Heterogeneity among Homologs of Cutinase-Like Protein Cut5 in Mycobacteria

Deepshikha Verma*, Lahari Das*, Vandana Gambhir, Kanak Lata Dikshit, Grish C. Varshney*

Cell biology and Immunology Division, CSIR-Institute of Microbial Technology, Chandigarh, India

* These authors contributed equally to this work.
* grish@imtech.res.in

Abstract

The study of genomic variability within various pathogenic and non-pathogenic strains of mycobacteria provides insight into their evolution and pathogenesis. The mycobacterial genome encodes seven cutinase-like proteins and each one of these exhibit distinct characteristics. We describe the presence of Cut5, a member of the cutinase family, in mycobacteria and the existence of a unique genomic arrangement in the cut5 gene of M. tuberculosis (Mtb) strains. A single nucleotide (T) insertion is observed in the cut5 gene, which is specific for Mtb strains. Using in silico analysis and RT-PCR, we demonstrate the transcription of Rv3724/cut5 as Rv3724a/cut5a and Rv3724b/cut5b in Mtb H37Rv and as full length cut5 in M. bovis. Cut5b protein of Mtb H37Rv (MtbCut5b) was found to be antigenically similar to its homologs in M. bovis and M. smegmatis, without any observed cross-reactivity with other Mtb cutinases. Also, the presence of Cut5b in Mtb and its homologs in M. bovis and M. smegmatis were confirmed by western blotting using antibodies raised against recombinant Cut5b. In Mtb H37Rv, Cut5b was found to be localized in the cell wall, cytosol and membrane fractions. We also report the vast prevalence of Cut5 homologs in pathogenic and non pathogenic species of mycobacteria. In silico analysis revealed that this protein has three possible organizations in mycobacteria. Also, a single nucleotide (T) insertion in Mtb strains and varied genomic arrangements within mycobacterial species make Rv3724/Cut5 a potential candidate that can be exploited as a biomarker in Mtb infection.

Introduction

Members of genus Mycobacterium are known to cause deadly diseases like tuberculosis (TB), leprosy and skin ulcers. Among these, TB is a major killer causing death of 2–3 million people per year. According to the WHO global tuberculosis report, 2013 (http://www.who.int/iris/bitstream/10665/91355/1/9789241564656_eng.pdf) the major limitation in TB control is the lack of rapid diagnostics owing to the delayed progress in biomarker discovery. Researchers have exploited the potential of mycobacterial cell wall proteins, secretory proteins, lipoproteins and enzymes, especially those involved in lipid metabolism pathways, in designing novel
biomarkers for TB [1–4]. Various growth phase dependent antigens of mycobacteria have also been considered for biomarker development [2, 5].

Despite the fact that _M. tuberculosis (Mtb)_ does not encounter cutin or any of its homologs amid its pathogenic life cycle or environments inside the host, the whole genomic sequence of _Mtb_ H37Rv unraveled seven cutinase genes namely _cut1/clp5/Rv1758, cut2/clp2/Rv2301, cut3/clp3/Rv3451, cut4/clp4/Rv3452, cut5/clp7/Rv3724, cut6/clp6/Rv3802c cut7/clp1/cfp21/Rv1984_ [6]. It is likely that these cutinases are involved in alternate functions. These cutinase proteins have already been identified, expressed and discussed in terms of various metabolic pathways and physiological functions in mycobacteria [7, 8].

Cutinases are present in both environmental and pathogenic strains of mycobacteria [9, 10]. Cutinases are α/β hydrolases, which possess a conventional catalytic triad with a serine residue located within the conserved pentapeptide G-X-S-X-G motif. Gamieldein et al., 2002 [11] proposed 19 genes from the cutinase family in _Mtb_ which may have been acquired from eukaryotes during evolution. Four mycobacterial cutinases, namely _Rv1758, Rv3451, Rv3452_ and _Rv1984c_ were thought to be acquired by horizontal gene transfer, as no bacterial orthologs were found for these mycobacterial cutinases. On the other hand, cutinases of oomycetes which are closely related to diatoms and brown algae have been speculated to be acquired from mycobacteria by lateral gene transfer. The absence of cutin in the mycobacterial environment is evocative of the divergence of mycobacterial cutinases from other genera bearing cutinases [9].

The cutinase motif is prevalent in environmental and pathogenic strains of mycobacteria. Various environmental mycobacterial species such as _Mycobacterium_ sp. _KMS_ and _M. vanbaalenii_ have been reported to contain homologs of cutinase encoding genes [9]. Phylogenetic analysis revealed: i) the presence of gene duplications among other members of mycobacterial cutinases [8], ii) strong bootstrap support for orthology between _cut6_ and _M. leprae_ cutinase and iii) the conserved nature of Cfp21/Cut7 protein in the genome of _Mtb_ H37Rv and _M. bovis (Mbovis_ 2006c), although this protein is deleted from most of the strains of BCG.

In spite of the availability of genome data of _Mtb_, and some other mycobacteria, the presence of cutinases in mycobacteria remained unknown for a decade until Parker et al. 2007 purified and characterized the phospholipase A (Cut4) from _M. smegmatis_ culture supernatant and associated this activity with the putative mycobacterial cutinase. Cut2 and Cut7 are secreted proteins that have been found as components of culture filtrate [12], whereas Cut6 has been shown localized in the cell wall of _Mtb_ and belongs to the gene cluster which is specifically found to encode proteins involved in mycolic acid synthesis [7]. With a predicted molecular weight of 23 kDa, Cut2 has previously been named Cfp23. Parker et al. 2007 demonstrated that Cut4 is secreted in the culture supernatant of _M. smegmatis_ and resides within the cell wall of _Mtb_.

Among all members of the mycobacterial cutinase family, Cut2 and Cut7 were found to be highly immunogenic and protective as protein vaccines [13, 14]. High titer antisera were obtained for Cut2, Cut3 and Cut7/Cfp21. Evaluation of a serological response along with immunoblot analysis of Cut2, Cut3, Cut6 and Cut7, with antiserum raised against the individual proteins, indicated the lack of cross-reactivity [14].

The secreted cutinases like Cut7 and Cut4 have also been reported to have potential as biomarkers in patients with active tuberculosis, thereby making these cutinases suitable candidates for the production of a TB vaccine [1, 14]. Transcripts of _Rv3451/cut3_ and _Rv3724/cut5a_ have been shown to be elevated during _in vivo_ survival of mycobacteria inside hypoxic foamy macrophages [15]. Recently, _Rv3451/Cut3_ has been reported as the primary trehalose dimyoceolate hydrolase in _Mtb_. It is induced in nutrient limiting conditions which helps in nutrient influx and simultaneously sensitizes the bacteria towards intracellular stresses encountered in the host [16]. Also, the role of Cut2 in host cell invasion has been reported, making it a potential
vaccine candidate [17]. All of these reports emphasize the relevance of the cutinase family of proteins in *Mtb* pathogenesis.

*Rv3724* in *Mtb* encodes two different protein products, Cut5a and Cut5b, while in other mycobacterial species, the *cut5* gene is transcribed as a single product. Here, we examined: i) the evolution of the *cut5* gene at the nucleotide and protein levels, ii) antigenic similarity and cross reactivity among Cut5 homologs in mycobacteria, iii) subcellular localization of Cut5b in *Mtb* H37Rv and iv) N-terminal sequences of Cut5 and its homologs in various mycobacterial species.

## Materials and Methods

The plasmid construct pET 19b-cut1 and antisera generated against Cut2, Cut6 and Cut7/cfp21 were kind gifts from Dr. Nicholas P. West, Centenary Institute, Sydney, Australia. The following reagent was obtained through the NIH Biodefense and Emerging Infections Research Resource Repository, NIAID, NIH: monoclonal anti- *Mycobacterium tuberculosis* HspX (Gene Rv2031c), Clone CS–49 (produced *in vitro*), NR-13814. Genomic DNA of *Mtb* H37Rv was a kind gift from Dr. G P S Raghava and Dr. Ashwani Kumar IMTECH, Chandigarh, India.

### Bacterial growth conditions

*Mtb* H37Rv (originally procured from University of Berkley, California was a kind gift from Dr. Ashwani Kumar, IMTECH, Chandigarh, India), *M. smegmatis* mc² 155 (ATCC 607) and *M. bovis* BCG (ATCC 35734) were grown in Middlebrook 7H9 broth supplemented with ADC (Difco Laboratories, Detroit, MI) and 0.5% pyruvate (in case of *M. bovis* BCG) for 14 days at 37°C. Bacterial cells were plated on Middlebrook 7H11 agar plates supplemented with OADC (Difco Laboratories, Detroit, MI) and were stored in 30% glycerol at -70°C after enumeration.

### Construction of recombinant plasmids, expression and purification of recombinant proteins

All target genes were amplified from *Mtb* H37Rv, *M. bovis* or *M. smegmatis* genomic DNA using a specific set of forward and reverse primers for each gene that incorporated a 5’NdeI and a 3’BamHI site to express a protein with a C-terminal histidine tag. The primers used were as follows (F) 5’–GATCCATATGGCACCGGGAGTCACCTGGTATT–3’ and (R) 5’–GATCGGATCCCTACAGGCGGCTGGCGGAATT–3’ for *Rv3724b/cut5b*; (F) 5’–GATTCATATGGATGTCATCAGATGGGCTCG–3’ and (R) 5’–GATCGGATCCCTACAGGCGGCTGGCGGGAATT–3’ for *Mb_3751/cut5*; (F) 5’–ATATATATCATATGATGAACGTT–3’ and (R) 5’–ATATATGGATCCCAAACCGGTC–3’ for *Msmeg_5878*. The PCR products were ligated into pET28c. These constructs were transformed into *E. coli* BL21 (DE3). Expression conditions included induction with 1 mM IPTG at O.D600nm 0.4 for 12 hours at 25°C in LB broth containing kanamycin (50μg/ml). Cells overexpressing the proteins were harvested by centrifugation at 2,500g, for 10 min, at 4°C and resuspended in a buffer containing 50 mM NaH2PO4, 20 mM NaCl, 10 mM imidazole and 2.5 mM CHAPS. Cells were lysed by sonication and the lysate was cleared by centrifugation at 12,000g for 10 min at 4°C. Lysate containing the soluble protein was purified using a Ni-NTA column (Qiagen) according to manufacturer’s instructions. The purity of the protein was checked on a 12% SDS-PAGE gel after coomassie staining. We could purify recombinant Cut5b of *Mtb* H37Rv (*MtbrCut5b*) and Cut5 of *M. bovis* (*MbovcCut5*). However, in case of *M. smegmatis* we could not get the purified recombinant protein. Accordingly, the lysate of *E. coli* overexpressing the protein was taken for further studies.
Animals and Antisera generation

Inbred BALB/c mice (6–8 weeks old) were obtained from the Central animal facility of CSIR--IMTECH, Chandigarh, India. Mice were originally procured from Jackson Laboratory, in Bar Harbor, USA and were reared under conventional conditions with a pellet diet and water ad libitum. Protocols involving immunization and sera collection were approved by the Institutional Animal Ethics Committee of Institute of Microbial Technology, Chandigarh, India under the project approval number: IAEC 10/8 and IAEC 13/18. Groups of 5–6 female BALB/c mice, 8–10 weeks old, were immunized with purified protein (25 g in Freund’s complete adjuvant/mouse). Pre-immune sera, which served as control, were first collected by tail bleeding of the mice before immunizations. Mice were immunized subcutaneously (IP/SC) with the above materials and boosters were given in Freund’s incomplete adjuvant at 3 week intervals. After 3–4 boosters, blood was collected by orbital puncture, pooled and kept at 4C for 1–2 hours. After breaking the clot, the blood was centrifuged at 400g at 4C for 10 min. The sera was collected, decomplemented at 56C for 30 min and stored in aliquots at -70C after adding sodium azide to a final concentration of 0.02%.

Subcellular fractionation of mycobacteria

*M. bovis* and *M. smegmatis* were cultured in Middlebrook 7H9 broth at 37°C with mild agitation. Log phase cultures (O.D600nm 0.5–0.6) were harvested by centrifuging at 2,500g for 15 min at 4°C. The pellets were washed three times with PBS. For lysing the cells, the final pellet was resuspended in a breaking buffer (containing 0.1% Tween 80, 1mM MgCl2, 1mM benzamidine and a protease inhibitor cocktail [Pierce] in PBS). The bacterial suspension was sonicated (Ultrasound homogenizer, Cole and Parmer Instruments, Chicago) in an ice bath for 25 minutes followed by centrifugation at 10,000g for 10 min. The supernatant containing the cell free extract was collected and stored at -70°C. For the preparation of the extract and the subcellular fractions of *Mtbc* H37Rv, the bacterium was cultured in Middlebrook 7H9 broth containing 10% ADC at 37°C with mild agitation in the Biosafety level 3 facility of the CSIR-IMTECH, Chandigarh, India. Log phase cultures (O.D600nm 0.5–0.6) were harvested by centrifuging at 2,500g for 15 min at 4°C. The pellet was washed three times with PBS. For lysing the cells, the final pellet was resuspended in breaking buffer. One ml of the suspension was added to 0.5ml of 0.1mm glass beads in a screw capped tube with an O- ring and then subjected to 3 × 1 min pulses with a 1 min rest on ice. The lysate was collected by centrifugation at 13,000g for 10 min at 4°C. The supernatant (or the cell free extract) was collected and centrifuged at 27,000g for 20 min at 4°C. The pellet containing cell wall proteins was resuspended in PBS containing 2% SDS and kept overnight at room temperature (RT). For obtaining the cytosolic and membrane fractions, the extract was subjected to ultracentrifugation at 100,000g for 1 hour at 4°C. The supernatant containing the cytosolic fraction was collected and the pellet containing the membrane fraction was resuspended in PBS containing 2% SDS. All fractions were stored at—80°C.

SDS—PAGE and Immunoblotting

Protein samples and molecular weight standards (BioRad) were separated on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane following a standard protocol [18]. Blots were probed with an appropriate dilution of polyclonal antisera or monoclonal antibodies. A goat- anti- mouse-HRP (BioRad) secondary antibody was used and a blot was developed with an ECL Plus chemilluminescent substrate (GE healthcare).
DNA and RNA extraction
Genomic DNA of *Mtb* H37Rv was used for PCR amplification of full length *Rv3724*, *Rv3724a* and *Rv3724b*. For the total RNA extraction, mid—log phase cultures of *Mtb* H37Rv and *M. bovis* BCG were harvested by centrifugation at 3000g for 10 minutes at 4°C. The bacterial pellet obtained was washed in a GTC buffer containing 5M guanidium thiocyanate (Sigma-Aldrich), 0.5% Tween-80 (Sigma-Aldrich) and 1% beta mercaptoethanol (Merck). FastRNA Pro (MP biomedicals) was added to the pellet and the RNA extraction was performed according to the manufacturer’s instructions. The RNA obtained was checked for its purity on a 1% non denaturing agarose gel.

RT-PCR analysis
For the RT-PCR analysis, RNA was extracted from mid—log phase cultures of *Mtb* H37Rv and *M. bovis* BCG. Synthesis of cDNA was carried out using a Thermo Scientific First Strand cDNA Synthesis Kit (Cat no.K1611) as per the manufacturer’s instructions. The cDNA obtained was directly used for PCR amplification of *cut5a*, *cut5b* and full length *cut5* using gene specific primers. The amplicons were visualized on a 1% agarose gel.

Immunoelectron microscopy
For sample preparation, standard protocols were followed with minor modifications [19–22]. Briefly, log phase cells of *Mtb* H37Rv were fixed overnight with in PBS containing 4% paraformaldehyde and 0.2% glutaraldehyde at 4°C. It was then washed twice with 0.1M cacodylate buffer and subjected to dehydration with an ethanol gradient (30%, 50%, 70%, 80% and 90%) for 30 min and absolute ethanol for 1h at 4°C. Embedding was done in LR-white acrylic resin (Sigma-aldrich) for 36h at 55°C. Ultrathin sections (80nm thick) were cut with a Leica EM UC7 ultramicrotome and collected on nickel grids and processed for immunogold labeling. Grids were blocked with PBS containing 0.01% Tween-20 and 2% skimmed milk for 30 min at room temperature followed by three washes in PBST. Primary antibody incubation was done with 1:100 dilution of anti-Cut5b antiserum or pre-immune serum for 16–18 h at 4°C. Grids were washed and incubated with a 1:5 dilution of 10nm gold nanoparticles conjugated goat-anti- mouse secondary antibody (Sigma) for 1h at room temperature. Three washes were performed with PBST and one with water before staining the grids with 2% aqueous uranyl acetate. Samples were then observed under JEOL 2100 TEM.

Bioinformatics analysis
**Sequence retrieval.** *Rv3724a/Cut5a* and *Rv3724b/Cut5b* sequences of *Mtb* H37Rv and its homologs in *M. bovis* and *M. smegmatis* were retrieved from a Tuberculist database (genolist.pasteur.fr/tuberculist). Gene sequences of mycobacterial homologs were retrieved from a KEGG genomic database (http://www.genome.jp/kegg/genes).

**Alignment studies and percentage GC calculation.** Nucleotide derived amino acid sequences were compared with a non-redundant database in PSI-BLAST or BLASTP (Basic Local Alignment Tool for protein sequences) program using a mail server at the NCBI website [23]. Gap opening and extension penalties of 10 and 0.02, respectively, were used during the alignments. Sequence alignment was initially performed using the multiple sequence alignment software programs ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/) (and ClustalOmega (www.ebi.ac.uk/Tools/msa/clustalo/)). The results were displayed with ESPIRIT [24] and adjusted manually. Genomic sequences of *Rv3724a*, *Rv3724b* and their homologs in different
mycobacterial species were analyzed for percentage GC content using the online available tool GC Calculator (www.genomicsplace.com/gc_calc.html)

Results

In silico analysis of \textit{M. tuberculosis} H37Rv \textit{cut5} (Rv3724)

NCBI, Tuberculist, tbdb and KEGG are few databases where a single gene \textit{Rv3724 (cut5)} has been annotated to encode two protein products Cut5a and Cut5b in \textit{Mtb} H37Rv. To understand the rationale behind such an arrangement, the genomic sequences of \textit{Rv3724} and its homologs from different mycobacterial species were studied. Its homolog, \textit{M. bovis} (\textit{Mb}_3751) showed 100\% identity with \textit{cut5a} and \textit{cut5b}. However, in \textit{Mtb} H37Rv a single nucleotide (T) insertion creates a stop codon within the gene that leads to the translation of Cut5a and Cut5b from different reading frames (Fig 1).

This single nucleotide (T) insertion is also present in the homologs of \textit{Cut5} in pathogenic \textit{Mtb} strains like CDC1551, KZN1435 and F11 (Fig 2). It is likely that this particular single nucleotide insertion is a unique molecular signature of \textit{Mtb} strains.

RT-PCR analysis of \textit{cut5} from \textit{M. tuberculosis} H37Rv and \textit{M. bovis} BCG

In order to understand the consequence of a mutation on the occurrence of Cut5 in mycobacteria, its expression at the mRNA level was checked in \textit{Mtb} H37Rv and \textit{M. bovis} using RT-PCR. The expression of \textit{Rv3724/cut5} gene and its homolog \textit{Mb}_3751 were checked with total RNA extracted from a mid log phase culture of \textit{Mtb} H37Rv and \textit{M. bovis} BCG respectively. Gene specific primers were used to amplify their respective transcripts. A transcript of \textit{Rv3724b} (confirmed by DNA sequencing) was detected but no transcripts for \textit{Rv3724a} and full length \textit{Rv3724} were obtained from the \textit{Mtb} H37Rv genome (Fig 3, panel A). However, the same primers could amplify \textit{Rv3724a} (243 bp), \textit{Rv3724b} (564 bp) and full length \textit{Rv3724} (702 bp) from the \textit{Mtb} H37Rv genomic DNA (S1 Fig). In case of \textit{M. bovis} BCG, only the transcript of full length \textit{Mb}_3751 could be detected (Fig 3, panel B). This observation was in accordance with the bioinformatics analysis which indicated that the \textit{Rv3724/cut5} gene is transcribed as \textit{Rv3724a/cut5a} and \textit{Rv3724b/cut5b} only in \textit{Mtb}. The expression of \textit{Rv3724a/cut5a} could not be detected under the given test conditions, although its transcript has been reported to be upregulated in dormant mycobacteria residing in lipid loaded macrophages [15].

Specificity of Cut5 homologs in mycobacteria

First, in order to study the antigenic similarities among Cut5 homologs in mycobacterial species recombinant proteins (\textit{Cut5b} of \textit{Mtb} H37Rv [\textit{Mtb}rCut5b] and \textit{Cut5} of \textit{M. bovis} [\textit{Mbov}-Cut5]) or \textit{E. coli} cell lysate containing recombinant Cut5 of \textit{M. smegmatis} (\textit{Ms}rCut5) were probed with anti-\textit{Mtb}rCut5b antisera (\textit{Mtb} Cut5b sera) and anti-\textit{Mbov}rCut5 antisera (\textit{Mb} Cut5 sera) in western blotting. Both these sera showed good cross reactivity among Cut5 homologs in \textit{Mtb} H37Rv, \textit{M. bovis} and \textit{M. smegmatis} (Fig 4, panels A & B). These results demonstrated the antigenic similarities among the Cut5 homologs in the mycobacterial species studied.

Next, in order to check whether some antigenic cross-reactivity exists between Cut5 and other cutinases of \textit{Mtb} H37Rv, comprehensive information pertaining to protein sequence homology of different \textit{Mtb} H37Rv cutinases was retrieved from the literature. Table 1 shows that among all cutinases, Cut1 bears a maximum sequence homology (~56\%) at protein level with Cut5 while other cutinases namely Cut2, Cut4 and Cut7/Cfp21 share nearly 45–50\% homology with Cut5. Accordingly, the possible existence of cross-reactive epitopes among these cutinases was checked by using antisera raised against \textit{Mtb}rCut2, \textit{Mtb}rCut7/Cfp21,
MtbCut6, MtbCut1 and MtbCut5b with the lysate of E.coli cells overexpressing MtbCut1 and MtbCut5b in immunoblotting. The results (Fig 4, panel C) indicate that in spite of sequence homology among MtbH37Rv cutinases, there was no antigenic cross-reactivity among Cut5 and other mycobacterial cutinases conferring the specificity of generated antibodies towards Cut5.

Presence of Cut5b homologs in mycobacteria and its subcellular localization in M. tuberculosis H37Rv

The presence of Cut5b and its homologs was demonstrated in the cell free extracts of MtbH37Rv, M. bovis and M. smegmatis. MtbCut5b sera was able to detect Cut5b in MtbH37Rv at ~24kDa and its homologs in M. bovis and M. smegmatis at ~24kDa and ~27kDa respectively (Fig 5, panel A). Also, in subcellular fractions (cell wall, cytosol and membrane) of Mtb H37Rv, Cut5b was recognized by MtbCut5b sera at ~24kDa (Fig 5, panel B). MtbCut6 sera, taken as a positive control, showed the presence of Cut6 at ~34kDa in cell wall and membrane fractions only (data not shown). Further, immunoelectron microscopy results (Fig 6) were consistent with above results and confirmed the presence of Cut5b in Mtb.

Cut6, a cell wall associated cutinase, has been reported to be essential for in vitro growth of mycobacteria [8, 25]. Initially the occurrence of Cut5b in different phases of in vitro growth of mycobacteria was investigated. Mtb H37Rv cultures were harvested at early exponential (O.D600 0.2), late exponential (O.D600 0.7) and stationary phase (O.D600 1.8). Cell free extracts and the cell wall fractions were prepared, immunoblotted and probed with MtbCut5b sera. The

Fig 1. Complete nucleotide sequences of the genes encoding Cut5a and Cut5b (in M. tuberculosis H37Rv) and Cut5 (in M. bovis).

doi:10.1371/journal.pone.0133186.g001
HspX (16 kDa), a highly abundant protein in the stationary phase of mycobacteria [26], was used as a positive control. Cut5b was found to be present at late exponential and stationary phases and was not detected in the early exponential phase (Fig 7). This observation is consistent with the fact that enzymes like cutinases (e.g. Cut4 and Cut6) can remain associated with

Fig 2. Clustal omega alignment of \textit{Rv3724b} in \textit{Mtb} H37Rv and its homologs.
doi:10.1371/journal.pone.0133186.g002

HspX (16 kDa), a highly abundant protein in the stationary phase of mycobacteria [26], was used as a positive control. Cut5b was found to be present at late exponential and stationary phases and was not detected in the early exponential phase (Fig 7). This observation is consistent with the fact that enzymes like cutinases (e.g. Cut4 and Cut6) can remain associated with

Fig 3. Expression of \textit{Rv3724} and its homolog \textit{Mb_3751} in \textit{Mtb} H37Rv and \textit{M. bovis} BCG respectively.
doi:10.1371/journal.pone.0133186.g003
cell wall where they may be involved in cell wall biosynthesis, its modification, lipid metabolism and host lipid scavenging that are essential for *in vitro* growth, intracellular replication and adaptation to dormant phases [8, 15, 27].

Study of *M. tuberculosis* H37Rv Cut5a and Cut5b homologs in other mycobacterial species

The unique genome arrangement of cut5a and cut5b and the presence of intact cut5 in some species indicate that there is a special pattern of phylogeny among mycobacterial species and different strains of *Mtb* (Fig 8, panel A). The presence of both cut5a and cut5b homologs in *M. canettii*, *M. africanum* and *M. bovis* indicates the possibility of the existence of the same
homolog in a most recent common ancestor (MRCA) as *M. canettii* got separated from the direct descendant of tubercle bacilli before the *M. africanum* and *M. bovis* lineage [28].

In order to check the distribution of *Mtb cut5b* and its homologs, mycobacteria species were divided into two groups: (i) human pathogenic *Mtb* strains CDC1551, F11, Harlem and KZN1435 and, (ii) other pathogenic species *M. canettii*, *M. africanum*, *M. marinum*, *M. ulcerans* and *M. avium*. Percentage identities of *cut5a* and *cut5b* were obtained using information

Table 1. Percentage homology based on amino acid sequences of cutinases of *MtbH37Rv*.

| Nomenclature | Percentage homology among *MtbH37Rv* cutinases*** |
|--------------|--------------------------------------------------|
|              | Cut1 | Cut2 | Cut3 | Cut4 | Cut5 | Cut6 | Cut7 |
| Rv1758*/Cut1*/Clp5** | 100  |      |      |      |      |      |      |
| Rv2301*/Cut2*/Clp2** | 39   | 100  |      |      |      |      |      |
| Rv3451*/Cut3*/Clp3** | 36   | 39   | 100  |      |      |      |      |
| Rv3452*/Cut4*/Clp4** | 45   | 54   | 63   | 100  |      |      |      |
| Rv3724*/Cut5*/Clp7** | 56   | 38   | 35   | 46   | 100  |      |      |
| Rv3802c*/Cut6*/Clp6** | 18   | 22   | 17   | 21   | 27   | 100  |      |
| Rv1984c*/Cut7(Clp21)/Clp1** | 49   | 44   | 49   | 53   | 49   | 20   | 100  |

* Tuberculist database (genolist.Pasteur.fr/Tuberculist), ** West et al., 2009 ** Adapted from West et al., 2008

doi:10.1371/journal.pone.0133186.t001

Fig 5. Existence of Cut5b and its homologs in mycobacteria.

doi:10.1371/journal.pone.0133186.g005
available in the KEGG genome database. The three best identical homologs were included for analysis as depicted in Table 2. In order to study the occurrence of horizontal gene transfer events in cut5 of Mtb the % GC contents of cut5a and cut5b were analyzed using the online available tool ‘GC Calculator’. Amino acid sequences of their homologs were retrieved from the KEGG database and analysis of these sequences revealed that Mtb H37Rv Cut5a and Cut5b were present in various virulent strains of Mtb as well as in other virulent mycobacterial species. Along with this, the cut5 (Rv3724) gene was found to have a unique genomic arrangement in different species and strains of mycobacteria. In a few strains of Mtb, annotated sequences indicated that two forms of the protein, Cut5a and Cut5b were possibly encoded by Rv3724a and Rv3724b. Similarly, in Mtb KZN1435 MT_03768 and MT_03769, were assigned to encode homologs of Cut5a and Cut5b. Surprisingly, the products of both MT_03768 and MT_03769 were found to bear 100% identity with Cut5a and Cut5b, respectively. In other strains of Mtb i.e. F11 and CDC1551, homologs encoding Cut5b were present, but homologs encoding Cut5a

| Extract          | Cell wall |
|------------------|-----------|
| Anti-HspX        | E.E       |
|                  | L.E       |
|                  | St        |
| MtbCut5b sera    | E.E       |
|                  | L.E       |
|                  | St        |
| Pre-immune sera  | E.E       |
|                  | L.E       |
|                  | St        |
| Anti-Hsp65       | E.E       |
|                  | L.E       |
|                  | St        |

**Fig 6.** Immunoelectron microscopy showing localization of Cut5b in Mtb H37Rv.

doi:10.1371/journal.pone.0133186.g006

**Fig 7.** Expression of Cut5b at different in vitro growth phases of Mtb H37Rv.

doi:10.1371/journal.pone.0133186.g007
Fig 8. Proposed evolutionary pathway of Rv3724a (cut5a) and Rv3724b (cut5b) (Panel A) and possible organizations (Org. 1, 2 & 3) of Cut5 protein and its homologs (Panel B) in mycobacteria.

Table 2. Homologs of M. tuberculosis H37Rv Cut5a/Rv3724a and Cut5b/Rv3724b present in various mycobacterial and bacterial species.

| Gene     | Size | GC Content | Mtb KZN1435; TBMG_03768 [100%] [80/80] | M. bovis; Mt_3751 [100%] [67/233] | M. africanum; M&F_37330 [100%] [67/233] | Kineococcus radiotolerance; Krad_4111 [53.2%] [62/294] | M. bovis; Mt_3751 [100%] [166/233] | Rhodococcus sp. RHA1; RHA1_RO00629 [54.8%] [168/247] | Rhodococcus sp. RHA1; RHA1_ro00629 [37.5%] [60/247] |
|----------|------|------------|---------------------------------------|-------------------------------------|-------------------------------------------|---------------------------------|-------------------------------------|---------------------------------|---------------------------------|
| Rv3724a  | 243 bp | 64.6%| Mtb KZN1435; TBMG_03768 [100%] [80/80] | M. bovis; Mt_3751 [100%] [67/233] | M. africanum; M&F_37330 [100%] [67/233] | Kineococcus radiotolerance; Krad_4111 [53.2%] [62/294] | M. bovis; Mt_3751 [100%] [166/233] | Rhodococcus sp. RHA1; RHA1_RO00629 [54.8%] [168/247] | Rhodococcus sp. RHA1; RHA1_ro00629 [37.5%] [60/247] |
| Rv3724b  | 564 bp | 64.4%| Mtb CDC1551; MT_3827 [99%] [187/207] | M. bovis; Mt_3751 [100%] [166/233] | M. africanum; M&F_37330 [100%] [166/233] | Gordonia bronchialis; Gbro_4311 [44.8%] [174/239] | M. bovis; Mt_3751 [100%] [166/233] | Frankia_Eul1c; FraEul1c_0562 [40.7%] [167/226] | Frankia_Eul1c; FraEul1c_0562 [40.7%] [167/226] |

Names of the homologs in the table are mentioned as; specie; gene; % identity; amino acid overlap/total amino acids, e.g. Kineococcus radiotolerance (specie); Krad_4111 (gene); (53.2%) (% identity); [62/294] (overlap/total amino acids)
were absent. Homologs of Cut5 from other human pathogenic species like *M. bovis*, *M. canetti* and *M. africanum* showed 99% identity with both Cut5a (Rv3724a) and Cut5b (Rv3724b). Homologs among non-mycobacterial species, members belonging to the class Actinobacteria such as *Rhodococcus sp. RHA1, Frankia_Eud 1c, Kineococcus radiotolerans, Gordonia brochialis* and *Nocardia farcinica*, were found to share 37–54% identity with *Mtbc* H37Rv Cut5a and Cut5b proteins.

**In silico** analysis of N-terminal sequence of *M. tuberculosis* H37Rv Cut5b and its homologs

Cut5b in human pathogenic *Mtbc* strains: *Mtbc* H37Rv, *Mtbc* C, CDC1551, Haarlem, KZN1435 and F11 and other mycobacterial species: *M. bovis*, *M. marinum*, *M. ulcerans*, *Agy99*, *M. avium* K10, *Mycobacterium sp. MCS*, *Mycobacterium sp. KMS*, *Mycobacterium sp. JLS* and *M. smegmatis* vary in N-terminal sequence and protein length (S3 Fig, panel A & S4 Fig, panel B).

Based on the variation in N-terminal sequence and length of the protein, Cut5b homologs in various mycobacterial species were classified into three major categories (Fig 8, panel B);

1. Cut5b as part of a putative bipartite system: The genomic sequence of *Mtbc* H37Rv encodes two different proteins: Cut5a (putative cutinase precursor) and Cut5b (putative cutinase) from a single gene. Surprisingly, such genomic arrangement, found in case of *Mtbc* H37Rv, could only be seen in *Mtbc* KZN1435, an MDR strain having a gene identical to cut5a which is present upstream of the cut5b homolog (S3 Fig, panel C and Table 2). In other mycobacterial species, homologs of cut5a are absent (S3 Fig, panel B).

2. Cut5b with an extra stretch of amino acids at an N-terminus: Extra stretch of highly charged 20 amino acids exist at the N-terminus of the protein in *Mtbc* (S3 Fig, panel A). However, these stretches show no significant identity with Cut5a of *Mtbc* H37Rv (S3 Fig, panel B), further emphasizing the unique genomic arrangement of cut5 in *Mtbc* H37Rv.

3. Cut5 as a single entity: In a few species like *M. sp* MCS, *M. sp KMS* and *M. smegmatis* etc., homologs of cut5b were predicted to encode a single protein having amino acid sequence variability ranging from 53 to 74%. Furthermore, N-terminus of this full length protein resembles neither that of Cut5a nor of Cut5b of *Mtbc* H37Rv (S4 Fig, panel A & B).

**Discussion**

*Mtbc* has been reported to have the highest number of eukaryotic and prokaryotic interkingdom gene fusions of all the sequenced bacterial genomes [29]. Four mycobacterial cutinase genes, namely Rv1758, Rv3451, Rv3452 and Rv1984c were thought to be acquired by horizontal gene transfer because no bacterial orthologues were found for mycobacterial cutinases. The coding regions of the mycobacterial genome have been affected by frame shifts owing to microsatellite InDels, which is an indication of gene fission/fusion, premature termination and length variation [30]. Genes affected by frame shift mutations encode for membrane proteins, cell wall synthesis proteins, transporters, PPE, PE_PGRS [31, 32] and hypothetical proteins. The ORF encoding Rv3724/Cut5 in *Mtbc* H37Rv has been observed to split into two ORFs (Rv3724a/cut5a & Rv3725b/cut5b) due to the presence of a single nucleotide (T) insertion in the coding region which creates a stop codon in the coding region (Fig 1). These mutations are very common in *Mtbc* and results in gene fission/fusion, premature termination and length variation. For instance, a single nucleotide deletion in the ORF, encoding functional isocitrate lyase, resulted in splitting of the ORF to produce two new ORFs (Rv1915 & Rv1916) [30, 33]. The single nucleotide insertion in Rv3724/cut5 does not seem to be the part of the core genome as the
insertion is absent in homologs from other mycobacterial species. Since it is present only in \textit{Mtb} strains (Fig 2) it can be speculated that this mutation has been acquired very recently.

It is reported that \textit{Mtb} is devoid of a DNA mismatch repair system mediated by \textit{mutS}, \textit{mutL} and \textit{mutH} genes [34, 35], rather plasticity to the genome is imparted through gene duplication and divergent evolution [10, 36]. Overlapping genes have unique evolutionary constraints, either to minimize genome size [37] or to elongate coding regions [38]. Moreover, RT-PCR analysis of \textit{Rv3724/cut5} from \textit{Mtb} H37Rv and its homolog \textit{Mb\_3751} from \textit{M. bovis} BCG has demonstrated that \textit{cut5} is transcribed as \textit{cut5a} and \textit{cut5b} only in \textit{Mtb} H37Rv (Fig 3). However, the presence of \textit{cut5a} transcript was not observed under the tested conditions. This may be due to the low copy number of its transcript or because of its condition specific transcription. Microarray analysis however revealed a four-fold excess of the \textit{cut5a} transcript in dormant mycobacteria residing in lipid loaded macrophages [15].

Recombinant \textit{Cut5} of \textit{M. bovis} appeared at ~24kDa in SDS-PAGE, similar to the size of recombinant \textit{Cut5b} of \textit{Mtb} H37Rv (Fig 4, panel A & B). However, the predicted size of \textit{Mtb}Cut5b, based on molecular weight analysed by generunner (www.chembio.uoguelph.ca), and intact mass analysis was around 19 kDa and 20.9 kDa respectively (data not shown). Such reduced mobility in SDS-PAGE has been observed for other \textit{Mtb} H37Rv cutinases \textit{Cut4} and \textit{Cut1} [8]. This may be due to post-translational modifications in the protein [39] or possible binding of different molar amounts of SDS with such proteins [8].

Previous studies [8, 13] and this study show that numbers of mycobacterial cutinases are capable of inducing a good antibody response. For instance, the recombinant proteins \textit{Cut2}, \textit{Cut3}, \textit{Cut6} and \textit{Cut7/Cfp21} elicited high titer antibodies (> 1:10000) [8, 13] while both \textit{Mtb}Cut5b and \textit{Mb}Cut5 sera showed similar titers (1:12,500) (data not shown). Furthermore, these antibody responses were quite specific to their respective proteins. \textit{Mtb}Cut5b and \textit{Mb}Cut5 sera reacted equally well with recombinant \textit{Cut5} of all the species tested i.e. \textit{Mtb} H37Rv, \textit{M. bovis} and \textit{M. smegmatis} analysed by ELISA (data not shown) and western blotting (Fig 4, panels A & B). This was expected knowing the high levels of sequence homology among these proteins. Also, different levels of sequence homology (17–63%) result in the induction of low titer antibodies which are capable of cross-reacting with different cutinases [14]. However, no cross-reactivity was found between \textit{Mtb} H37Rv cutinases (Fig 4, panel C). Taken together these results with previous reports [8, 13], it is likely that each cutinase has a unique immunodominant and specific epitope(s), besides sharing a few common epitopes. Further, cutinases \textit{Cut7/Cfp21}, \textit{Cut2} and \textit{Cut6} have been shown to confer a moderate yet reproducible and significant level of protection in the murine model of an \textit{Mtb} H37Rv infection [14]. Although the vaccine potential of \textit{Mtb}Cut5b was not explored in this study, the observed antibody response, compared to those of the above cutinases, makes it a good candidate for further investigation into its protective (defensive) efficacy.

Attempts have been made earlier to localize various \textit{Mtb} H37Rv cutinases. West \textit{et al.}, 2009 showed the presence of ~18kDa \textit{Cut2} and ~21kDa \textit{Cut7/cfp21} in culture filtrate, and ~32 kDa \textit{Cut6} in cell wall preparations by immunoblotting, while \textit{Cut4} has been reported to be localized in the cell wall of \textit{Mtb} [40]. Reactivity of \textit{Mtb}Cut5b sera in immunoblotting with cell extracts of \textit{Mtb} H37Rv, \textit{M. bovis} and \textit{M. smegmatis} revealed that \textit{Cut5b} and its homologs exist at ~24kDa in \textit{Mtb} H37Rv and \textit{M. bovis} and at ~27kDa in case of \textit{M. smegmatis} (Fig 5, panel A). Such phenomena of the recognition of protein bands of different molecular size in different mycobacterial species has been previously noted in immunoblott analysis using \textit{Mtb}Cut6 sera [7]. In few experiments, low molecular weight products e.g. ~22kDa, ~18kDa were also observed while checking the reactivity of \textit{Mtb}Cut5b sera with the extract or membrane fraction (data not shown). It is likely that these lower bands were result of proteolytic degradation as reported for mycobacterial LprA [39]. In \textit{Mtb} H37Rv, immunoelectron microscopy and
immunoblotting revealed Cut5b to be associated with the cell wall, membrane and cytosol (Fig 5, panel B and Fig 6) under the given test conditions, indicating that like Cut6 & Cut4 it may have role in cell wall processes. Mycobacterial cutinases are classified under lipid metabolism enzymes and a role of some of these cutinases like Cut6 and Cut4 have been implicated in scavenging host lipids to provide a carbon source for the intracellular replicating mycobacteria [8, 27, 41]. Cut5b was found to be associated with the \textit{Mtb} H37Rv cell wall at late exponential and stationary phases rather than in the early exponential phase (Fig 7) which may indicate that enzymes like cutinases involved in lipid metabolism can remain associated with the cell wall where they may be involved in cell wall modeling during intracellular growth and dormant phases [8].

According to Brosch et al., 2002, the most recent common ancestor of the tubercle bacilli (MRCA) resembles \textit{Mtb} or \textit{M. canettii} and the lineage of \textit{Mtbc} separated from the direct descendant of tubercle before the \textit{M. africanum} and \textit{M. bovis} lineage. All mycobacterial cutinases were found to have their homologs in \textit{M. canettii}, \textit{M africanum} and \textit{M. bovis}, implying their presence in the common ancestor MRCA (Fig 8, panel A). Aberration in mean GC content has been used to study horizontal gene transfer (HGT) events in mycobacteria [42]. No observed difference between percentage GC content of cut5a and cut5b and mean GC content of mycobacteria i.e 65–66% (Table 2) indicated the absence of HGT events. Furthermore, no drastic changes could be seen in the sequences of these cutinases. Taken together, these observations suggest the ancient acquisition of these genes from \textit{M. prototuberculosis} (MTBC) through vertical gene transfer.

Cutinases exist in both pathogenic and environmental strains of mycobacteria [9]. Analysis of \textit{Rv3724/cut5} homologs from pathogenic and non-pathogenic strains of mycobacteria revealed its unique genomic arrangement (Table 2). In pathogenic mycobacterial species other than \textit{Mtbc}, like \textit{M. bovis}, \textit{M. africanum} and \textit{M. canettii}, the homolog of \textit{Rv3724/cut5} encoding for the full length protein product showed 99% identity with both \textit{Rv3724a/Cut5a} and \textit{Rv3724b/Cut5b}. Interestingly, there are some differences in the genomic arrangement of \textit{Rv3724/cut5} within \textit{Mtbc} strains too. Unlike \textit{Mtbc} H37Rv, where two forms of the protein are encoded by the same gene \textit{Rv3724/cut5}, \textit{Mtbc} KZN1435 has two different genes for encoding homologs of Cut5a and Cut5b (having 100% identity). Surprisingly, homologs of \textit{Rv3724a/cut5a} were absent in \textit{Mtbc} F11 and CDC1551 but those of \textit{Rv3724b/cut5b} were present. Thus, the presence of cut5a in \textit{Mtbc} KZN1435 may serve as a biomarker for this MDR strain and may be further exploited in studying other such strains as well.

Homologs of Cut5 were found to exhibit length variation, while the functional domains and catalytic triad remained conserved (S2 and S3 Figs). A peculiar 20 amino acid long N-terminus was observed in Cut5 homologues of strains belonging to \textit{Mtbc} except H37Rv (S4 Fig). It would be of interest to explore whether this has any functional significance. An \textit{in silico} analysis revealed that the deleted N-terminal region of MT2802.1 possesses greater probability to act as a signal peptide suggesting that the protein is secreted. On the other hand, its longer counterparts in \textit{Mtbc} H37Rv and \textit{M. bovis} possess negligible propensities of being signal peptides and thus are non-secretory. While in the case of mammalian cell entry (mce) family virulence proteins and some PPE proteins, length variation did not affect the function of the translated protein while keeping the functional domains well conserved [30].

In conclusion, this study demonstrates the existence of Cut5b in \textit{Mtbc} where it is present in the cell wall, membrane and cytosol. It has three possible organisations in different mycobacteria species. A single nucleotide (T) insertion in the \textit{cut5} gene was found to be unique for \textit{Mtbc} strains. Studies are now required to further characterize Cut5 in order to evaluate its role in mycobacterial physiology and pathogenesis. Additionally, it is likely that the unique genomic arrangement in \textit{cut5} can serve as a biomarker for differentiating \textit{Mtbc} strains from other mycobacteria species.
Supporting Information

S1 Fig. PCR amplification of Rv3724/cut5, Rv3724a/cut5a and Rv3724b/cut5b from M. tuberculosis H37Rv genomic DNA. Agarose gel (1%) showing Rv3724/cut5, Rv3724a/cut5a and Rv3724b/cut5b in lanes 1, 2 and 3 respectively, along with a 2log DNA ladder (NEB) in lane 4. (TIF)

S2 Fig. Multiple sequence alignment of Cut5 amino acid sequences from different mycobacterial species. Clustal X (1.81) was used for the alignment of Cut5 sequences of M. tuberculosis H37Rv (MtbRv), M. tuberculosis C (CG), M. tuberculosis CDC1551 (CDC), M. tuberculosis Haarlem (HG), M. tuberculosis KZN1435 (KZN), M. tuberculosis F11 (FG), M. bovis (bovis), M. marinum (marinum), M. ulcerans Agy99 (ulcerans), M. avium K10 (avium), Mycobacterium sp. MCS (MCS), Mycobacterium sp. KMS (KMS), Mycobacterium sp. JLS (JLS) and M. smegmatis (smeg). (TIF)

S3 Fig. Amino acid sequence alignment of Mtb H37Rv Cut5a and Cut5b with pathogenic M. tuberculosis strains. Clustal X (1.81) was used for the alignment of Cut5 sequences of M. tuberculosis H37Rv (MtbRv), M. tuberculosis C (CG), M. tuberculosis CDC1551 (CDC), M. tuberculosis Haarlem (HG), M. tuberculosis KZN1435 (KZN), M. tuberculosis F11 (FG). Panel A: Alignment of MtbCut5b with the Cut5 of virulent species mentioned above shows the presence of N-terminal 20 amino acid conserved sequence (depicted in box) in virulent strains of M. tuberculosis. Panel B: Alignment of MtbCut5a with Cut5 of human pathogenic strains of M. tuberculosis. Panel C: Clustal omega alignment showing 100% identity among Cut5a of M. tb H37Rv and M. tb KZN1435. Gaps introduced by Clustal, to optimize the alignment, are indicated by ‘-’. Identical amino acids are represented by asterisk (*), conserved residues by colon (:) and semi conserved residues by dot (.). Lower bar graphs represent the homology between the aligned sequences. (TIF)

S4 Fig. Amino acid sequence alignment of Cut5 from Mycobacterium sps. other than M. tuberculosis. Clustal X (1.81) was used for the alignment of Cut5 sequences of M. marinum (marinum), M. ulcerans Agy99 (ulcerans), M. avium K10 (avium), Mycobacterium sp. MCS (MCS), Mycobacterium sp. KMS (KMS), Mycobacterium sp. JLS (JLS) and M. smegmatis (smeg). Panel A: Alignment of MtbCut5b with Cut5 of the mycobacterial species mentioned above. Panel B: Alignment of MtbCut5a with Cut5 of pathogenic species mentioned above. Gaps introduced by Clustal, to optimize the alignment, are indicated by ‘-’. Identical amino acids are represented by asterisk (*), conserved residues by colon (:) and semi conserved residues by dot (.). Lower bar graphs represent the homology between the aligned sequences. (TIF)

Acknowledgments

The plasmid construct pET 19b-cut1 and antisera generated against cut2, cut6 and cut7/cfp21 were kind gifts from Dr. Nicholas P. West, Centenary Institute, Sydney, Australia. We thank Dr. Gajendra P. S. Raghava and Dr. Ashwani Kumar for providing the M. tuberculosis H37Rv genomic DNA. The following reagent was obtained through the NIH Biodefense and Emerging Infections Research Resource Repository, NIAID, NIH: monoclonal anti-M. tuberculosis HspX (Gene Rv2031c), Clone CS-49 (produced in vitro), NR-13814. We thank Dr. Manoj Raje, Mr. Randeep Sharma and Mr. Anil Theophilus for their help rendered in performing electron
microscopy. Inputs provided by Dr. Prabhu B. Patil during preparation of this manuscript are greatly acknowledged. We are thankful to Prof. Alok Mondal, School of Life Science, JNU, New Delhi and Dr. Ashutosh Upadhyay and Ms. Kimberly Arnett, Department of Microbiology, Immunology and Pathology, Colorado State University, USA for their help in editing and critical reading of the manuscript. This work was supported by funds provided by the Council of Scientific and Industrial Research (projects SIP 10, SPenDID BSC0104 and GENESIS BSC0121), Govt. of India. This is IMTECH communication number; 073/2014

Author Contributions
Conceived and designed the experiments: GCV DV LD KLD. Performed the experiments: DV LD VG. Analyzed the data: GCV DV LD KLD. Contributed reagents/materials/analysis tools: GCV DV LD VG. Wrote the paper: GCV DV LD VG KLD.

References
1. Brust B, Lecoufle M, Tuailion E, Dedieu L, Canaan S, Valverde V, et al. Mycobacterium tuberculosis lipolytic enzymes as potential biomarkers for the diagnosis of active tuberculosis. PloS one. 2011; 6(9): e25078. Epub 2011/10/04. doi:10.1371/journal.pone.0025078 PMID: 21966416; PubMed Central PMCID: PMC3178603.

2. Schuck SD, Mueller H, Kunitz F, Neher A, Hoffmann H, Franken KL, et al. Identification of T-cell antigens specific for latent mycobacterium tuberculosis infection. PloS one. 2009; 4(5):e5590. Epub 2009/05/15. doi:10.1371/journal.pone.0005590 PMID: 19440342; PubMed Central PMCID: PMC2680040.

3. Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. Nature reviews Immunology. 2011; 11(5):343–54. Epub 2011/04/09. doi: 10.1038/nri2960 PMID: 21475309.

4. Sartain MJ, Slayden RA, Singh KK, Laal S, Belisle JT. Disease state differentiation and identification of tuberculosis biomarkers via native antigen array profiling. Molecular & cellular proteomics: MCP. 2006; 5(11):2102–13. Epub 2006/08/11. doi: 10.1074/mcp.M600089-MCP200 PMID: 16899542.

5. Chegou NN, Black GF, Loxtan AG, Stanley K, Essone PN, Klein MR, et al. Potential of novel Mycobacterium tuberculosis infection phase-dependent antigens in the diagnosis of TB disease in a high burden setting. BMC infectious diseases. 2012; 12:10. Epub 2012/01/21. doi: 10.1186/1471-2334-12-10 PMID: 22260319; PubMed Central PMCID: PMC3282638.

6. Cole ST, Brosch R, Parkhill J, Garnier C, Churcher C, Harris D, et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 1998; 393(6685):537–44. Epub 1998/06/20. doi:10.1038/31159 PMID: 9634230.

7. Parker SK, Barkley RM, Rino JG, Vasil ML. Mycobacterium tuberculosis Rv3802c Encodes Phospholipase/Thioesterase and Is Inhibited by the Antimycobacterial Agent Tetrahydrolipstatin. PloS one. 2009; 4(1):e4821 doi:10.1371/journal.pone.0004281.g001

8. West NP, Chow FM, Randall EJ, Wu J, Chen J, Ribeiro JM, et al. Cutinase-like proteins of Mycobacterium tuberculosis: characterization of their variable enzymatic functions and active site identification. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2009; 23(6):1694–704. Epub 2009/02/20. doi: 10.1096/fj.08-114421 PMID: 19225166; PubMed Central PMCID: PMC2698861.

9. Belbahri L, Calmin G, Mauch F, Andersson JO. Evolution of the cutinase gene family: evidence for lateral gene transfer of a candidate Phytophthora virulence factor. Gene. 2008; 408(1–2):1–8. Epub 2007/11/21. doi: 10.1016/j.gene.2007.10.019 PMID: 18024004.

10. Skamnioti P, Furlong RF, Gurr SJ. Evolutionary history of the ancient cutinase family in five filamentous Ascomycetes reveals differential gene duplications and losses and in Magnaportha grisea shows evidence of sub- and neo-functionalization. The New phytologist. 2008; 180(3):711–21. Epub 2008/08/21. doi: 10.1111/j.1469-8137.2008.02598.x PMID: 18713314.

11. Gamieldien J, Pittsyn A, Hide W. Eukaryotic genes in Mycobacterium tuberculosis could have a role in pathogenesis and immunomodulation. Trends in genetics: TIG. 2002; 18(1):5–8. Epub 2001/12/26. PMID: 11750687.

12. Weldingh K, Rosenkrands I, Jacobsen S, Rasmussen PB, Elhay MJ, Andersen P. Two-dimensional electrophoresis for analysis of Mycobacterium tuberculosis culture filtrate and purification and characterization of six novel proteins. Infection and immunity. 1998; 66(8):3492–500. Epub 1998/07/23. PMID: 9673225; PubMed Central PMCID: PMC1083778.
13. Grover A, Ahmed MF, Verma I, Sharma P, Khuller GK. Expression and purification of the Mycobacterium tuberculosis complex-restricted antigen CFP21 to study its immunoprophylactic potential in mouse model. Protein expression and purification. 2006; 48(2):274–80. Epub 2006/05/24. doi: 10.1016/j.pep.2006.03.010 PMID: 16716602.

14. West NP, Wozniak TM, Valenzuela J, Feng CG, Sher A, Ribeiro JM, et al. Immunological diversity within a family of cutinase-like proteins of Mycobacterium tuberculosis. Vaccine. 2008; 26(31):3853–9. Epub 2008/06/21. doi: 10.1016/j.vaccine.2008.05.007 PMID: 18565629; PubMed Central PMCID: PMC2671993.

15. Daniel J, Maamar H, Deb C, Sirakova TD, Kolattukudy PE. Mycobacterium tuberculosis uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. PLoS pathogens. 2011; 7(6):e1002093. Epub 2011/07/07. doi: 10.1371/journal.ppat.1002093 PMID: 21731490; PubMed Central PMCID: PMC3121879.

16. Yang Y, Kulka K, Montelaro RC, Reinhart TA, Sissons J, Aderem A, et al. A hydrolase of trehalose dimycolate induces nutrient influx and stress sensitivity to balance intracellular growth of Mycobacterium tuberculosis. Cell host & microbe. 2014; 15(2):153–63. doi: 10.1016/j.chom.2014.01.008 PMID: 24528862; PubMed Central PMCID: PMC3974621.

17. Ocampo M, Rodriguez DM, Curtidor H, Vanegas M, Patarrayo MA, Patarrayo ME. Peptides derived from Mycobacterium tuberculosis Rv2301 protein are involved in invasion to human epithelial cells and macrophages. Amino acids. 2012; 42:2067–77. doi: 10.1007/s00726-011-0938-7 PMID: 21594640.

18. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America. 1979; 76(9):4350–4. Epub 1979/09/01. PMID: 388439; PubMed Central PMCID: PMC411572.

19. Vizcaino C, Restrepo-Montoya D, Rodriguez D, Nino LF, Ocampo M, Vanegas M, et al. Computational prediction and experimental assessment of secreted/surface proteins from Mycobacterium tuberculosis H37Rv. PLoS computational biology. 2010; 6(6):e1000824. doi: 10.1371/journal.pcbi.1000824 PMID: 20585611; PubMed Central PMCID: PMC2891697.

20. Bhanduri A, Misra R, Maji A, Bhetaria PJ, Mishra S, Arora G, et al. Mycobacterium tuberculosis cyclophilin A uses novel signal sequence for secretion and mimics eukaryotic cyclophilins for interaction with host protein repertoire. PLoS one. 2014; 9(2):e88090. doi: 10.1371/journal.pone.0088090 PMID: 24505389; PubMed Central PMCID: PMC3913756.

21. Bashiri G, Perkowski EF, Turner AP, Felcher ME, Braunstein M, Baker EN. Tat-dependent translocation of an F420-binding protein of Mycobacterium tuberculosis. PLoS one. 2012; 7(10):e45003. doi: 10.1371/journal.pone.0045003 PMID: 23110042; PubMed Central PMCID: PMC3478262.

22. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic acids research. 1997; 25(17):3389–402. Epub 1997/09/01. PMID: 9254694; PubMed Central PMCID: PMC1469171.

23. Gouet P, Courcelle E, Stuart DI, Metoz F. ESPript: analysis of multiple sequence alignments in PostScript. Bioinformatics. 1999; 15(4):305–8. Epub 1999/05/13. PMID: 10320398.

24. Sassetti CM, Boyd DH, Rubin EJ. Genes required for mycobacterial growth defined by high density mutagenesis. Molecular microbiology. 2003; 48(1):77–84. Epub 2003/03/27. PMID: 12657046.

25. Hu Y, Movahedzadeh F, Stoker NG, Coates AR. Deletion of the Mycobacterium tuberculosis alphacrystallin-like hspX gene causes increased bacterial growth in vivo. Infect Immunn. 2006; 74(2):861–8. Epub 2006/01/24. doi: 10.1128/IAI.74.2.861-868.2006 PMID: 16428728; PubMed Central PMCID: PMC1360292.

26. Schue M, Maurin D, Dhouib R, Bakala N’Goma JC, Delorme V, Lambeau G, et al. Two cutinase-like proteins secreted by Mycobacterium tuberculosis show very different lipolytic activities reflecting their physiological function. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2010; 24(6):1893–903. Epub 2010/01/28. doi: 10.1096/fj.09-144766 PMID: 20103719.

27. Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglemeier K, et al. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99(6):3684–9. Epub 2002/03/14. doi: 10.1073/pnas.052548299 PMID: 11981304; PubMed Central PMCID: PMC122584.

28. Wolf YI, Kondrashov AS, Koonin EV. Interkingdom gene fusions. Genome biology. 2000; 1(6): RESEARCH0013. Epub 2001/02/24. doi: 10.1186/gb-2000-1-6-research0013 PMID: 11178267; PubMed Central PMCID: PMC16144.
30. Sreenu VB, Kumar P, Nagaraju J, Nagarajaram HA. Microsatellite polymorphism across the M. tuberculosis and M. bovis genomes: implications on genome evolution and plasticity. BMC genomics. 2006; 7:78. Epub 2006/04/11. doi:10.1186/1471-2164-7-78 PMID: 16603092; PubMed Central PMCID: PMC1501019.

31. Brennan MJ, Delogu G, Chen Y, Bardarov S, Kriakov J, Alavi M, et al. Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. Infection and immunity. 2001; 69(12):7326–33. Epub 2001/11/14. doi:10.1128/IAI.69.12.7326-7333.2001 PMID: 11705904; PubMed Central PMCID: PMC98818.

32. Banu S, Honore N, Saint-Joanis B, Philpott D, Prevost MC, Cole ST. Are the PE-PGRS proteins of Mycobacterium tuberculosis variable surface antigens? Molecular microbiology. 2002; 44(1):9–19. Epub 2002/04/23. PMID: 11967065.

33. Honer Zu Bentrup K, Miczak A, Swenson DL, Russell DG. Characterization of activity and expression of isocitrate lyase in Mycobacterium avium and Mycobacterium tuberculosis. Journal of bacteriology. 1999; 181(23):7161–7. Epub 1999/11/26. PMID: 10572116; PubMed Central PMCID: PMC103675.

34. Springer B, Sander P, Sediacek L, Hardt WD, Mizrahi V, Schar P, et al. Lack of mismatch correction facilitates genome evolution in mycobacteria. Molecular microbiology. 2004; 53(6):1601–9. Epub 2004/09/03. doi: 10.1111/j.1365-2958.2004.04231.x PMID: 15341642.

35. Wanner RM, Guthlein C, Springer B, Bottger EC, Ackermann M. Stabilization of the genome of the mismatch repair deficient Mycobacterium tuberculosis by context-dependent codon choice. BMC genomics. 2008; 9:249. Epub 2008/05/30. doi: 10.1186/1471-2164-9-249 PMID: 18507851; PubMed Central PMCID: PMC2430213.

36. Walsh JB. Sequence-dependent gene conversion: can duplicated genes diverge fast enough to escape conversion? Genetics. 1987; 117(3):543–57. Epub 1987/11/01. PMID: 3692140; PubMed Central PMCID: PMC1203229.

37. Krakauer DC. Stability and evolution of overlapping genes. Evolution; international journal of organic evolution. 2000; 54(3):731–9. Epub 2000/08/11. PMID: 10937248.

38. Fukuda Y, Washio T, Tomita M. Comparative study of overlapping genes in the genomes of Mycoplasma genitalium and Mycoplasma pneumoniae. Nucleic acids research. 1999; 27(8):1847–53. Epub 1999/04/02. PMID: 10101392; PubMed Central PMCID: PMC149392.

39. Pecora ND, Gehring AJ, Canaday DH, Boom WH, Harding CV. Mycobacterium tuberculosis LprA is a lipoprotein agonist of TLR2 that regulates innate immunity and APC function. J Immunol. 2006; 177 (1):422–9. Epub 2006/06/21. PMID: 16765538.

40. Parker SK, Curtin KM, Vasil ML. Purification and characterization of mycobacterial phospholipase A: an activity associated with mycobacterial cutinase. Journal of bacteriology. 2007; 189(11):4153–60. Epub 2007/04/10. doi: 10.1128/JB.01909-06 PMID: 17416658; PubMed Central PMCID: PMC1913378.

41. Dedieu L, Serveau-Avesque C, Canaan S. Identification of Residues Involved in Substrate Specificity and Cytotoxicity of Two Closely Related Cutaınases from Mycobacterium tuberculosis. PloS one. 2013; 8(7):e66913. Epub 2013/07/12. doi: 10.1371/journal.pone.0066913 PMID: 23843969; PubMed Central PMCID: PMC3699616.

42. Rosas-Magallanes V, Deschavanne P, Quintana-Murci L, Brosch R, Gicquel B, Neyrolles O. Horizontal transfer of a virulence operon to the ancestor of Mycobacterium tuberculosis. Molecular biology and evolution. 2006; 23(6):1129–35. doi: 10.1093/molbev/msj120 PMID: 16520339.