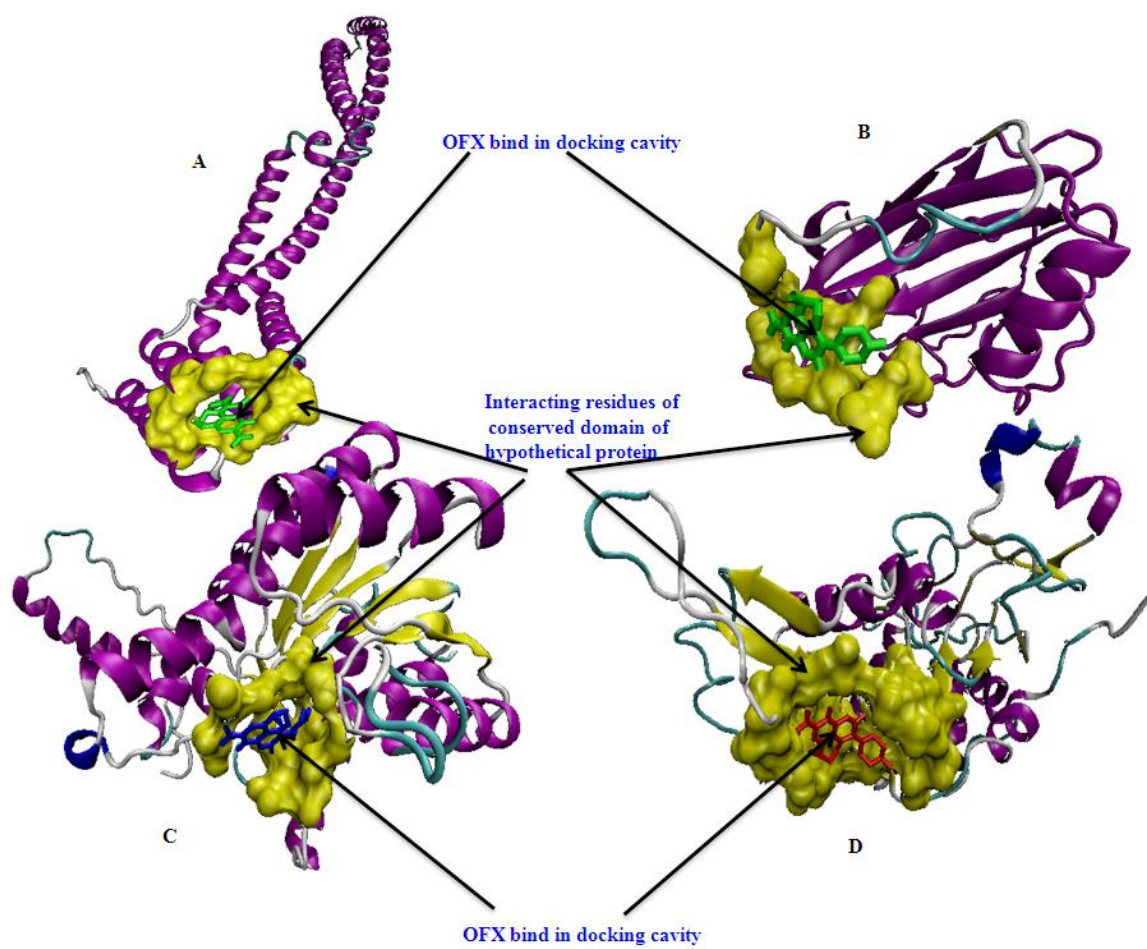


Figure 3

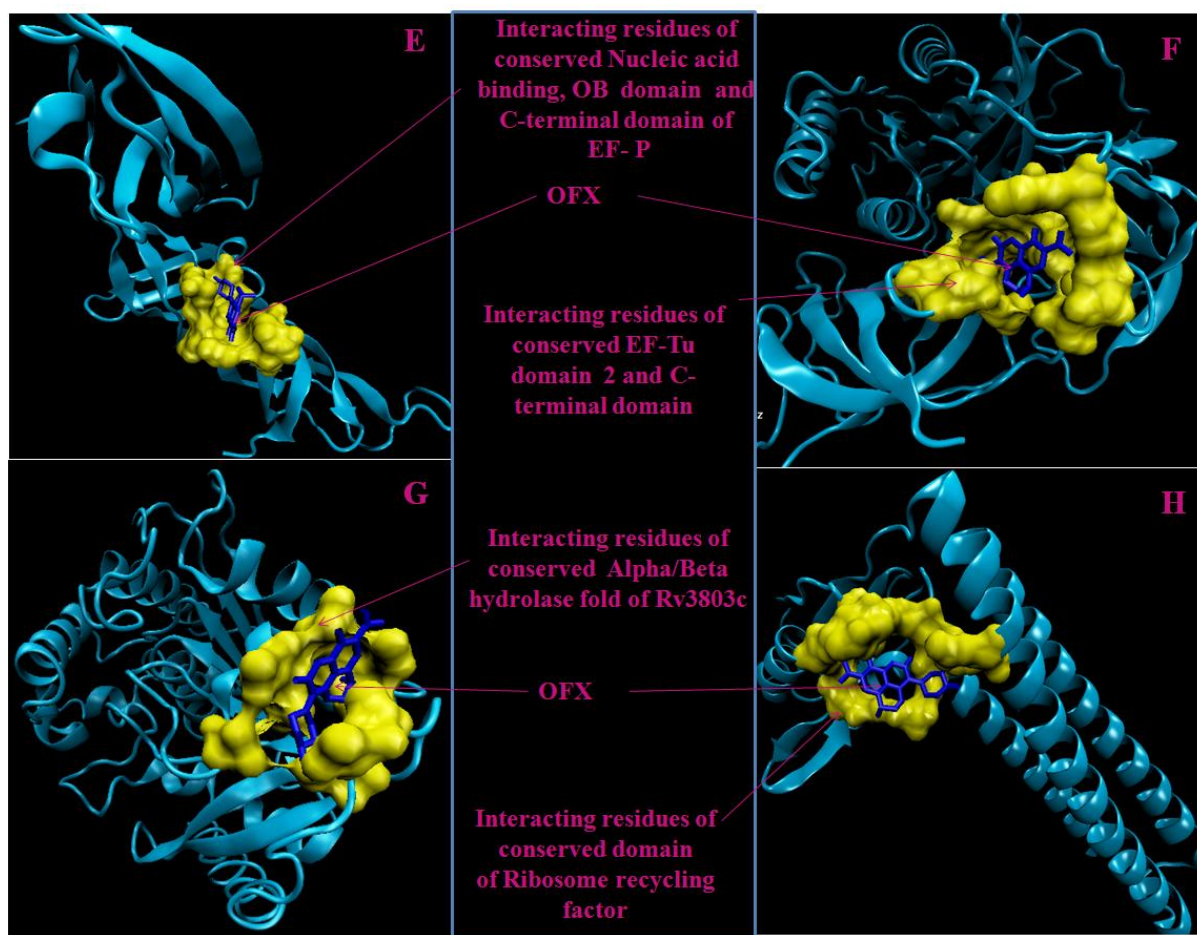


Figure 3 cont.

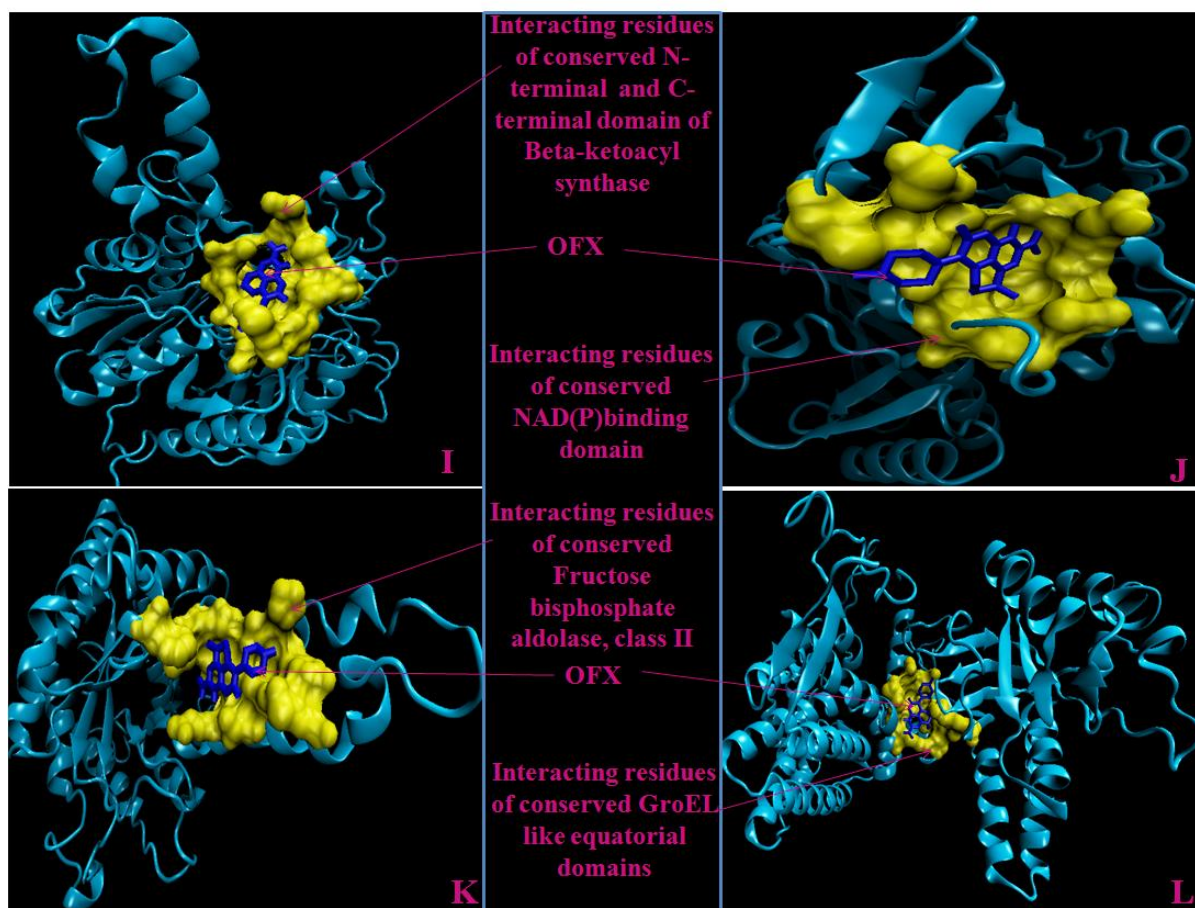


Figure 3 cont.

**Table 1: Details of overexpressed proteins identified by MS**

Spot no.	Accession number	Protein identified	Mascot score	Nominal mass (da)	PI	Sequence coverage %	ORF number	Fold change Resistant	Fold change Resistant induced	Functional category
M1	P0A558 (EFTU_MYCTU)	Elongation factor Tu	193	43566	5.28	60%	Rv0685	2.3 (+)	2.5 (+)	Information pathways
M2	P67475 (ALF_MYCTU)	Fructose-bisphosphate aldolase	120	36750	5.49	44%	Rv0363c	1.8 (+)	1.7 (+)	Intermediary metabolism and respiration
M3	P0C5C4 (35KD_MYCTU)	35 kDa protein	65	29240	5.71	22%	Rv2744c	1.7 (+)	1.9 (+)	Conserved hypotheticals
M4	P0A4V6 (MPT51_MYCTU)	MPT51/MPB51/fbpC 1 antigen	54	31069	6.13	22%	Rv3803c	1.6 (+)	1.8 (+)	Lipid metabolism
M5	P64034 (EFP_MYCTU)	Elongation factor P	50	20395	5.54	41%	Rv2534c	1.5 (+)	1.6 (+)	Information pathways
M6	P67226 (Y2140_MYCTU)	Conserved hypothetical protein TB18.6	91	18622	5.41	30%	Rv2140c	1.9 (+)	1.7 (+)	Conserved hypotheticals
M7	O53166 (O53166_MYCTU)	Aconitate hydratase	123	102385	4.95	19%	Rv1475c	1.8 (+)	2.3 (+)	Intermediary metabolism and respiration
M8	P0A520 (Y2623_MYCTU)	60 kDa chaperonin 2,	85	56692	4.85	27%	Rv0440	1.5 (+)	1.9 (+)	Virulence, detoxification, adaptation
M 9	P63454 (FAB1_MYCTU)	3-oxoacyl-[acyl-carrier-protein] synthase 1	42	43389	5.11	21%	Rv2245	1.5 (+)	1.7 (+)	Lipid metabolism
M 10	P64178 (G3P_MYCTU)	Glyceraldehyde-3-phosphate dehydrogenase	63	35934	5.19	21%	Rv1436	1.6 (+)	1.5 (+)	Intermediary metabolism and respiration
M11	P71850 (Y3551_MYCTU)	Putative CoA-transferase subunit alpha	52	31703	5.64	26%	Rv3551	2.1 (+)	1.9 (+)	Intermediary metabolism and respiration
M 12	P96825 (P96825_MYCTU)	Hypothetical protein	127	29760	5.26	58%	Rv0148	2.2 (+)	2.3 (+)	Intermediary metabolism and respiration
M 13	P66734 (RRF_MYCTU)	Ribosome-recycling factor	113	20815	5.71	58%	Rv2882c	1.8 (+)	1.9 (+)	Information pathways
M 14	P69440 (KAD_MYCTU)	Adenylate kinase	152	20113	5.02	58%	Rv0733	2.2 (+)	1.9 (+)	Intermediary metabolism and respiration

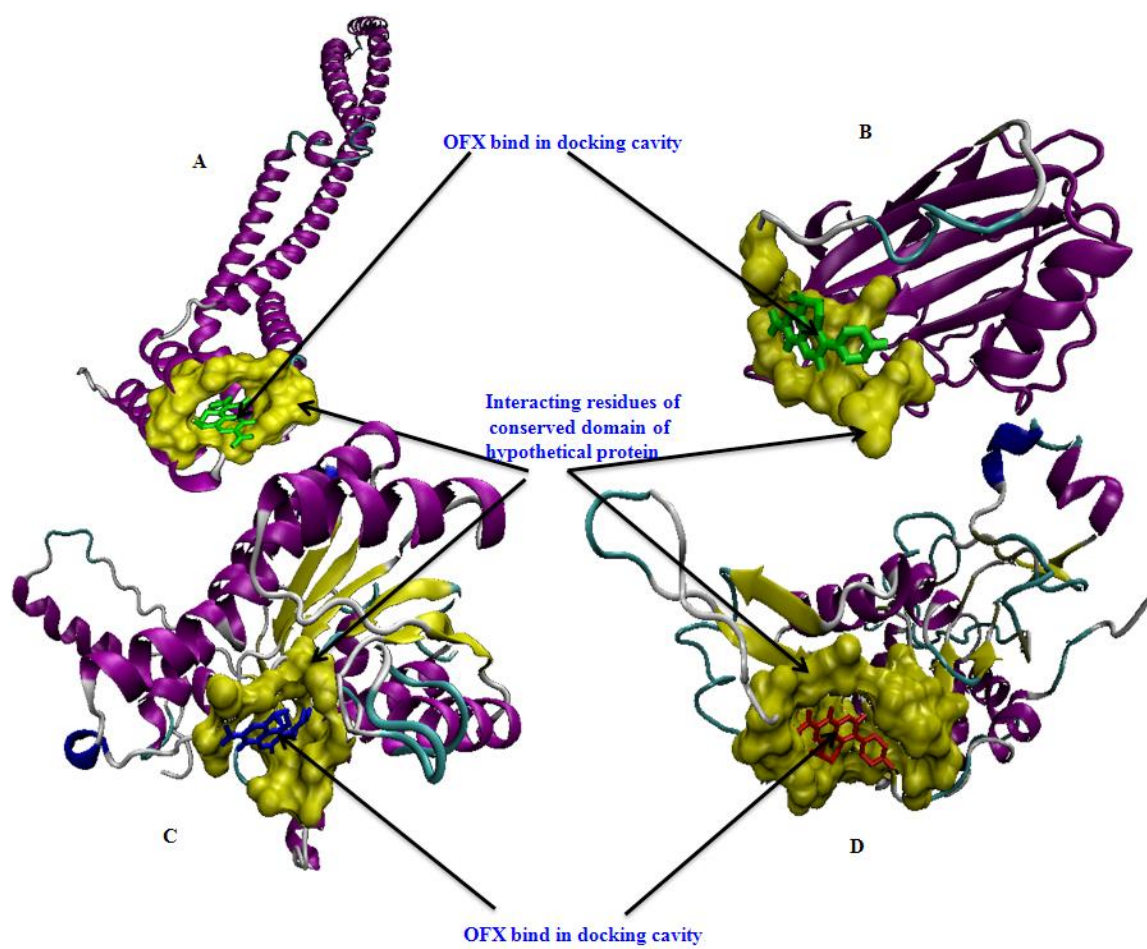
**Table 2: Multiple sequence alignment of the identified protein with defined set of organisms**

	<b>mbo</b> <i>(M. bovis)</i>	<b>Maf</b> <i>(M. africanum)</i>	<b>Mav</b> <i>(M. avium)</i>	<b>Mle</b> <i>(M. leprae)</i>	<b>Has</b> <i>(Homo. sapiens)</i>
<b>Rv2744c</b>	99.6%	99.3%	88.0%	26.6%	24.6%
<b>Rv2140c</b>	100%	100%	83.0%	86.3%	26.4%
<b>Rv3551</b>	99.7%	99.7%	87.2%	25.2%	24.8%
<b>Rv0148</b>	100%	100%	87.6%	33.5%	50.0%

**Table 3: 3D modelling and docking parameters used for bioinformatic analysis of hypothetical proteins**

ORF No.	TM-score	RMSD value (Å)	Drug	Global Energy	Attractive Vander wall forces	Repulsive Vander wall forces	ACE	Interacting amino acids	Remarks
<b>Rv2744c</b>	0.30±0.10	15.3±3.4Å	OFX	-39.60	-17.54	1.81	-11.41	23, 24, 25, 26, 27, 30, 243, 245, 249, 253	OFX binds properly within the region of conserved domain
<b>Rv2140c</b>	0.97±0.05	1.7±1.4Å	OFX	-35.40	-16.03	6.01	-11.85	1, 69, 70, 71, 72, 74, 75, 127, 128, 129	OFX interactet with USP domain of hypothetical protein
<b>Rv0148</b>	0.81±0.09	4.6±3.0Å	OFX	-35.24	-16.79	5.46	-10.25	13, 16, 18, 99, 101, 102, 103, 200, 201,202	OFX binds the amino acid residues of conserved domain
<b>Rv3551</b>	0.87±0.07	3.8±2.6Å	OFX	-36.41	-15.21	5.63	-13.46	18, 19, 20, 48, 68, 70, 98, 137, 138, 139, 140, 159, 159, 160, 161,163	OFX binds the amino acid residues of conserved domain
<b>Rv2534c</b>	0.86±0.07	3.1±2.2Å	OFX	-39.63	-17.34	3.03	-11.69	98, 101, 123, 124, 125, 126, 127, 175, 176, 177	OFX binds conserved Nucleic acid binding, OB and C-terminal domain of Protein
<b>Rv0685</b>	0.99±0.04	2.6±1.9Å	OFX	-39.68	-19.55	3.79	-8.98	210, 211, 213, 215, 234, 300, 301, 335, 336, 337, 338, 370, 372, 375	OFX interactet with conserved EF-Tu domain- 2 and C-terminal domain
<b>Rv3803c</b>	0.69±0.12	6.5±4.0Å	OFX	-38.77	-16.91	3.50	-11.96	30, 31, 36, 74, 78, 79, 82, 83, 84, 85	OFX binds to conserved Alpha/Beta hydrolase fold of Rv3803c
<b>Rv2882c</b>	0.78±0.10	4.2±2.8Å	OFX	-21.19	-14.71	3.75	-2.62	26, 27, 28, 39, 40, 85, 86, 87, 116	OFX Interact with residues of conserved domain of the RRF
<b>Rv2245</b>	0.99±0.04	2.9±2.1Å	OFX	-37.48	-15.07	4.59	-13.15	115, 116, 170, 171, 205, 206, 209, 210, 213, 277, 278, 347, 404, 405	OFX interact with conserved N and C-terminal domain of Beta ketoacyl synthase
<b>Rv1436</b>	0.99±0.04	2.4±1.8Å	OFX	-42.94	-16.99	2.09	-12.58	18, 21, 22, 24, 29, 30, 32, 33, 34, 61, 73, 74	OFX binds to residues of conserved NAD(P) binding domain
<b>Rv0363c</b>	0.95±0.05	3.1±2.2Å	OFX	-34.91	-18.65	2.32	-6.43	29, 30, 32, 33, 59, 280, 283, 284, 287, 291, 313, 316, 317, 324	OFX bind to conserved Fructose bisphosphate aldolase, class II
<b>Rv0440</b>	0.76±0.10	6.7±4.0Å	OFX	-36.63	-16.64	1.80	-10.08	171, 172, 173, 175, 192, 395, 398, 399, 402	OFX binds to residues of conserved GroEL like equatorial domain





Graphical abstract

## Highlights

- Proteins showed increased intensity in *M.tuberculosis* of OFX resistant (Six) and OFX resistant induced with OFX (Fourteen).
- Out of them four proteins (Rv2744c, Rv2140c, Rv3551 and Rv0148) were with unknown functions.
- Molecular docking revealed that OFX interacts with conserved motifs of these hypothetical proteins.
- We hypothesized that these proteins might be neutralizing the effect of OFX & contributing to OFX resistance.

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