In silico characterization of hypothetical proteins obtained from *Mycobacterium tuberculosis* H37Rv

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Received: 28 September 2016 / Revised: 13 December 2016 / Accepted: 24 January 2017 / Published online: 4 February 2017
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Abstract Tuberculosis is one of the oldest diseases with a death rate of 1.5 million per year. Tuberculosis spreads from one person to another through *Mycobacterium tuberculosis*. This bacteria belongs to the family *Mycobacteriaceae*, genus *Mycobacterium*, member of the tuberculosis complex. *Mycobacterium tuberculosis* is an acid-fast, aerobic, rod-shaped bacteria, ranging from 2 to 4 μm in length and 0.2 to 0.5 μm in width. Tuberculosis spreads through infected people via sneezing, coughing, etc., with humans acting as the host for the bacteria. The genome of *Mycobacterium tuberculosis* H37Rv encodes 3906 proteins, of which 1055 are hypothetical proteins (HPs), wherein the functions of the proteins are unknown. The sequences of 1055 HPs of *Mycobacterium tuberculosis* were analyzed and the functions of 578 HPs were subsequently predicted with a high level of confidence. Several enzymes, transporters and binding proteins of 1055 HPs in *M. tuberculosis* were analyzed and potential targets were discovered which contribute to the overall survival of the bacteria. The analysis will be of relevance in understanding the mechanism of the bacteria and will prove to be beneficial in the discovery of new drugs.

Keywords *Mycobacterium* · Hypothetical proteins · Functional annotation · ROC analysis

1 Introduction

Tuberculosis is one of the oldest and most dangerous diseases in the world. Death rate due to tuberculosis is more than 1.5 million per year despite various vaccines being used worldwide. The causative organism responsible for this disease was identified as *Mycobacterium tuberculosis*, a bacteria which belongs to the genus *Mycobacterium*.

Mycobacterium tuberculosis is a bacteria related to the family *Mycobacteriaceae* and is the main organism responsible for causing tuberculosis (Munksgaard 2004), which is spread from one person to another through the air. This bacteria is normally found in air in the form of aerosol droplets of size 1–5 μm in diameter. This aerosol droplet nuclei is dispersed into the air when a person suffering from tuberculosis sneezes or coughs. The bacteria has a waxy coating on its cell surface, which is due to the presence of mycolic acid. *Mycobacterium tuberculosis* is gram positive (Fu and Fu-Liu 2002) and is immobile. It is an acid-fast aerobic rod of length 2–4 μm and width 0.2–0.5 μm . It divides over a time period of 15–20 h, spreading very slowly in the human lung (Munksgaard 2004).

The advancement of the field of genomics especially next generation sequencing analytical techniques have been brought about by the collection of large number of sequences obtained from various different bacterial strains (Mazandu and Mulder 2012; Kamal et al. 2016a; Gupta et al. 2016). Though nearly 40–50% of genes within a genome are classified in the category of ‘unknown’, ‘hypothetical’ or ‘conserved hypothetical’ or ‘orphan’ genes,

Electronic supplementary material The online version of this article (doi:10.1007/s13721-017-0147-8) contains supplementary material, which is available to authorized users.

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constraining our comprehension of virulence and pathogenicity of the various species of bacteria (Kumar and Shanmughavel 2011; Kamal and Nimmy 2016), the genome sequence of *Mycobacterium tuberculosis* is available in the NCBI database which contains 4019 genes encoding 3906 proteins. Out of 3906 proteins, the function and characterization of 1055 proteins are unknown and are thus considered as hypothetical proteins. Various bioinformatics tools were employed for predicting and understanding the function of these hypothetical proteins. The current protocol used in this study can likewise be connected to annotate the uncharacterized proteins from other prokaryotic and eukaryotic life forms (Chowdhury et al. 2016). This analysis will prove to be beneficial in understanding the mechanism of the bacteria; beneficial to the discovery of new drugs and could significantly impact TB control and vaccine development strategies.

2 Related work

The functional characteristic of various *Mycobacterium tuberculosis* H37Rv proteins annotated as hypothetical proteins are yet to be performed. The current study includes various in silico approaches to identify and annotate the biological role and function of unknown *Mycobacterium tuberculosis* H37Rv hypothetical proteins. The presence of HPs in genomes is crucial for the overall comparative as well as functional genomics analyses. Specifically for pathogenic organisms, these HPs hinder the search for novel drug targets, and diminish the scope of our understanding to study various virulence and pathogenic properties (Gupta et al. 2016). Earlier studies reported the functions of only 250 HPs of *Mycobacterium tuberculosis* using various Bioinformatics web tools, BLAST, INTERPROSCAN, PFAM and COGs (Kumar and Shanmughavel 2011). But in-depth study and analysis is yet to be carried out for the function prediction and characterization of 1055 HPs obtained from *Mycobacterium tuberculosis* H37Rv.

3 Materials and methods

3.1 Sequence retrieval

From the NCBI database (<http://www.ncbi.nlm.nih.gov/genome>), the genome of *Mycobacterium tuberculosis* was retrieved in the FASTA format. There are 3906 proteins present, out of which 1055 proteins are characterized as hypothetical proteins because the function and characterization of these proteins are unknown. The sequences of these 1055 HPs have been collected from the above-mentioned NCBI database in the form of FASTA sequences

which were further validated from UNIPROT database and the redundant proteins were excluded. Only unique HPs were taken further for the functional annotation and characterization through various analysis such as physiochemical properties calculation, subcellular localization and sequence comparisons, protein function and domain analysis, virulence factor analysis, etc. The overall methodology has been mentioned in the form of schematic representation as shown in Fig. 1. This was further explained by making a flowchart of various steps involved in the computational and functional annotation of HPs.

3.2 Physicochemical characterization

Data related to these hypothetical proteins were found to be insufficient, and thus the ExPASy's ProtParam server (Gasteiger et al. 2003; Raj et al. 2015) was used to determine the physicochemical properties of a protein that can be obtained from its sequence like molecular weight, extinction coefficient, isoelectric point, instability index, aliphatic index and grand average of hydropathicity (GRAVY). The information obtained from the server was found to be essential for analyzing the function of the HPs. The predicted physicochemical properties of 1055 HPs are listed in S1 Table.

3.3 Subcellular localization

After predicting the physicochemical property of the hypothetical proteins, the location of proteins will be analyzed. Subcellular localization is more helpful for prediction of the drug target and vaccine target. HPs can be present anywhere in the cell, for instance the cytoplasm, periplasm, extracellular, inner membrane, and outer membrane. Proteins present in the cytoplasm play an essential role in the prediction of drug target, while outer or inner localized proteins work as vaccine targets. PSORTb (Nancy et al. 2010) and CELLO (Yu et al. 2006) were used for subcellular localization of the protein. PSORTb is a database which describes the location of proteins in the cell and provides a more accurate collection of experimental data as compared to CELLO. PSORTb requires a sequence of the protein to be submitted in the FASTA format, after that the organism type as well as its gram stain was selected. As we have taken *Mycobacterium tuberculosis* H37Rv strain, we selected Gram stain as gram +ve in this case. SignalP 4.1 (Emanuelsson et al. 2007) was used for predicting signal peptide and TMHMM (Krogh et al. 2001) was used for the prediction of the transmembrane helices in protein. The subcellular localization predictions of 1055 HPs are listed in the S2 Table. The inbuilt parameter of the cut-off values used in the case of SignalP 4.1 is chosen to optimize the performance which can be measured as

Fig. 1 Schematic representation of the various steps involved in annotation of HPs

Matthews Correlation Coefficient (MCC). This results in a lower sensitivity (true positive rate) than SignalP 4.0 possess. One of the peculiar factors of using TMHMM is that it becomes possible to model helix length, which has just been done reasonably roughly in most different techniques, by fixing upper and lower limits for the length of a membrane helix.

3.4 Sequence comparisons

In subcellular localization, the location of the HPs was defined. The sequences of HPs were analyzed by using BLASTP (Altschul et al. 1990; Raj et al. 2015) and HHPred (Söding et al. 2005). BLASTP is an algorithm for the prediction of similar sequences of HPs which provides 100 similar sequences of HPs. Proteins with high identity values are considered as more identical with HPs, while less identity values are considered as remote homologues. We have used the non-redundant database for the similarity search using the default parameters of the BLASTP. The *E* value should be filled with a number, such as 1, 10, 0.01 or 1.0E−5. This setting specifies the statistical significance threshold for reporting matches against database sequences. If the statistical significance ascribed to a match is greater than the EXPECT threshold, the match will not be reported. Lower EXPECT thresholds are more stringent, leading to fewer chance matches being reported. HMMSCAN (Gough et al. 2001) was used to search the

Pfam, SUPERFAMILY, TIGRFAMs and Gene3D databases for protein with similar families and domains. The list of predicted similar sequence and function values of 1055 HPs are given in S3 Table.

3.5 Function prediction

Proteins are classified into families and superfamily on the basis of their sequence, structure and function by various protein classification tools like CATH, SCOP, etc. Based on sequence similarity, Pfam database was used to predict the function of proteins. INTERPROSCAN is a tool to describe the function, domain, family, location of protein at a time. To group proteins along these categories, InterPro uses predictive models, k/a as signatures, provided by few unique databases (referred to as part databases) that make up the InterPro consortium. SUPERFAMILY (Gough et al. 2001) was used to predict the functional and structural annotation of hypothetical protein. Results of function prediction based on these tools are listed in the S4 Table.

3.6 Virulence factors analysis

Virulence factor plays an important role in the prediction of drug target. Virulence factor gives a description about more active proteins which play important roles for the function and survival of pathogens. VICMPred (Saha and Raghava 2006) and VirulentPred (Garg and Gupta 2008) were

employed for the analysis of virulence factor. The VICMPred server allows the prediction on the basis of three different approaches, i.e., patterns based; patterns based and amino acid composition; patterns based and amino acid composition and dipeptide composition. In this case, we have selected pattern-based approach. The tool VirulentPred gives a more accurate prediction of virulence factors as compared to VICMPred. Results of virulence factor prediction based on these tools are listed in the S5 Table.

3.7 Protein annotation prediction

Receiver operating characteristics (ROC) analysis was used for verifying the predicted function of HPs in *Mycobacterium tuberculosis*. The first 100 protein sequences were selected for statistical analysis (S6 Table), while the first 10 are displayed in Table 1. Prediction of these protein functions was made and the efficacy for these diagnostics was evaluated at five levels. True positives and true negatives predicted were classified as “0” and “1”. The confidence rating for the predicted functions was given a rank from 1 to 5 (Kamal et al. 2016b). In this curve, the true positive rate (sensitivity) is plotted in function of the false positive rate (100-specificity) for different cut-off points of a parameter. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold.

4 Results and discussion

The genome of the H37Rv strain containing 4 million base pairs was published in 1998 (Ioerger et al. 2010). The genome sequence of *Mycobacterium tuberculosis* is available in the NCBI database containing 4019 genes, encoding 3906 proteins. Out of 3906 proteins, the function and characterization of 1055 proteins are unknown and are classified as hypothetical proteins. Tools such as Pfam, INTERPROSCAN, SUPERFAMILY, HMMSCAN, CELLO, PSORTb, SignalP, Expasy, VirulentPred, VICMPred and TMHMM were used for the prediction of function (Raj et al. 2016), family, domain, superfamily subcellular localization, physicochemical properties and virulence factor of HPs of *Mycobacterium tuberculosis*, out of which 578 HPs were predicted with high level of confidence (S6 Table and S7 Table) while 477 HPs of low level of confidence were also predicted. All of these 578 HPs were categorized into various functional classes containing 158 enzymes, 36 binding proteins, 66 transporters, 32 cellular process/regulatory proteins, 53 virulent proteins, 20 ATP proteins, 31 membrane proteins and the rest as miscellaneous proteins as depicted in Fig. 2.

4.1 Enzymes

Details of enzymes are important in understanding the function and activities of microorganisms in order to predict drug targets (Raj et al. 2015). 158 enzymes were characterized, out of which 81 oxidoreductase enzymes were identified. These enzymes are necessary for the transfer of electrons from one part of the bacteria to the other (Nambi et al. 2015). 7 proteins were identified as radical SAM (S-adenosylmethionine) proteins (Cantoni 1952). SAM is an enzyme which plays an important role in the transfer of methyl group within the body. 11 HPs with kinase activity were also identified, whose roles are to transfer phosphate group.

Similarly, 47 transferase enzymes which provide the pathway for the transfer of methyl or glycosyl functional groups were identified. 4 proteins were identified as lactamase, which decrease the function of antibiotics like penicillin. 31 hydrolase enzymes, responsible for hydrolysis of bonds were annotated. 1 lyase enzyme playing a significant role in the breaking of new bonds and creating ring structures was found to be present. 18 cyclase enzymes involved in the formation of cyclic compounds were identified.

4 isomerase and 5 ligase proteins were also identified. Isomerase enzymes are involved in structural rearrangement of molecule (Noda-García et al. 2015), while ligase enzymes are involved in combining two large molecules into a new molecule. 14 reductase enzymes were also present, which are involved in reduction reaction within the bacteria. 4 lipase enzymes which are important in hydrolysis of fat in human body were also identified.

Presence of 6 endonuclease and 5 mutase enzymes were found. Mutase enzymes are involved in the transfer of functional groups from one ring to another (Mishra et al. 2016).

4.2 Transporters

Transporters play a significant role for the survival of pathogens as they are involved in the transfer of nutrients to the bacteria. 66 different transporter proteins are present in *Mycobacterium tuberculosis*, out of which 5 ABC (ATP-binding cassettes) transporters hydrolyze the ATP and transfer the nutrient to the bacteria. 31 membrane proteins are present which are important for the survival of pathogens. 4 lipoproteins are essential for the transfer of fats into the blood. 20 ATPase type transporter proteins are present which utilize energy and transfer it to bacteria (Geider and Hoffmann-Berling 1981). Such transporter proteins are important for the survival of the bacteria and may be beneficial for drug discovery.

Table 1 List of annotated function of ten proteins with known function from *Mycobacterium tuberculosis* H37Rv using BLASTp, HMMER, INTERPROSCAN, SUPERFAMILY and Pfam for ROC analysis

S. no.	Protein name	HMMER/HMMSCAN	Major blast hit	INTERPROSCAN	Pfam (family/domain)	SUPERFAMILY
1.	HP Rv0004	Protein of unknown function (DUF721) 1 4	RNA-binding protein 1 4	Protein of unknown function (DUF721) 1 4	Protein of unknown function (DUF721) 1 4	Metallo-dependent hydrolases 1 4
2.	HP Rv0021C	Nitronatemonooxygenase 1 5	Oxidoreductase 1 3	Nitronatemonooxygenase 1 5	Nitronatemonooxygenase 1 5	Inosine monophosphate dehydrogenase (IMPDH) 1 3
3.	HP Rv 0025	Domain of unknown function (DUF4226) 1 4	Biofilm regulator 1 5	Domain of unknown function (DUF4226) 1 4	Domain of unknown function (DUF4226) 1 4	No hit found 0 1
4.	HP Rv0026	Domain of unknown function (DUF4226) 1 4	Biofilm regulator 1 5	Domain of unknown function (DUF4226) 1 4	Domain of unknown function (DUF4226) 1 4	No hit found 0 1
5.	HP Rv0027	Excreted virulence factor EspC, type VII ESX diderm 1 5	ESX-1 secretion-associated protein 1 5	ESX-1 secretion-associated protein 1 5	Excreted virulence factor espc, type VII ESX diderm 1 5	Sigma3 and sigma4 domains of RNA polymerase sigma factors 0 5
6.	HP Rv0028	Protein of unknown function (DUF2694) 0 1	DUF2694 domain containing protein 0 1	Protein of unknown function (DUF2694) 0 1	Protein of unknown function (DUF2694) 0 1	No hit found 0 1
7.	HP Rv0030	Protein of unknown function (DUF2710) 0 1	Transposase 1 4	Protein of unknown function (DUF2710) 0 1	No hit found 0 1	No hit found 0 1
8.	HP Rv0034	SnoaL-like domain 1 5	ACP 1 3	SnoaL-like domain, NTF2-like domain 1 5	Protein of unknown function (DUF2710) 0 1	Ybab-like 1 3
9.	HP Rv0036	Mycothiolmaleylpyruvate isomerase N-terminal domain 1 4	TIGR03084 family protein 1 4	Mycothiolmaleylpyruvate isomerase N-terminal domain, actinobacterial-type 1 4	Snoal-like domain 1 3	NTF2-like 1 4
10.	HP Rv0038	Uncharacterized ACR, COG1678 1 4	Transcriptional regulator 1 5	Protein of unknown function UPF0301 0 1	Mycothiolmaleylpyruvate isomerase N-terminal domain 1 4	Dinb/yfit-like putative metalloenzymes 1 3

4.3 Binding proteins

Out of 578 functionally annotated HPs, 36 proteins were predicted to be binding proteins with high level of confidence. Out of these 36 binding proteins, 10 DNA-binding proteins were identified, which are involved in the repair, storage, replication and transcription of DNA (Zhang and Rowley 2006). 7 RNA-binding proteins, which play

significant roles in cellular function, transport, localization of proteins in bacteria, were identified. The presence of 4 protein binding, 12 domain containing and 3 exporter proteins were observed. Exporter proteins are similar to mRNA whose main functions are to transport the information from the nucleus to the cytoplasm (Pemberton and Paschal 2005). The information obtained from these proteins may prove to be valuable in the design of new drugs.

Fig. 2 Flowchart showing the overall methodology used in the annotation of HPs

Fig. 3 Performance analysis of functional prediction tools using ROC analysis

Table 2 List of accuracy, sensitivity, specificity and ROC area of various bioinformatics tools/softwares used in this study

S. no.	Software name	Accuracy of prediction (%)	Sensitivity (%)	Specificity (%)	ROC area
1.	HMMER	98	97.9	100	0.989
2.	BLAST	100	100	100	1
3.	INTERPROSCAN	98	97.8	100	0.989
4.	Pfam	98	96.6	100	0.983
5.	SUPERFAMILY	98	96.8	100	0.984

4.4 Cellular processes/regulatory proteins

32 regulatory proteins were observed out of 578 HPs in *Mycobacterium tuberculosis*. Out of these 32 regulatory proteins, 25 transcription proteins and 3 biofilm regulator proteins were found to be present, whose main function is the control over the binding of regulatory protein sequences, decreasing the rate of RNA polymerase (Latchman 1997).

4.5 Virulent proteins

Virulence factor plays a significant role in the survival of pathogens. VICMPred and VirulentPred were used for analyzing the virulence factor. 53 more virulent proteins were found to be present in 578 of the HPs (S8 Table). Knowledge of these virulent proteins could be beneficial for the discovery of new drugs.

4.6 Performance assessment

The prediction of functions for HPs were done using five tools, specifically HMMER, BLAST, INTERPROSCAN, Pfam and SUPERFAMILY. These approaches were then evaluated using ROC analysis (Rai et al. 2016; Gupta et al. 2015) (Fig. 3).

The results from the ROC analysis as obtained from Fig. 3 indicate high reliability for the given bioinformatics tools (Mazandu and Mulder 2012; Ripon et al. 2016) (Table 2). The confidence of prediction for each HP was considered high if three or more of the tools give the same output. Using the pipeline, the accuracy for average HP was found to be 98.4%. The pie chart in Fig. 4 shows the distribution of function across 578 HPs, of which 158 proteins act as enzymes, 66 HPs as transporters, 36 proteins for binding, 32 regulatory proteins, 53 virulent proteins, 20 ATP proteins, 31 membrane proteins and 182 being miscellaneous proteins, classified based on the flowchart represented in Fig. 2.

5 Conclusions

The occurrence of HPs in genomes constitutes a key issue for both the comparative as well as functional genomics investigations. Specifically for pathogenic living beings, these uncharacterized HPs obstruct the search for novel and more potent drug targets, and debilitate progress towards the development of research on these life forms and enhancement of our understanding of their virulence and pathogenicity. Analysis of the function, family and domain of 1055 HPs was conducted using various bioinformatics

Fig. 4 Statistical analysis of 578 HPs processed using functional annotations from various bioinformatics tools

tools and the prediction of function of 578 HPs was made with high level of confidence. Various types of enzymes, transporter proteins, binding proteins, regulatory proteins and virulent proteins which play essential roles in the survival of *Mycobacterium tuberculosis* were identified and discussed. The information gathered from this study would be helpful in understanding the functions of hypothetical proteins present in *Mycobacterium tuberculosis*. The conclusions drawn for *Mycobacterium* proteins are expected to be generally valid for all pathogens. Hence, an in silico approach for functional annotation of HPs can facilitate further studies on these HPs as novel putative drug targets for other clinically important pathogens (Fig. 4).

Acknowledgements The authors acknowledge the Department of Applied Sciences, Indian Institute of Information Technology, Allahabad, for providing computing facility.

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