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# Biophysical and biochemical characterization of Rv3405c, a tetracycline repressor protein from *Mycobacterium tuberculosis*

Akilandeswari Gopalan [a], Raghu Bhagavat [b], Nagasuma Chandra [b], Savithri Handanahal Subbarao [b], Alamelu Raja [a], Ramalingam Bethunaickan [a, *]

[a] Department of Immunology, National Institute for Research in Tuberculosis, Chennai, India
[b] Department of Biochemistry, Indian Institute of Science, Bangalore, India

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ABSTRACT

*Mycobacterium tuberculosis*, the causative agent of tuberculosis disease, is one among the deadliest pathogens in the world. Due to long treatment regimen, HIV co-infection, persistence of bacilli in latent form and development of XDR and TDR strains of *Mtb*, tuberculosis has posed serious concerns for managing the disease, and calls for discovery of new drugs and drug targets. Using a computational pipeline involving analysis of the structural models of the Mtb proteome and an analysis of the ATPome, followed by a series of filters to identify druggable proteins, solubility and length of the protein, several candidate proteins were shortlisted. From this, Rv3405c, a tetR family of DNA binding protein involved in antibiotic resistance, was identified as one of the good drug targets. Rv3405c binds to the upstream non-coding region of Rv3406 and causes repression of Rv3406 activity there by affecting the downstream processes involved in antibiotic resistance was further characterized.

The Rv3405c gene was cloned; the gene product was over-expressed in *E. coli* and purified by Ni NTA chromatography. DNA binding studies by EMSA showed that the recombinant Rv3405c protein binds to the DNA sequence corresponding to the promoter region of Rv3406 and upon addition of tetracycline, the DNA binding activity was lost. β-galactosidase reporter assay in *E. coli* using both wild type and a DNA binding defective mutant protein indeed proved that Rv3405c acts as a repressor.



## 1. Introduction

Tuberculosis (TB) is one of the ancient and chronic infectious diseases, continues to pose a persistent threat to humans and animals. *Mycobacterium tuberculosis* (Mtb)- the causative agent of TB, infects about two-thirds of the world population. World Health Organisation (WHO) reports that Mtb causes nearly two million deaths and 10.3 million new cases annually which place the disease among the top three fatal infections [1]. Being an intracellular pathogen, Mtb has the ability to dwell inside the alveolar macrophages evading the immune responses of the host. This results in establishing a proper niche in macrophages in the form of a closed structure called a granuloma. Depending upon the immune state of the host, the infection may proceed to an active disease state (active TB) or remain dormant without showing any symptoms (latent TB). This shows the pathogen's plasticity in gene expression which is mostly regulated by transcription factors. Though the current treatment regimen is being used for more than 40 years [2,3], its safety and efficiency rate is high, the long duration of treatment and adverse effects are still major challenges in TB management. The emergence of drug resistant forms of TB especially the XDR and TDR-TB cases has made the problem even worse [4,5]. Identification of new biomarkers for diagnosis, vaccine candidates, drug targets and drugs to combat the infection in order to achieve the “END TB 2030” vision is a primary aim of research in this area.

Tetracyclines (TCN) come under the broad spectrum of antibiotics which are proposed to inhibit polypeptide elongation during protein synthesis by binding to ribosomes [6,7]. Currently, the application of broad spectrum antibiotics for treating infectious diseases has become less due to the emergence of wide-spread resistance mechanisms [8]. The resistance is achieved by dynamic changes that occur inside the pathogens leading to an alteration in gene expression patterns and an exchange of gene segments

* Corresponding author. Department of Immunology, National Institute for Research in Tuberculosis (ICMR), No.1. Mayor Satyamoorthy Road, Chetpet, Chennai 600 031, India.
*E-mail address:* ramalingam.b@nirt.res.in (R. Bethunaickan).

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through transposons or plasmids. This adaptation to the environmental changes aids in the easy survival of the organism inside a host.

Tetracyline resistance is common among Gram negative prokaryotes where the drug is actively transported out of the cell. The resistance mechanism is based on the expression of tetA gene which indeed is regulated by the Tet repressor (tetR). TetR family of transcriptional regulators (TFTRs) function as homodimers which contain a conserved N-terminal helix-turn-helix (HTH) motif which acts as a DNA binding domain and a variable regulatory domain in its C-terminus which is involved in ligand binding and dimerization [9]. The binding of the ligand (TCN) causes conformational changes in the N-terminal region of the protein and thus the protein loses its affinity for DNA [10]. Though TFTRs are involved in antibiotic effluxing, they do engage in different functions like activator or repressor of gene activity, peptide ligand binding and regulation of enzymatic activity. Analysis by *Balhana* et al. [9] showed that in mycobacteria, majority of the TFTRs aid in the regulation of enzymes involved in energy and cellular metabolism which indicates that these proteins are involved in cellular adaptation. Thus, targeting TFTRs may pave the way in disrupting the adaptation strategy of the pathogen and aid in its clearance inside the host.

In this paper we have characterized Rv3405c; a member of TFTRs. Rv3405c has a typical HTH motif in its N terminal domain and a C-terminal ligand binding domain. Rv3405c protein is divergently oriented to its adjacent gene Rv3406 [9], an iron and α-ketoglutarate dependent sulphur ester dioxygenase, which is involved in scavenging sulphur from medium-chain alkyl sulphates particularly 2-ethylhexyl sulphate (2-EHS) [11]. Binding of Rv3405c protein to the palindromic inverted repeat region present upstream of Rv3406 causes repression of Rv3406 activity thereby disruption of sulphur scavenging mechanism. As tetR proteins, the most abundant transcription factors in mycobacteria, play an important role in antibiotic resistance and regulation of efflux mechanism [12–14], studying the biophysical and biochemical function of one such protein may give clue about its role in pathogenesis and survival inside host.

## 2. Materials and methods

### 2.1. Bioinformatics analyses for selection of target

TargetTB, a multi-level target identification pipeline in Mtb, which was carried out previously, was used to list out all the proteins that posses ideal drug target characteristics [15]. From another study carried out by us, a genome-scale analysis to identify ATP binding proteins in Mtb resulted in 1768 proteins [16], which were used as a second filter to choose ATP-binding proteins for further characterization.

For binding site scan against drug binding proteins, 348 drugs complexed to their macromolecular receptors in the Protein Data Bank [17] were considered. A set of all known binding sites for 348 drugs was prepared from a list of all proteins complexed with drug molecules, available from PDB. PocketMatch [18], an in-house site-matching algorithm that captures shape and chemistry of the pocket, was used for comparing query Mtb binding pockets with drug binding sites prepared from PDB. Mtb pocketome [19] was used to obtain all the putative pockets for Mtb.

### 2.2. Cloning, over-expression and purification

The gene coding Rv3405c was amplified with gene specific primers from H37Rv genomic DNA and cloned into pET22b vector digested with *NdeI* and *HindIII* by ligation independent cloning. Positive clones were verified by colony PCR, restriction digestion and commercial sequencing (Eurofins, India). The pET22b-Rv3405c constructs having C terminal hexa histidine tag were transformed into BL21 (DE3) *E. coli* expression strain. Single colony of transformed cells was picked up and inoculated in LB broth with ampicillin (100 μg/ml) overnight at 37 °C. 1% of overnight primary culture was inoculated in ampicillin selective LB broth, grown for 3–4 h till the OD reached 0.6–0.7 and induced with 1 mM IPTG for 4 h at 37 °C. The cells were pelleted and lysed by sonication and the supernatant was collected after centrifuging the sample at 12,000 rpm for 15 min at 4 °C. The crude lysate was mixed with 1 ml of fresh Ni-NTA agarose (Invitrogen) and allowed for binding for 1–2 h at 4 °C in an end-to-end rotor. Purification of the recombinant Rv3405c protein (rRv3405c) was done as described in Ref. [20] excluding the endotoxin removal step. The final eluted fractions were analysed by 12% SDS-PAGE. The fractions containing pure recombinant protein were pooled and dialysed against 10 mM Tris HCl pH8.0, 100 mM NaCl overnight at 4 °C and used for further biophysical and biochemical assays.

### 2.3. Construction of triple mutant of Rv3405c

Another construct having mutations (triple mutant) in the DNA binding region of the protein (H52A-R57A-K62A) was carried out using PCR based site-directed mutagenesis (SDM). Primers were synthesized by including the region to be mutated in the centre and flanking regions that match the plasmid DNA and overlapping PCR (gradient) was done with the recombinant plasmid of Rv3405c as template. 1 μl of Dpn1 was added to the PCR product directly and incubated at 37 °C for 2 h for digestion of methylated (parent) DNA. Following incubation, the reaction mixture was transformed into chemically competent DH5α cells and the transformants were inoculated for plasmid isolation. The recombinant plasmids were then checked for the presence of mutation by DNA sequencing commercially (Eurofins, Bangalore).

### 2.4. Circular dichroism (CD) spectroscopy

The binding ability of tetracycline (TCN) and metal ions to the purified rRv3405c protein was checked using CD. All the reagents were prepared freshly before every experiment. 0.1 mg/ml of rRv3405c protein was incubated with increasing molarity of TCN or ampicillin at 4 °C for 20 min and then CD spectrum was recorded at a scanning speed of 100 nm/min with 1 nm wavelength steps from 200 to 290 nm at 25 °C with three accumulations. CD spectrum was monitored using a JASCO J175 spectropolarimeter. 10 mM Tris pH 8.0 with respective antibiotic was used as blank. The secondary structure of the native protein and protein incubated with various ligands was calculated using K2D2 structural analysis tool. The extent of structural change of the protein upon binding of ligands compared to that of the native protein was assessed.

### 2.5. Fluorescence spectroscopy

For the measurement of TCN binding, increasing concentration of TCN (10 μM-100 μM) was added to 0.1 mg/ml of rRv3405c protein and emission fluorescence was recorded between 300 nm and 400 nm upon excitation at 280 nm. The scanning was performed at a speed of 100 nm/min. The emission and excitation slits used were 2.5 and 5 nm respectively. The binding constant and stoichiometry of tetracycline binding to the protein were calculated with the fluorescence values obtained at 340 nm. The data were fitted into double logarithm plot with the equation: