DU Journal of Undergraduate Research and Innovation

Building a repository of potential drug targets in M*ycobacterium tuberculosis* by crowdsourcing

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ABSTRACT

The objective of this study was to i) clone and express a selected number of genes from *Mycobacterium tuberculosis* H37Rv and purify the recombinant proteins, which shall further be tested for their potential as drug targets, ii) to initiate undergraduate students in to research. The list of genes was prepared by Open Source Drug Discovery (OSDD) team. These genes are known to be essential for the pathogen survival, growth, persistence and infection and hence have potential to be tested as target for the new drugs against tuberculosis. Genes were cloned and expressed in suitable vectors followed by purification of the recombinant proteins by affinity chromatography and refolding of the proteins isolated as inclusion bodies was done by urea denaturation. So far, nineteen genes have been cloned and expressed and fifteen proteins have been purified successfully.

Keywords: Cloning, Gene expression, *Mycobacterium tuberculosis*, Drug targets, Crowd Sourcing, Undergraduates

INTRODUCTION

Mycobacterium tuberculosis (Mtb) is the causative pathogen of tuberculosis, a major public health threat worldwide. According to World Health Organization (WHO), tuberculosis is the second leading cause of death among infectious diseases (1, 2). Co-infection with human immunodeficiency virus in TB patients and the emergence of extensively drug-resistant Mtb strains make treatment of the diseases a major

challenge (3). Hence, there is an urgent requirement to discover new drugs against Mtb. With the release of whole genome sequence of Mtb and data on annotation of the genome available, new drug targets can be identified and validated. Open Source Drug Discovery (OSDD) is a CSIR (Council of Science and Industrial Research) Team India Consortium with Global Partnership with a vision to provide affordable healthcare to the developing world. OSDD initiative is taken to provide a platform where the scientists, students, faculty members can collaborate & collectively endeavor towards the conquest of tuberculosis by innovative and robust drug discovery programs.

Through computational approach, the OSDD team made an effort to identify several proteins that play important role in survival, growth and pathogenicity of Mtb and hence could act as potential drug targets (4). The next step in the drug discovery program is the experimental validation of significance of these targets. This study was undertaken for meeting two major objectives: i) To isolate and purify a set of identified potential drug target proteins from *Mtb* and to make them available to OSDD community and other scientists working on Mtb biology and ii) To train undergraduate students in basic molecular techniques required in cloning, expression and purification of recombinant proteins. The purified target proteins are available for further characterization, for development of enzyme assays and screening of inhibitors. The target proteins that were cloned and purified are Sigma factors (B, C, D, E C, F), transcriptional regulators such as WhiB3, PhoP, DosR, repressor proteins (IdeR, LexA), heat shock protein (HspX) and enzymes involved in cell division (FtsZ) and in various metabolic pathways such as Glyoxalate pathway (Isocitrate lyase), Propionyl CoA metabolism (Methyl citrate synthase).

METHODOLOGY

To ensure uniformity in methods and quality of results, standard operating procedures (SOPs) were developed (Fig. I). However, each protein being unique, further optimization was required.

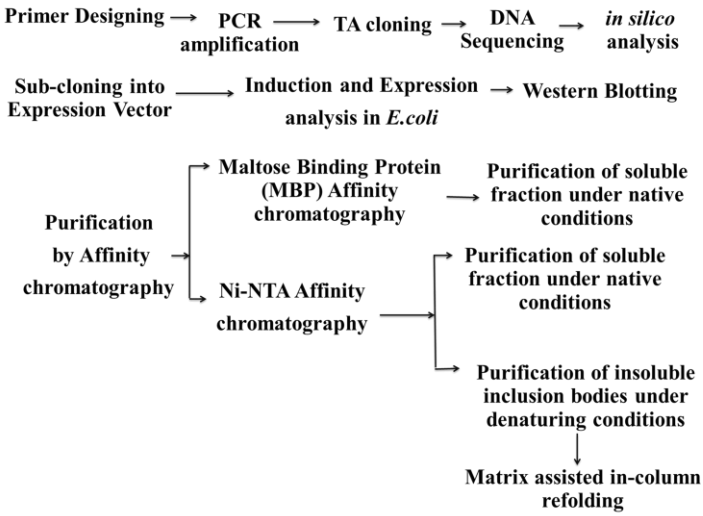

Fig-I Schematic representation of standard operating procedures used in the study

Bacterial strains, Plasmids and Chemicals

Escherichia coli DH5α was used for general cloning procedures and *Escherichia coli* BL21 (DE3) was used for expression studies. T4 DNA ligase, Restriction enzymes and Amylose resin were obtained from New England Biolabs (USA). Synthesis of gene-specific oligonucleotide primers was done by Sigma, Inc. (USA). Plasmid pET28a-c was kindly gifted by Dr.Ramachandran (IGIB, New Delhi), pMALc2x was kindly gifted by Dr.Vinay K. Nandikoori (National Institute of Immunology, New Delhi) and pGEM-T easy vector was purchased from Promega (USA). Ni-NTA agarose and other PCR purification/ Plasmid isolation kits were obtained from Qiagen (Germany). Phenylmethanesulfonylfluoride (PMSF), Media for growing E.coli and all analytical grade chemicals were purchased from HiMedia laboratories, India. Standard operating protocols were developed based on Recombinant DNA techniques described elsewhere (5).

Designing of gene-specific primers

A set of 20 genes was selected for the study (table I). Sequence of the genes was retrieved from Tuberculsit (http://genolist.pasteur.fr/TubercuList/genome.cgi), a server for H37Rv genome. The primers were synthesized in a way that the flanking regions of the coding region were carefully altered by making two or three base substitutions to create restriction sites available in the multiple cloning site (MCS) of pET vectors. Suitable reading frame of the expression vector pET28a-c was considered while designing the primers. The melting temperatures (Tm) of the primers were adjusted such that the difference between the Tm of the forward and reverse primers did not exceed 5ºC. Further primer sequence was analysed by "Oligo analyzer" tool (http://eu.idtdna.com/calc/analyzer) for Self dimerization, Loop formation and Primer dimer formation.

Table-I List of genes identified as potential drug targets in Mtb

Rv ID/Gene Name	Function	Size of the gene (bp)
Rv3574 (TetR)	Tetracycline transcriptional regulator	612
Rv3414c (SigD)	Sigma factor D	664
Rv2069 (SigC)	Sigma factor C	569
Rv0014c (pknB)	Protein kinase B	1898
Rv0757 (PhoP)	Transcriptional regulatory protein	760
Rv0182c (SigG)	Sigma factor G	1140
Rv3286c (SigF)	Sigma factor F	841
Rv2711(IdeR)	Iron dependent repressor	704
Rv3133c (DosR)	Transcriptional regulatory protein	665
Rv3223c (SigH)	Sigma factor H	691
Rv2031c (HspX)	Heat shock protein	470
Rv2720 (LexA)	Repressor lexA	722
Rv2710 (SigB)	Sigma factor B	998
Rv1354c (Hypothetical)	Unknown function	1872
Rv0467 (Icl)	Isocitrate lyase	1297
Rv3130 (tgs1)	Triglycerate synthase	1581
Rv1131 (Mcs)	Methylcitrate synthase	1182
Rv2150c (FtsZ)	Cell division protein FtsZ	1158

Rv1221 (SigE)	Sigma factor E	795
Rv3416 (WhiB3)	WhiB-like regulatory protein	321

PCR (Polymerase chain reaction) amplification of genes

Primers designed specific to gene of interest were synthesized commercially and their sequence are listed in table II. The genes were amplified using genomic DNA of Mtb H37Rv as template. Since Mtb DNA has high percentage of GC content, DMSO were added in the PCR reaction mixture to lower the melting temperature (Tm). The PCR for the genes were carried out using Phusion High Fidelity Polymerase and the program includes initial denaturation at 95ºC, 5 mins, 30 cycles of denaturation at 95ºC, 1 min, annealing for 1min, extension at 72ºC, 1min, and a final extension at 72ºC, 10 mins. PCR products were analysed by Agarose gel electrophoresis. Preparative gel electrophoresis was done to purify PCR products from agarose gel using Qiagen Gel Purification Kit. The purified products were quantified using Alpha imager gel documentation system.

Table-II Primers designed in the study

Name	Sequence
Rv3574 (TetR)	F - 5´-GGGATCCTGAAAGTGGCGGTA-3´ R - 5´-CGTAAGCTTTCTAGGCGCTGTC-3´
Rv3414c (SigD)	F - 5´-GGATCCGCCGTTATGGTCGAT-3´ R - 5´-AAGCTTGTCCTTCAGCCGCTGAAG-3´
Rv2069 (SigC)	F - 5´-GGATCCTGCCCATGACCGCGA-3´ R - 5´-AAGCTTCTAGCCGGTGAGGTCGTC-3´
Rv0014c (pknB)	F - 5´-GGATCCGAGATAGCGCAATGAC-3´ R - 5´-AAGCTTCTACTGGCCGAACCTCAG-3´
Rv0757 (PhoP)	F - 5´-GTGGATCCAATGCGGAAAGG-3´ R - 5´-CCAAGCTTCATCGAGGCTC-3´
Rv0182c (SigG)	F - 5´-GGATCCGAGATAGCGCAATGAC-3´ R - 5´-AAGCTTCTACTGGCCGAACCTCAG-3´
Rv3286c (SigF)	F - 5´-CGGGATCCAGCAGGTGACG-3´ R - 5´-GTCGACACCCAGGGCACGGTG-3’
Rv2711(IdeR)	F - 5´-GAGGATCCTGAATGAACGAG-3´ R - 5´-GTGAAGCTTAGCTCAGACTTTCT-3´
Rv3133c (DosR)	F - 5´-TGGATCCCTGGTGGTAAAGG-3´ R - 5´-AAGCTTGTCATGGTCCATCACCG-3´
Rv3223c (SigH)	F - 5´-GAAGGGATCCAGTTAATCAAG-3´ R - 5´-AAGCTTGACACCGGGCTGCTC-3´
Rv2031c (HspX)	F - 5´-AGGATCCATCAAATGGCCACC-3´ R - 5´-AAGCTTAGTGGTCAGTTGGTGGAC-3´
Rv2720 (LexA)	F - 5´-CATGAATTCCCATGAACGACAG-3´ R - 5´-AAGCTTCATCAGACCTTGCGGATC-3
Rv2710 (SigB)	F - 5´-GGGAATTCGCTATGGCCGA-3´ R - 5´-GCTAAGCTTGTCCAGCTTCA-3´
Rv1354c (Hypothetical)	F - 5´-GGATCCGAGATGTGCAACGAC -3´ R - 5´-AAGCTTCCGTCGTTGACGCTTG-3´
Rv0467 (Icl)	F - 5´-GGATCCGTCTATGTCTGTCGTC-3´ R - 5´-AAGCTTCTAGTGGAACTGGCCCTC -3´
Rv3130 (tgs1)	F - 5´-GGAATTCGCTGACCATGAATC-3´ R - 5´-AAGCTTCGTCACACAACCAGC-3´
Rv1131 (Mcs)	F - 5'-ATTCCACCAGGATCCTTTCGATG -3' R - 5'-GAGAAAGCTTATGGCCCATAAGAG-3'
Rv2150c (FtsZ)	F - 5´-GGGATCCGAACGATGACC-3´ R - 5´-AAGCTTTCAGCGGCGCATG-3´

Rv1221 (SigE)	F - 5´-GGGAATTCCCATGGAACTC-3´ R - 5´-CGTCGACTAGTTCAGCGAACT-3’
Rv3416 (WhiB3)	F - 5´-AGGAATTCAGCAATGCCAC-3´ R - 5´-AAGCTTTTAAGCTGTGCGGCGGAT-3´

Cloning in pGEM-T easy vector

The PCR products were cloned using the pGEM-T easy vector system. The amplified PCR products were A-Tailed for pGEM-T cloning and then ligated to pGEM-T easy vector (Fig. IIa). To calculate the appropriate amount of PCR product (insert) to be included in the ligation reaction, following formula was used, as suggested in the Promega pGEM-T easy vector manual:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{Size of the vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

The ligated PCR products were then transformed into *E.coli* DH5α strain using the method described by Sambrook et al (5).

Blue/White Screening for recombinants & Colony PCR of the recombinants

Blue white screening (on agar plates) containing 100µg/ml of X-gal and 0.5mM of IPTG was done to screen positive colonies of the recombinant pGEM-T clones. Colony PCR using M13 primers was carried out for screening white colonies. The white colonies were patched on agar plate containing 100µg/ml ampicillin and few cells from the patch were scraped and added to the tube containing the PCR reaction mixture. The PCR was carried out as mentioned earlier.

Isolation of the recombinant plasmids & Restriction analysis of positive clones

Recombinant plasmids were isolated using alkaline lysis method (6). The isolated plasmids were then subjected to restriction digestion by respective enzymes whose sites were included in the primer sequence. Positive clones showing desired gene product were then isolated using Qiagen plasmid isolation kit and their DNA Sequence was verified commercially.

Cloning and expression in pET28a-c and in pMALc2x vectors

Sequence confirmed inserts were re-cloned cloned into pET28a-c vectors (Fig. IIb). pET vectors contain N-terminal 6xHis-Tag and facilitates purification by Nickel affinity chromatography. The recombinant plasmids containing the genes were used to transform *E.coli* BL21 (DE3) strain for expression studies where 1mM IPTG was used as inducer. Induction was carried out at 0.6 OD_{600} at 37^{o}C for three hours and analyzed by SDS-PAGE (12%) (7). The genes expressed as insoluble inclusion bodies were then cloned in another expression vector pMALc2x (Fig. IIc), which contains Maltose Binding Protein (MBP) tag and facilitates purification by Amylose affinity chromatography for soluble expression.

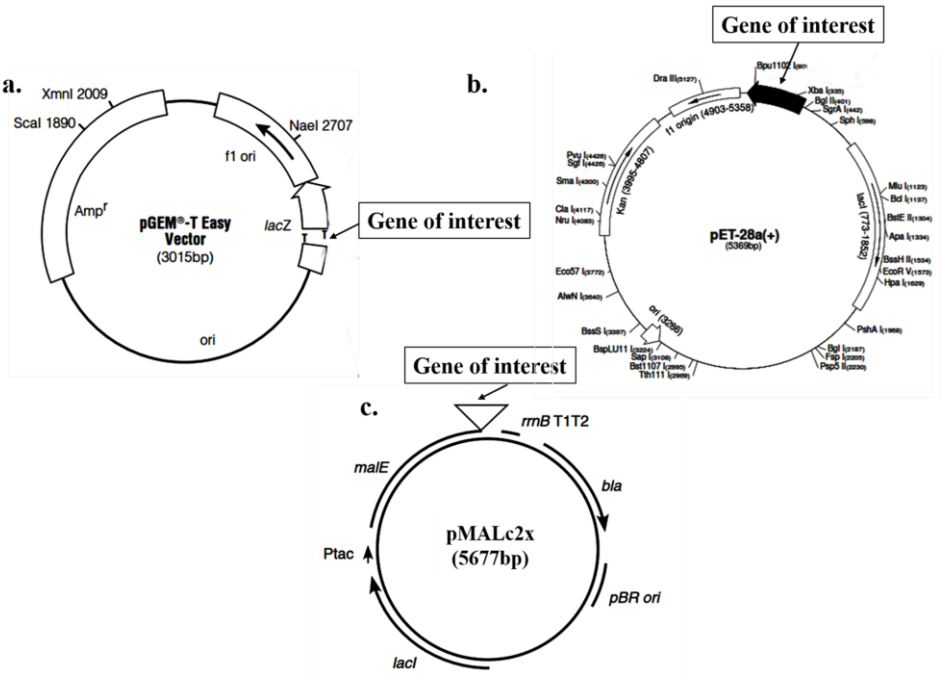

Fig-II Vectors used in the study. a. pGEM-T easy vector; b. pET28a vector; c. pMALc2x vector

Western Blotting

The recombinant proteins were then confirmed by Western blotting using anti-HIS antibodies. Western blotting was carried out by following Towbin method (8). Briefly, the recombinant proteins were transferred to PVDF membrane and then blocked using BSA (5%). Primary antibody was added and incubated overnight at 4^{o}C (Mouse anti-HIS IgG,) and after washing the blots with 1x PBS, secondary antibodies (goat anti mouse IgG,) was added and incubated for two hours at room temperature. The bands were developed using 3,3'-diaminobenzidine (DAB).

Solubility analysis of proteins

E. coli BL21 (DE3) cells containing recombinant proteins were re-suspended in lysis buffer (25 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole, pH 8.0) containing 1mM PMSF and lysed by sonication (10cycles at 30secs pulse and 30secs pause) to analyze the solubility of the expressed proteins. The sonicated cells were centrifuged at 13,000rpm for 30 minutes and the resulting supernatant and pellet was analyzed by SDS-PAGE (12%). The proteins found in the supernatant fraction were purified by affinity chromatography method and the proteins that were found in insoluble pellet fraction were optimized for soluble expression under different expression conditions (lower incubation temperature, various concentration of inducer and time duration of induction) to yield the protein in soluble fraction. The final optimized conditions obtained for soluble expression of the proteins are given in table III.

For proteins expressed as MBP fusion, the cells were re-suspended in column buffer (25mM Tris-HCl, 200mM NaCl, 1mM EDTA) and lysed by sonication. The sonicated cells were then analyzed by SDS-PAGE (12%) as described before.

Table-III a. Purification under native conditions

Gene Name	Purification Method	Conditions for Soluble Expression
Rv3574 (TetR)	Ni-NTA Affinity chromatography	IPTG (1mM), 37°C for 3 hours
Rv2069 (SigC)	Ni-NTA Affinity chromatography	IPTG (1mM), 37°C for 3 hours
Rv0757 (PhoP)	Ni-NTA Affinity chromatography	IPTG (0.1mM), 16°C for 18 hours
Rv2711(IdeR)	Ni-NTA Affinity chromatography	IPTG (1mM), 37°C for 3 hours
Rv3133c (DosR)	Ni-NTA Affinity chromatography	IPTG (1mM), 37°C for 3 hours
Rv3223c (SigH)	Ni-NTA Affinity chromatography	IPTG (0.1mM), 16°C for 18 hours
Rv2031c (HspX)	Ni-NTA Affinity chromatography	IPTG (1mM), 37°C for 3 hours
Rv2720 (LexA)	Ni-NTA Affinity chromatography	IPTG (1mM), 37°C for 2 hours
Rv2710 (SigB)	MBP Affinity chromatography	IPTG (0.3mM), 37°C for 3 hours
Rv0467 (Icl)	Ni-NTA Affinity chromatography	IPTG (0.3mM), 16°C for 18 hours
Rv1131(Mcs)	Ni-NTA Affinity chromatography	IPTG(0.3mM), 30°C for 6 hours
Rv2150c (FtsZ)	Ni-NTA Affinity chromatography	IPTG (1mM), 37°C for 3 hours

Table-III b. Purification under denaturing conditions (8M Urea)

Gene Name	Purification Method	Conditions for Expression
Rv34i4c (TetR)	Ni-NTA Affinity chromatography	IPTG (1mM), 37°C for 3 hours
Rv1221c (SigC)	Ni-NTA Affinity chromatography	IPTG (1mM), 37°C for 3 hours
Rv3416 (PhoP)	Ni-NTA Affinity chromatography	IPTG (1mM), 37°C for 3 hours

Affinity chromatography

*Purification under native conditions***:** The recombinant proteins were purified using Affinity chromatography either by Ni-NTA method or by MBP affinity method. Purification was carried out using 100ml of *E.coli* BL21 (DE3) cell culture. The supernatant containing the soluble proteins were incubated with Ni-NTA agarose matrix overnight at 4ºC and then loaded into the column. After washing the column with wash buffer I (25mM Tris-HCl, 500mM NaCl, 20mM imidazole, pH 8.0) and wash Buffer II (25 mMTris-HCl, 500mM NaCl, 40mM imidazole, pH 8.0) the bound proteins were eluted using elution buffer (25mM Tris-HCl, 500mM NaCl, 200mM imidazole, pH 8.0).

For MBP affinity chromatography, the supernatant containing the soluble proteins were incubated with amylose resin overnight at 4ºC and then loaded into the column. After washing the column with column buffer (25mM Tris-HCl, 200mM NaCl, 1mM EDTA) the bound proteins were eluted using column buffer containing 10mM Maltose.

The eluted fractions were analyzed by SDS-PAGE. The purity of the proteins was assessed by analyzing eluted fractions in SDS-PAGE (12%) and quantitative

estimation of protein was done using the Bradford method.

Purification under denaturing conditions: For refolding of proteins from inclusion bodies, the method followed was as described previously (9). The pellet fraction containing the inclusion bodies was treated with denaturation buffer (25mM Tris-HCl, 500mM NaCl, pH 8.0) containing 8M urea overnight at 4^{o}C and then centrifuged at 13,000rpm for 30 minutes to collect the denatured proteins. The proteins were incubated with Ni-NTA agarose matrix for 2 hours and then loaded into the column. The column was washed with wash buffer (25mM Tris-HCl, 500mM NaCl, 20mM imidazole, pH 8.0) containing gradient amounts of urea (8M -0M). The bound proteins were eluted using elution buffer (25mM Tris-HCl, 500mM NaCl, 200mM imidazole, pH 8.0) and the eluted fractions were analyzed by SDS-PAGE (12%).

RESULTS

PCR amplification and gene cloning

The genes listed in table I were successfully amplified and the size of the amplified product was verified by 1.2% agarose gel electrophoresis (Fig. III).

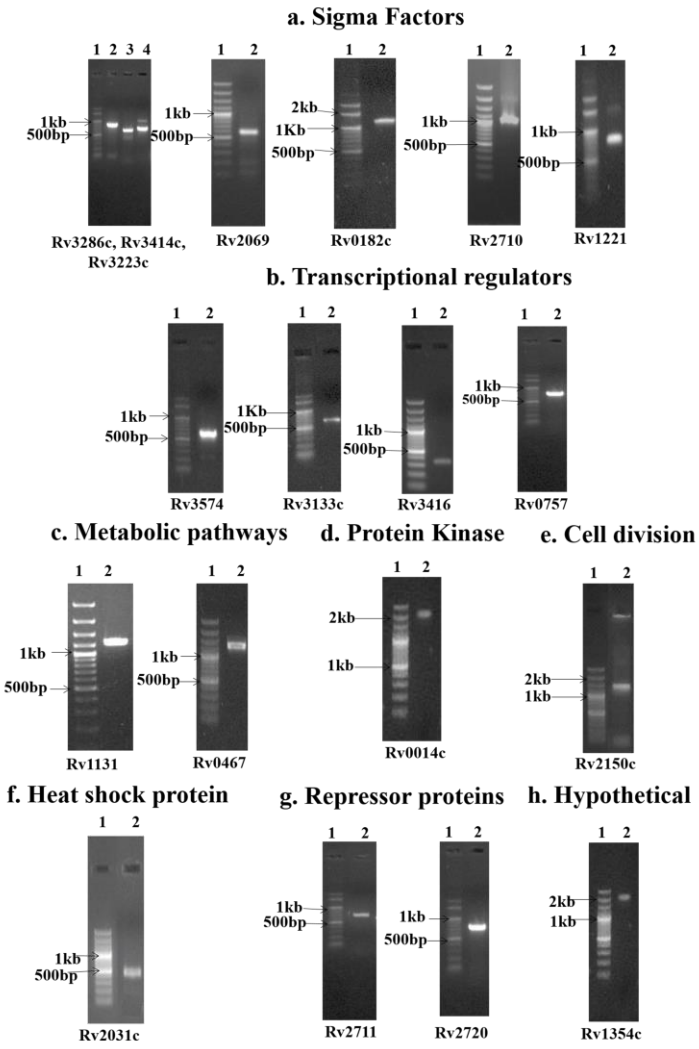

Fig-III Agarose Gel (1.2%, containing ethidium bromide) analysis of PCR amplified genes. Lane 1: 100bp DNA marker and Lane 2: PCR product.

Expression analysis of recombinant proteins

E. coli BL21 (DE3) cells transformed with recombinant plasmids were induced for expression by adding IPTG to a final concentration of 1mM at 37ºC for 3 hours. The induced proteins were analyzed by 12% SDS-PAGE stained with Coomasie Brilliant Blue R250 and the expression was verified by comparing the size of the expressed proteins with molecular weight markers (Fig. IV).

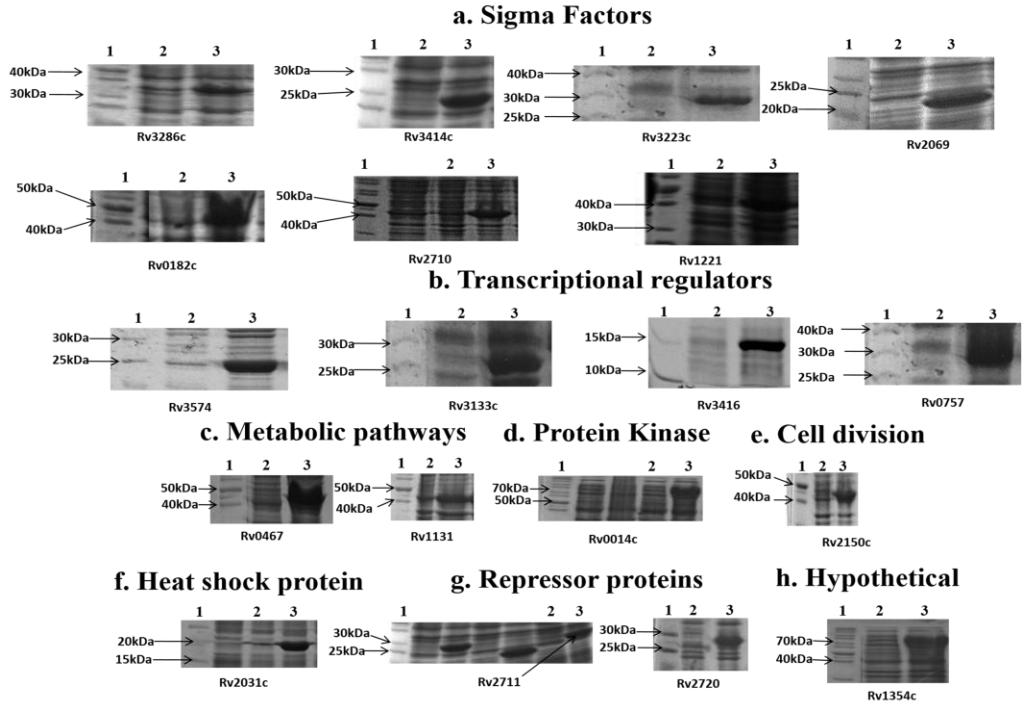

Fig-IV SDS-PAGE analysis of Recombinant Proteins. Lane 1: Unstained Molecular weight standards, Lane 2: Un-induced cell lysate and Lane 3: Induced cell lysate. Gels were stained by Coomasie Brilliant Blue R250.

The solubility and cellular localization of the proteins were analyzed by sonication of the cell pellet and subjecting the soluble and insoluble fractions to SDS-PAGE. The proteins expressed as insoluble inclusion bodies were further optimized for its soluble expression. The final optimal conditions for soluble expression of each proteins are given in table III.

Purification of recombinant proteins

*Purification under native conditions***:** Under native conditions, the proteins were purified from the soluble fraction. The supernatant was incubated with Ni-NTA agarose matrix overnight at 4ºC and then loaded into the column. After washing the column with wash buffer, the bound proteins were eluted using elution buffer containing 200mM imidazole. The eluted fractions were then analyzed by SDS-PAGE. Similarly, the purification by MBP affinity chromatography was carried out by incubating the supernatant fraction with amylose resin overnight at 4ºC and after

washing the column with column buffer, the bound proteins were eluted using column buffer containing 10mM maltose. The analysis showed single bands of the purified proteins (Fig. V).

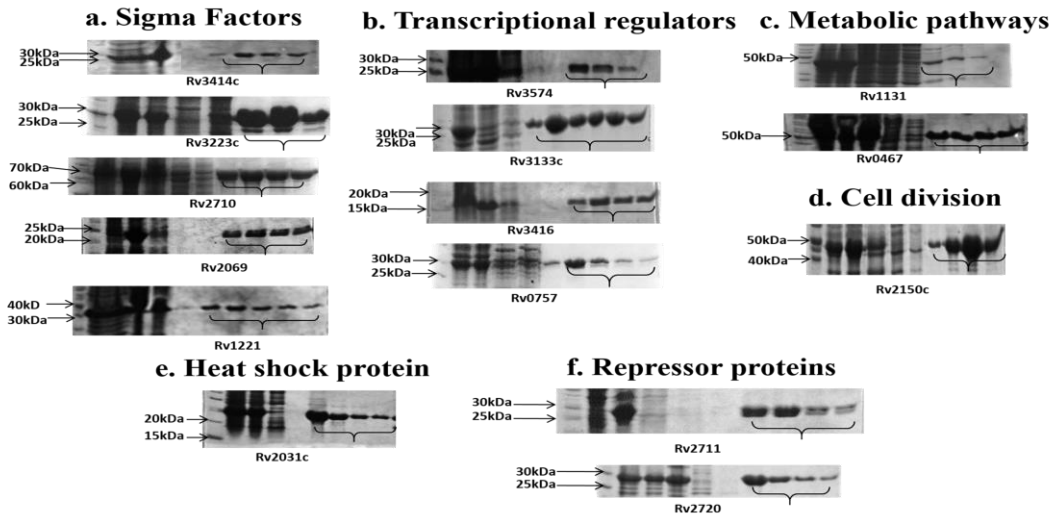

Fig-V. SDS-PAGE analysis of purified recombinant proteins. Lane 1: Unstained Molecular weight standards, the last lanes containing the purified proteins are marked. Gels were stained by Coomasie Brilliant Blue R250.

Purification under denaturing conditions: Under denaturing conditions, the pellet fraction containing the inclusion bodies was treated with 8M urea overnight at 4^{o}C and then centrifuged to collect the denatured proteins. Then they were applied to the column and washed with wash buffer containing gradient amounts of urea (8M -0M). The last wash (containing no urea) was to displace contaminant proteins that are generally loosely bound to column. The bound proteins were eluted using elution buffer and analyzed by SDS-PAGE. The analysis showed single bands of the purified proteins (Fig. V).

All the eluted proteins were confirmed by western blotting using anti-His IgG antibodies (Fig. VI) but for the protein purified by MBP affinity chromatography.

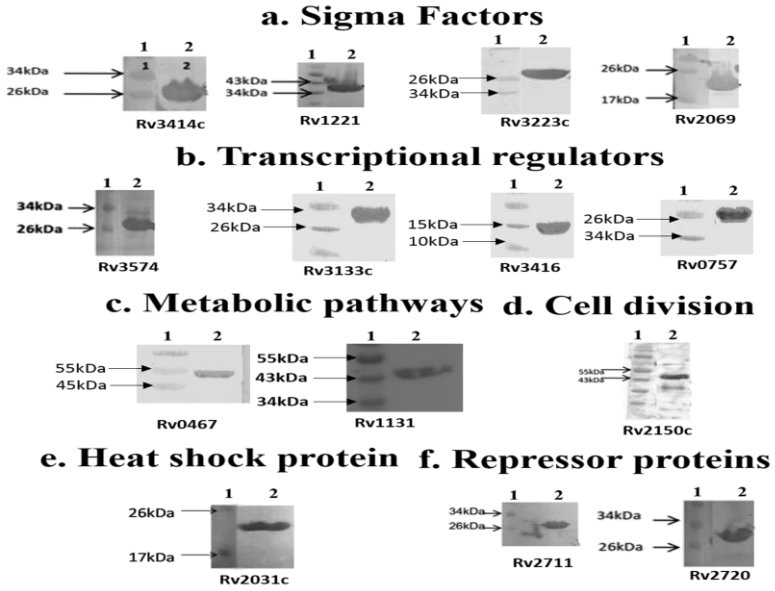

Fig-VI. Western Blotting of purified recombinant proteins using anti-His_6-tag antibodies. Lane 1: Pre-stained molecular weight standards, Lane 2: Purified proteins.

DISCUSSION

Since the present work was carried out by a number of students (31) in different batches, emphasis was laid on maintaining consistency in the followed protocols and in using same reagents/kits throughout this study. It was also ensured to procure Genomic DNA H37Rv from one source only.

While designing gene-specific primers, restriction sites were inserted within the primers keeping reading frame of pET28a-c vectors. Different types of PCR such as Touch Down, Hot start and gradient PCR were tested for optimization of the annealing temperature to get the desired specific PCR product. All the genes were first cloned in T-vector (by TA cloning) and then were cloned again in expression vector. Though it was an extra step, but was done to make the repository uniform (with respect to the choice of cloning vector). The sequence of the cloned genes was verified prior to expression analysis. Expression of genes (cloned in pET28a-c vector series) was initially carried out at 37ºC and at 1mM concentration of IPTG. Seven proteins (Rv3574, Rv2069, Rv2711, Rv3133c, Rv2031c, Rv2720, and Rv2150c) were obtained in soluble form under these conditions of temperature and concentration of inducer (IPTG), while the remaining proteins were found to be present as insoluble inclusion bodies. When lower incubation temperature (30ºC and 16ºC) was tested to get these recombinant proteins in soluble form, Rv1131 at 30ºC and Rv0757, Rv0467 & Rv3223c at 16ºC could be isolated in soluble form. Rv2710 still could not be obtained in soluble form and was cloned in another expression vector pMALc2x vector and was found to be expressed in soluble form at 37ºC and 0.3mM IPTG. The remaining three genes (Rv3414c, Rv1221c, Rv3416) which expressed as insoluble inclusion bodies were not cloned in pMALc2x vector because the amplified genes were not in frame with the vector, hence these proteins were purified by refolding of the inclusion bodies in to their native conformation. Urea denaturation method (9) was followed and proteins could be yielded in soluble form. However, in the absence of assay methods for these proteins (Rv3414c, Rv1221c, Rv3416), it could not be ascertained if the refolded soluble proteins were in their native conformation or not and requires further characterization. Proteins were obtained at a purity of about 90-99% with a yield ranging 2-10 mg/ml per 100 ml culture.

CONCLUSIONS

A repository of 19 gene clones and 15 recombinant proteins from *Mycobacterium tuberculosis* H37Rv has been developed. These genes are identified as potential drug targets.

ACKNOWLEDGMENTS

We thank OSDD for conceptualizing the project and CSIR for research funds and the Principal Dr.Savithri Singh for her constant support and encouragement. We are also thankful to administrative & laboratory staff of the college.

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