Phenylalanine-Rich Peptides Potently Bind ESAT6, a Virulence Determinant of *Mycobacterium tuberculosis*, and Concurrently Affect the Pathogen’s Growth

Krishan Kumar1, Megha Tharad1, Swetha Ganapathy1, Geeta Ram1, Azeet Narayan2, Jameel Ahmad Khan2, Rana Pratap4, Anamika Ghosh1, Sachin Kumar Samuchiwal1, Sushil Kumar1, Kuhulika Bhalla1, Deepi Gupta3, Krishnamurthy Natarajan3, Yogendra Singh5, Anand Ranganathan1*

1 Recombinant Gene Products Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India, 2 Institute of Genomics and Integrative Biology, Delhi, India, 3 Immunology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India, 4 School of Life Sciences, Jawaharlal Nehru University, New Delhi, India

Abstract

**Background:** The secretory proteins of *Mycobacterium tuberculosis* (*M. tuberculosis*) have been known to be involved in the virulence, pathogenesis as well as proliferation of the pathogen. Among this set, many proteins have been hypothesized to play a critical role at the genesis of the onset of infection, the primary site of which is invariably the human lung.

**Methodology/Principal Findings:** During our efforts to isolate potential binding partners of key secretory proteins of *M. tuberculosis* from a human lung protein library, we isolated peptides that strongly bound the virulence determinant protein Esat6. All peptides were less than fifty amino acids in length and the binding was confirmed by *in vivo* as well as *in vitro* studies. Curiously, we found all three binders to be unusually rich in phenylalanine, with one of the three peptides a short fragment of the human cytochrome c oxidase-3 (Cox-3). The most accessible of the three binders, named Hcl1, was shown also to bind to the *Mycobacterium smegmatis* (*M. smegmatis*) Esat6 homologue. Expression of *hcl1* in *M. tuberculosis* H37Rv led to considerable reduction in growth. Microarray analysis showed that Hcl1 affects a host of key cellular pathways in *M. tuberculosis*. In a macrophage infection model, the sets expressing *hcl1* were shown to clear off *M. tuberculosis* in much greater numbers than those infected macrophages wherein the *M. tuberculosis* was not expressing the peptide. Transmission electron microscopy studies of *hcl1* expressing *M. tuberculosis* showed prominent expulsion of cellular material into the matrix, hinting at cell wall damage.

**Conclusions/Significance:** While the debilitating effects of Hcl1 on *M. tuberculosis* are unrelated and not because of the peptide’s binding to Esat6—as the latter is not an essential protein of *M. tuberculosis*—nonetheless, further studies with this peptide, as well as a closer inspection of the microarray data may shed important light on the suitability of such small phenylalanine-rich peptides as potential drug-like molecules against this pathogen.

Introduction

Tuberculosis (TB), a disease caused by *M. tuberculosis*, is completely curable, and yet, two million succumb to it every year [1,2]. In India, that along with sub-Saharan Africa has the largest number of TB cases, partial adherence to directly observed drug treatment regimen, coupled with non-availability of the drugs in remote areas combine devastatingly to exacerbate the problem, resulting in multi-drug resistant strains that then *de facto* necessitate the scientific community’s search for newer anti-TB molecules [3–5]. Tied with this seemingly intractable predicament is the lengthy anti-TB therapy that lasts on average six to eight months, giving ample opportunity for poor patients to play truant [6]. The search for new drug-like molecules against TB that can reduce the length of therapy as well as address the problem of resistance is, therefore, an urgent one.

As a starting point for our own efforts towards addressing this urgency, we decided to identify potent protein-protein interactions that must take place between the secretory proteins of *M. tuberculosis*, the carrier of TB, and their human counterparts at the primary site of infection, the human lung, in order for the infection to either take root, or, as in many cases, be cleared [7]. Our aim was, once such interactions had been identified, to use *de novo* protein/peptide libraries and screen for entities that are able to disrupt such interactions [8,9]. That some *M. tuberculosis* proteins, mostly those that are found in the culture filtrate of *M. tuberculosis*—and thus termed ‘the culture-filtrate’ proteins or CFPs—
that many among this set interact with human lung proteins has been known for some time [10–14]. A few of such interactions have also been studied in detail [14–16]; however, no comprehensive picture of the infection process has as yet emerged.

Here, we report our investigations with one such secretory protein, the Esat6 protein that has been implicated in the virulence and pathogenesis of *M. tuberculosis* [17–20]. Briefly, Esat6 is a 100 aa long protein that normally exists, indeed is secreted, as a 1:1 complex with its cohort the Cfp10 protein, the latter being also of roughly equal length [21]. Both proteins are encoded from the RD1 region of the *M. tuberculosis* genome, a region that has been found to be absent in the non-pathogenic strains of mycobacteria, esp. BCG [19]. Numerous structural and biochemical studies on this Esat6:Cfp10 complex bear out their all alpha-helical tertiary fold, the interaction itself a result of hydrophobic-hydrophobic interactions between the two partners spread over an 1800 angstrom wide surface [22]. Although these proteins are not crucial for the survival of *M. tuberculosis*, their absence potently reduces, in many cases abrogates, the pathogenesis of the bacteria [18,19,23,24]. More recently, it has been proposed that the 1:1 complex dissociates post secretion, thus leaving Esat6 free to perform its functions, among which also include induction of host apoptosis [25,26].

Our aim was to identify any host proteins that interact with free Esat6 either in a macrophage or in the extracellular matrix, once it has dissociated from its complex with Cfp10. In this context, we chose to screen the human lung proteins against Esat6, given that the primary site of infection is the human lung [7]. Herein, we report the identification of peptides that strongly bind Esat6 and, separately, appreciably affect the growth of the pathogen. None among these peptides were complete human proteins (and could thus be classified as inhibitors in their own entities); one was a portion of the mitochondrial Cox-3 protein, while the other two were formed as a result of aberrant ligation products during cDNA library construction. Nonetheless, the genes coding for all three peptides were sequenced and their integrity confirmed. A striking feature common to all three peptides was the presence of a high percentage of phenylalanine residues. One of the peptides, Hc11, was constitutively expressed inside mycobacterium and its effects on the bacterial host investigated.

**Results**

**Isolation of Peptides That Bind Esat6**

In order to search for host interacting partners of Esat6, bacterial two-hybrid reporter strain was co-transformed with esat6pBTtm and human lung cDNA library cloned in pTRG. We isolated three distinctly blue-coloured colonies, the colour being an indication that *in vivo* interaction had occurred. Employing repeated rounds of plasmid segregations, co-transformations, and in two cases PCR-amplification of the host gene followed by its re-ligation with the pTRG plasmid, we were able to confirm the interaction of the isolated host clones with Esat6 (Fig. 1A). To further validate the interaction, we performed liquid β-galactosidase assays and found that one of the host genes, named Hc11, was a much stronger binder of Esat6 compared with the other two (Hc12 and Hc13; Fig. 1B). When sequenced, Hc11 indicated an open reading frame (ORF) corresponding to a 27 amino acid long peptide (rich in phenylalanine) (Table 1). Interestingly, the ORF itself was a result of an incorrectly placed adaptor during the cDNA library construction, when the library members were being ligated to the recipient pTRG plasmid. A BLAST search showed that Hc11 protein shared no homology with any known protein in the available databases.

**Figure 1. Interaction of Esat6 with human cDNA library (HCL) clones.**

(A) BacterioMatch two-hybrid reporter strain was co-transformed with (A) hcl1pTRGnn+pBTtm (negative control); (B) hcl1pTRGnn+esat6pBTtm; (C) hcl2pTRGnn+esat6pBTtm; (D) hcl3pTRGnn+esat6pBTtm; and (E) gal11pTRG+lgf2pBT (positive control). Two individual colonies from each co-transformant were patched on X-gal indicator plates. (B) Confirmation of the *in vivo* interaction between Hc11 and Esat6 using the liquid β-galactosidase assay. Assays were performed in duplicates with appropriate controls. Mean±s.d values are displayed here. A similar pattern was observed each time the experiment was repeated. doi:10.1371/journal.pone.0007615.g001
Since Esat6 from \textit{M. smegmatis} (MSMEG_0066) and Esat6 from \textit{M. tuberculosis} (Rv3875) share 70\% identity, we decided to explore if Hcl1 could bind \textit{M. smegmatis} Esat6 as well. Indeed, in an \textit{in vivo} bacterial two-hybrid experiment, we found that Hcl1 interacted with \textit{M. smegmatis} Esat6 almost as potently as it did with \textit{M. tuberculosis} (results not shown). The other two binder peptides, Hcl2 and Hcl3 (Table 1) also displayed an unusually high number of the Phenylalanine residues. While Hcl3, like Hcl1, was also formed from an aberrant adapter ligation, analysis of Hcl2 confirmed that it was part of a larger protein, the human Cox-3. To investigate whether the full-length human Cox-3 interacts with Esat6, we synthesized the wild type \textit{cox-3} gene and cloned it in pTRGnn plasmid. We did not detect any interaction between the full-length Cox-3 and \textit{M. tuberculosis} Esat6 (results not shown).

**Pull-Down Studies**

To further validate the interaction between Esat6 and Hcl1, \textit{in vitro} pull-down assays were performed. For these assays, Esat6 was expressed as, separately, FLAG-tagged and His-tagged proteins, the proteins purified using DEAE-sepharose column under denaturing conditions and successfully resolubilized. To confirm the structural and conformational integrity of FLAG-Esat6 protein, we first studied its interaction with \textit{M. tuberculosis} Cfp10. Cfp10 was expressed as containing an N-terminal 6X His-tag and purified as a soluble protein under native conditions using Ni-NTA agarose column. Additionally, Hcl1 was expressed and purified as a GST-fusion protein under native conditions using a glutathione-sepharose column. Equal amounts (10 \( \mu \)g) of the interacting proteins were used for pull-down experiments. We found that purified Esat6 interacted with both Cfp10 as well as Hcl1-GST proteins (Fig. 2A and 2B).

**Effect of Hcl1 on the Intracellular Survival of \textit{M. tuberculosis}**

Once it was confirmed that Hcl1 interacts with Esat6, we decided to investigate if the peptide has any effect on the intracellular survival of \textit{M. tuberculosis} within macrophages. However, first, we cloned \textit{hcl1} in another shuttle vector, gppVV16 that has a C-terminal GFP-tag, to see if this peptide was expressed in mycobacteria in the first place. Cells were harvested from log phase cultures of \textit{M. tuberculosis} H37Rv, harbouring \textit{hcl1}gppVV16 and examined under a fluorescent microscope. The green fluorescence, compared with its complete absence in the pVV16-harbouring control cells clearly demonstrated to us the expression of Hcl-GFP, also suggesting that peptides like Hcl1 could be synthesized in mycobacterium (Fig. 3). Furthermore, a BLAST scan of phenylalanine-rich proteins within \textit{M. tuberculosis} revealed that mycobacteria are abundant with proteins rich in phenylalanine (results not shown).

To study the effect of Hcl1 on the survival of mycobacteria within human macrophages, THP1 cells were infected with

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**Table 1.** Primary amino acid sequences and characteristics of the peptide binders that interact with Esat6.

| Peptide | Sequence            | Amino Acids | Molecular Weight (kDa) | pI | Hydrophobic Content (%) |
|---------|---------------------|-------------|------------------------|----|------------------------|
| Hcl1    | AARRIRHEGELVSSFFFFFIENKFDNY* | 27          | 3.3                    | 5.5 | 41                     |
| Hcl2    | AAHEGESTQYQHTTPQVKLGILFITSEVFFAGFF* | 40          | 4.5                    | 6.2 | 33                     |
| Hcl3    | AARRIRGTESSLFFFFFPKATLMLSCS5VH* | 32          | 3.7                    | 9.3 | 41                     |

Phenylalanine residues are shown in bold.

**Footnotes:**
- aa: amino acids; MW: molecular weight; pI: isoelectric point; % hydr.: percentage hydrophobic content.
- *stop codon.

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**Figure 2.** \textit{In vitro} confirmation of the protein-protein interactions. (A) 16.5\% Tricine-SDS polyacrylamide gel stained with coomassie blue showing interaction between purified Esat6 and Cfp10. Lane 1: pre-stained protein marker, Lane 2: purified His-tagged Cfp10; Lane 3: purified FLAG-tagged Esat6; Lane 4: 10 \( \mu \)g Cfp10 alone incubated with Ni-NTA agarose and eluted; Lane 5: 10 \( \mu \)g Esat6 alone incubated with 1\% PVP blocked Ni-NTA agarose beads and subsequently eluted. (B) 16.5\% Tricine-SDS polyacrylamide gel stained with coomassie blue showing interaction between Esat6 and Hcl1. Lane 1: protein marker; Lane 2: purified His-tagged Esat6; Lane 3: purified Hcl1-GST; Lane 4: 5 \( \mu \)g Esat6 incubated with Ni-NTA agarose beads and subsequently eluted; Lane 5: beads initially bound with Esat6 and further incubated with purified GST protein; Lane 6: purified GST protein.

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**Reference:**
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mycobacteria harboring either hcl1pVV16 (Mtb/Hcl1) or pVV16 (Mtb/pVV16) at an MOI of 10 for 4 hours at 37 °C in an atmosphere of 5% CO2. Infected THP1 cells were harvested either immediately after infection (0 hours) or after 24 hours. THP1 cells were lysed and the mycobacteria plated on Middlebrook 7H11 agar plates for colony forming unit (CFU) analysis. Mycobacterial colonies were counted and mean ± s.d values from one experiment were plotted against time (in hours). Similar pattern was observed each time the experiment was repeated. A significant reduction in the mycobacterial CFUs was observed in the sets where THP1 cells were infected with Mtb/Hcl1, in comparison to where they were infected with Mtb/pVV16 (Fig. 4): approximately a 5-fold reduction in the number of colonies obtained from mycobacteria containing hcl1pVV16 in comparison to those harbouring just the vector control, pVV16, immediately after infection (Day 0, Fig. 4A). Although there was a significant decrease in colony count of mycobacteria one day after infection (Day 1), a more pronounced, approximately 10-fold reduction in CFUs was observed in mycobacteria expressing Hcl1, in comparison to the vector controls (Fig. 4B).

Figure 3. Expression of Hcl1 as a GFP fusion protein in M. tuberculosis. Three individual colonies each from mycobacteria harbouring either hcl1gfppVV16 (Mtb/GFP-Hcl1) or pVV16 (Mtb/pVV16) were grown till mid-log phase. Cells were examined under a fluorescent microscope. Panel A: Mtb/GFP-Hcl1 cells; Panel B: Mtb/pVV16 cells. First column in each panel shows bright field image of the sample.

Figure 4. Effect of Hcl1 on survival of mycobacteria within macrophages. THP1 cells were infected with mycobacteria harbouring either hcl1pVV16 (Mtb/Hcl1) or pVV16 (Mtb/pVV16). The figure shows result of CFU counts, immediately after 4 hours of infection (A), called Day 0 and 24 hours post infection (B), called Day 1. Experiment was performed in triplicates and mean ± s.d values are displayed here. A significant decline in the survival of mycobacteria carrying hcl1pVV16 was observed in comparison to the vector controls.
Effect of Hc1 on the In Vitro Growth of *M. tuberculosis*  
To study the effect of Hc1 on general growth of mycobacteria, mycobacterial cells were transformed with either hcl1pVV16 (Mtb/Hc1) or just the vector control, pVV16 (Mtb/pVV16). Three colonies from each transformant were picked for growth curve analysis. Optical density of each culture was measured after every 24 hours and plotted against time (in days) using mean±s.d (standard deviation) values from one experiment. Similar pattern was observed each time the experiment was repeated. It was found that the mycobacteria harbouring hcl1pVV16 showed a significant retardation in growth when compared with the vector controls (Fig. 5). The difference became evident as soon as the cells entered log phase and lasted till the culture reached stationary phase.

Effect of Hc1 on the Cell Morphology of *M. tuberculosis*  
Taking into consideration the effect of Hc1 on the growth and intracellular survival of mycobacteria, we decided to investigate whether Hc1 has any effect on cell shape and morphology. Electron micrographs of *M. tuberculosis* H37Rv cells harbouring either hcl1pVV16 or pVV16 alone were compared (Fig. 6). Cells harbouring hcl1pVV16 showed an extensive shedding of extracellular matrix from the surface (Fig. 6B and 6D). These cells with altered morphology seemed to be devoid of cell coat and appeared bare.

Effect of Hc1 on Global Gene Profile of *M. tuberculosis*  
Having seen the effect of Hc1 on *M. tuberculosis* growth and cell morphology, we next performed microarray experiments to further explore the effects of Hc1 on mycobacterial cells. Microarray experiments were performed with three biological replicates and four on-chip replicates. In order to identify genes that were differentially regulated in presence of Hc1, we compared gene expression profiles of mycobacteria harbouring either hcl1pVV16 or pVV16 alone. Total RNA was isolated from log phase cultures of mycobacteria containing either hcl1pVV16 or pVV16, labelled with Cy3/Cy5 dyes and hybridized to microarray slides containing 4750 oligonucleotides (70-mer), each representing a unique *M. tuberculosis* H37Rv gene. Raw intensity data were transferred to excel spread sheet for analysis using the Z score method as described previously [29]. Raw intensity data was normalized using Z score transformation that produced Z score values for all the genes in each array (Fig. 7). The difference in average Z score values for all the genes in treated (Mtb/Hc1) and control (Mtb/pVV16) was used to calculate the Z ratio. Genes having a Z ratio >1.5 and p value <0.05 were considered significant.

We found differential expression of 176 genes, of which 38 were found to be significantly upregulated and 138 genes significantly downregulated (Please see Supplementary Table S1). Of the genes that were upregulated, there were 7 genes coding for hypothetical proteins of unknown function, 6 conserved transmembrane proteins, 3 belonging to PE/PPE_PGRS family, 2 membrane transporters—one among them being Rv3877, part of Esp-1 secretion system, 2 transposases, 1 transcriptional regulator, 16 involved in various metabolic processes, and a protein kinase (pkaG). Genes that were downregulated contain 84 genes coding for hypothetical proteins of unknown function, 22 genes involved in DNA replication, transcription, and translation, 4 belonging to PE/PPE_PGRS family, 20 genes involved in various metabolic pathways including cell wall synthesis, 2 genes coding for conserved transmembrane proteins of unknown function, one heat shock protein (*hspA*), 3 genes coding for putative secretory proteins: Rv0559c, EsxH, and EsxG, one twin-arginine translocase protein A (tatA), and a putative protein transporter, secE2.

The differentially expressed genes were compared with the Rubin’s list of essential genes for *in vitro* growth and growth within macrophages [31,32]. We found one gene (*pknG*) that was found to be upregulated in our microarray experiment to be an essential gene required for *in vitro* growth of *M. tuberculosis* according to Rubin and co-workers (Fig. 8). Another gene, Rv3868, coding for a hypothetical protein, that was found to be upregulated has been shown to be essential for the growth of mycobacteria within macrophages (Fig. 8). Similarly, 19 genes that were downregulated in our experiments were found to be essential for *in vitro* growth of mycobacteria according to Rubin and co-workers (Fig. 9); some of these genes are involved in various metabolic processes including cell wall synthesis.

Quantitative PCR  
To validate our microarray experiments, we carried out real time PCR analysis of some of the identified genes. The genes chosen for such analysis were *groES* (co-chaperonin) and *bfrB* (bacterioferritin). The 16S rRNA gene was used as an internal control to compare the relative expression levels of these genes between treated (Mtb/Hc1) and control (Mtb/pVV16) samples. Both the genes showed approximately 2-fold reduction in Mtb/Hc1 samples in comparison to Mtb/pVV16 cells (Fig. 10).

Discussion  
In this study, we report isolation and characterization of phenylalanine-rich peptides that bind Esat6 protein of *M. tuberculosis*. Our aim was to study host-pathogen protein-protein interactions. For this purpose, we screened human lung cDNA libraries for potential interacting partners of the *M. tuberculosis* secretory protein Esat6. Using bacterial two hybrid system, we were able to isolate three strong binders against Esat6. Two among them,
named Hc11 and Hc13, when sequenced, showed that their ORFs had resulted from incorrectly placed adaptors during the cDNA library construction, while the third, named Hc12, was a fragment of human Cox-3 protein. Table 1 shows the three isolated peptides and their primary sequence characteristics. Without exception, they are hydrophobic in nature, possessing at least six to seven phenylalanine residues. While Hc11 and Hc13 display no homology to any known protein in the database—by virtue of the manner in which their corresponding ORFs have been formed—Hc12 on the other hand is a fragment of the 260 aa long human Cox-3 protein, that, we showed, does not itself interact with Esat6. Human Cox-3 shares a very high degree of homology with other Cox-3 proteins, and prominently with the bovine counterpart, where the Hc12 region is 86% identical to the human Cox-3. Interestingly, Hc11 and Hc13 display an all-helical fold, as seen in their threading models (results not shown), while Hc12 fragment is also all-helical in the bovine Cox-3 protein (pdb code 2EIJ). We showed that the full-length human Cox-3 does not interact with Esat6, while Hc12 fragment in isolation does. A closer look at the bovine Cox-3 crystal structure shows that Hc12 forms part of a seven-bundle helix that is the Cox-3 protein; further, Hc12 is partially embedded within the whole structure. It should be noted that both, Esat6 and Cfp10 are all-helical in character and their interaction is principally a hydrophobic one [22]. Additionally, peptides and proteins rich in phenylalanine have been seen to adopt helical folds, largely to do with the aromatic ring stacking [33].

That Esat6 plays an important role in M. tuberculosis virulence is well studied [17–20]. We decided to investigate the effect of Hc11 binding to Esat6 and consequently its outcome on the survival of mycobacteria within macrophages. We noticed a significant

![Figure 6. Effect of Hc11 on cell shape and morphology of M. tuberculosis.](image)

Figure 6. Effect of Hc11 on cell shape and morphology of M. tuberculosis. Transmission electron micrographs of mycobacterial cells harbouring either pVV16 (Mtb/pVV16) or hcl1pVV16 (Mtb/Hc11). Arrow heads in Panel B and D show the difference in cell morphology of mycobacteria containing Hc11 when compared with the control (see Panel A and C).

![Figure 7. Scatter plot of average Z score values from treated (Mtb/Hc11) and control (Mtb/pVV16) samples obtained after Z score transformation.](image)

Figure 7. Scatter plot of average Z score values from treated (Mtb/Hc11) and control (Mtb/pVV16) samples obtained after Z score transformation. Data points represent the average Z score values of genes obtained from six arrays.

![Graph](image)
decline in the survival of mycobacteria expressing Hcl1 within macrophages as indicated by the mycobacterial colony count post infection. Having studied the binding of Esat6 and Hcl1, both in vitro and in two-hybrid experiments, it was rational to assume that the observed effect could be because of this interaction resulting in the sequestration of Esat6. Esat6 and Cfp10 are part of the same operon, are co-transcribed and secreted as a tight 1:1 complex by the Esx-1 secretion system [34,21]. It is possible that the binding of Hcl1 to Esat6 poses steric hindrance for the interaction between Esat6 and Cfp10. A second possibility is that the Hcl1-Esat6 complex, when formed, interferes with the normal functioning of Esat6. Another interesting observation made during this study was that THP1 cells infected with mycobacteria containing pVV16 alone, showed extensive cell death as indicated by the size of the cell pellet obtained after the cells were harvested post-infection, in comparison to the THP1 cells infected with mycobacteria containing Hcl1. Live M. tuberculosis bacilli and Esat6 as such are known to induce apoptosis in macrophages [26]. It is possible that binding of Hcl1 to Esat6 or to some other molecule(s) inside the cell inhibits this process, although at present there is little proof to ascertain this conjecture and studies are currently underway to ascertain the possibility of the above-mentioned scenarios.

Next, we studied the effect of Hcl1 on the in vitro growth of mycobacteria. We found that mycobacteria containing Hcl1 showed a significant retardation in growth when compared to the controls. We were initially intrigued by this finding, since Esat6 is not known to play a defined role during the in vitro growth of mycobacteria [35]. Seeing the effect of Hcl1 on in vitro growth, we then checked its effect on cell morphology. We found that mycobacteria containing Hcl1 displayed an altered phenotype (Fig. 6). These cells showed extensive shedding of extracellular matrix and appeared bare.

We performed microarray experiments to further study the effect of Hcl1 on mycobacteria. We found a differential expression

Figure 8. Comparison with Rubin’s lists of genes that were found to be upregulated in microarray experiments. Red circle shows the genes upregulated; yellow and green circles show the genes essential for in vitro growth and for survival of mycobacteria within macrophages, respectively.
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Figure 9. Comparison with Rubin’s lists of genes that were found to be downregulated in microarray experiments. Red circle shows the genes upregulated; yellow and green circles show the genes essential for in vitro growth and for survival of mycobacteria within macrophages, respectively.
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of 176 genes, of which 38 genes were significantly upregulated and 138 genes significantly downregulated. Using the elegant technique of transposon-based mutagenesis, Rubin and coworkers have identified sets of genes essential for in vitro growth of M. tuberculosis as well as for survival within macrophages [31,32]. We compared the genes differentially regulated in our microarray experiment with Rubin’s list of genes essential for in vitro growth and for growth within macrophages. On comparison, we found that, of the upregulated genes, pknG is essential for in vitro growth of mycobacteria, and another gene, Krv3868 (coding for a hypothetical protein), essential for survival of mycobacteria within macrophages. Similarly, 19 genes that were downregulated in our microarray experiment were found to be common to Rubin’s list of genes essential for in vitro growth of mycobacteria. Among these were two genes for putative fatty acid desaturases (desA1 and desA2), four hypothetical proteins, five ribosomal proteins, PE_PGRS family protein (PE5), atpF (ATP synthase subunit B), groES (co-chaperonin), folB (probable dihydroyeopterin aldolase), nadA (quinolinate synthetase), fdaC (probable ferredoxin), thiS (sulfur carrier protein), and kasB (β-ketoacyl ACP synthase).

Mycobacteria contain a thick protective cell wall chiefly consisting of an extensively modified fatty acid complex, called mycolic acid [36]. It has been suggested that acyl desaturases play a role in the biosynthesis of mycolic acids by introducing position-specific double bonds that can subsequently be modified [37,38]. Considering the debilitating effect of Hcl1 on cell surface of mycobacteria, it is tempting to speculate that a downregulation of desA1 and desA2 could affect mycolic acid biosynthesis and hence the cell wall integrity. Similarly, it has been shown that a deletion of kasB (involved in the biosynthesis of meromycolic acids) causes a change in mycobacterial colony morphology and loss of acid-fastness [39]. This again correlates well with the observed alteration in cell envelope of mycobacteria in this study. However, the effect may not exactly be of the same type as in case of the kasB strain, for in our case, it is not a deletion kasB but mere downregulation. Also, in a recent study, kasB and desA2 were shown to be significantly downregulated in mycobacteria treated with vancomycin [40]. The authors suggest that under conditions of growth arrest, bacteria slow down the synthesis of energy expensive cell wall components. M. tuberculosis contains one cnp10 (groES) gene present upstream of cnp60.1 (groEL1), which codes for a 10 kDa partner protein, Cnp10 [41]. Interestingly, it has been demonstrated that in M. smegmatis, GroEL1 interacts with KasA and modulates mycolic acid synthesis during biofilm formation, and a groEL1 mutant of M. smegmatis showed significant reduction in the levels of KasA and KasB [42]. Since M. tuberculosis encodes a similar groEL protein and Cnp10 as a helper protein, it is possible that downregulation of cnp10 could affect the functioning of Cnp60.1 during mycolic acid synthesis.

Other repressed genes, namely, thiS, fdaC, nadA, atpF, and folB code for proteins known to be involved in various metabolic pathways like siderophore biosynthesis, electron transport, NAD synthesis, ATP synthesis, and folate biosynthesis, respectively. It seems likely that all genes that were differentially regulated are directly/indirectly responsible for the observed effects of Hcl1 on growth and survival of mycobacteria. These findings suggest that Hcl1 is either working as a general antimicrobial peptide or interacting with some other molecule(s) inside the cell and hence interfering with normal cellular processes. As has already been mentioned, a threading-based modelling predicted a helical structure for Hcl1, a common feature of many antimicrobial peptides, and many such peptides have been found to be rich in phenylalanine residues [43]. However, whereas Hcl1 showed adverse effects on the growth of M. tuberculosis, it had no visible effect on the growth of Escherichia coli (E. coli), strengthening our belief that Hcl1 is interacting with molecule(s) specific to the mycobacterial cell.

In conclusion, this study demonstrates that Hcl1, a small peptide unusually rich in phenylalanine residues, affects the growth and pathogenesis of M. tuberculosis. What remains to be seen is whether the presence of Hcl1 in mycobacteria hinders Esat6 secretion. This could be assessed by comparing the levels of Esat6 protein in the culture filtrate of mycobacteria with and without Hcl1. Also, a pull-down assay from mycobacterial cell lysate is likely to shed some light on what might be many mycobacterial interacting partners of Hcl1. The localization of Esat6, in the presence and absence of Hcl1, within infected macrophages would also, we believe, shed new light on the possibility that Esat6 is involved in a hitherto unknown mechanism that helps the mycobacteria evade the host immune apparatus to a large extent. Finally, we hope that these studies might provide important clues regarding the mechanism of action of Hcl1 and other such phenylalanine-rich peptides and the information thus obtained could be further utilized in designing/improving peptide-based inhibitors against M. tuberculosis.

Materials and Methods

Bacterial Two-Hybrid Studies

Bacteriormatch™ two-hybrid system vector kit and Bacteriormatch™ Reporter strain competent cells were purchased from Stratagene, USA. The Bacteriormatch™ kit was supplied with bacterial two-hybrid system plasmids pBT, pTRG and control plasmids pBT-LGF2 and pTRG-Gall1™. The human lung cDNA library (cloned in pTRG vector) was purchased from Stratagene, USA. The bait and target expression vectors were modified to have a unique SnaBI site in its MCS for blunt end clonings [8].
The full length gene for esat6 (Rv3875) was amplified from *M. tuberculosis* H37Rv genomic DNA using forward (5'-CCT ACG TAA TGA CAG AGC AGT GG A A-3') and reverse (5'-CCT ACG TAT GCC AAG ATC GCA GTG ACG T-3') primers, and cloned in pBTnn vector. Also, the corresponding homologue of esat6 (MSMEG_0060) from *M. smegmatis* LR222 was amplified from genomic DNA using the forward (5'-CCG ATT TCT ACG TAA TGA CAG AGC AGT GAT ATT GC (reduced GST tag) and reverse primers (5'-CCG ATT TCT ACG TAG GCA AAC ATT CCC GTG ACG CCG GCC-3') and cloned in pBttn. Reporter strain competent cells were co-transformed with equal amounts (250 ng each) of esat6pBTtn and human lung cDNA library and plated on X-Gal indicator plates containing kanamycin (50 µg/ml), chloramphenicol (50 µg/ml), tetracycline (12.5 µg/ml), X-Gal (80 µg/ml), IPTG (25 µM), and X-Gal inhibitor (200 µM). Plasmids pBT-LGF2 (αCL-FLAG fusion) and pTRG-Gal11p (RNAAP subunit-Gal11p fusion) provided the positive controls, whereas empty pBttn and pTRGm plasmids served as negative controls for all experiments. Positive interactions were judged by the blue colour of the colonies obtained and further verified by repeated clonings and cotransformations. The interactions were further verified by performing liquid β-galactosidase assay as described earlier [27].

**Cloning of *hcl1*, *Esat6*, and *Cfp10* for Protein Expression**

Forward (5'-AGG GAT CCT ACG TAA GAA TTC GGC AGC AG-3') and reverse (5'-AGG GAT CCT ACG TAA TGA TTA TTA AAC TT-3') primers were used to amplify the ORF corresponding to Hcl1 protein from the original lung cDNA library clone. The PCR product was digested and cloned in *Sma*I site in pGEX4T3uun vector [8] resulting in a fusion protein with a C-terminal GST tag. The esat6pBTtn plasmid DNA was digested with *Nsi*I/BglII and the resulting 346 bp fragment was cloned in *Npl*/BamHI-digested pFLAGCMV6a vector to obtain esat6pFLAGCMVM6a. The esat6pFLAGCMVM6a plasmid DNA was digested with *Nco*I/EcoRI and a 359 bp fragment was cloned in *Npl*/EcoRI-cut pET28a (Novagen) vector. The clone, flagesat6pET28a, resulted in expression of Esat6 as a fusion protein with an N-terminal FLAG-tag but no His-tag. The ORF corresponding to Cfp10 protein was PCR amplified from *M. tuberculosis* H37Rv genomic DNA using forward (5'- CCT ACG TAA TGA CAG AGA TGA AGA CCG A-3') and reverse (5'- CCT ACG TAA GTA CTT AAG CCC ATT TTG CGC GAG A-3') primers. The PCR product was digested with *Sna*I and cloned in *Sma*I-cut pBADHisAnn vector. The clone, cpp10pBADHisAnn, resulted in expression of Cfp10 as a fusion protein with a C-terminal 6x His-tag. Similarly, for the expression of Esat6 with a C-terminal 6X His-tag, the ORF was amplified as described above from H37Rv genomic DNA and cloned in pBADHisAnn vector.

**Protein Expression and Purification of *Hcl1-GST***

*E. coli* BL21 (DE3) (Novagen) cells harbouring hcl1pGEX3T3m were grown till mid-log phase and induced with 1 mM IPTG for 3 hours with constant shaking at 37°C. Cells were harvested and the pellet was washed with PBS (40 mM K₂HPO₄, 10 mM KH₂PO₄, 150 mM NaCl, pH 7.2). Cell pellet was resuspended in (1% of the original culture volume) lysis buffer (100 mM EDTA, 1 mM PMSF, 0.5 mM DTT in PBS) and sonicated till a clear lysate was obtained. Triton X-100 was added to a final concentration of 1% and the tubes were incubated at 4°C for 30 minutes with constant shaking. Lysate was then centrifuged and the clear supernatant was transferred to fresh tubes. Glutathione-agarose beads (Amersham) were washed thrice with PBS. Desired amount of glutathione-agarose beads were added to the supernatant and incubated at 4°C for 3 hours with constant shaking. Flow-through was collected and beads were washed thrice with PBS containing 1% Triton X-100 followed by a single wash of 50 volume of TN buffer (50 mM tris-Cl, 150 mM NaCl, pH 7.5). Resin bound proteins were eluted using elution buffer (10 mM reduced glutathione in 50 mM tris-Cl, pH 8.0) and analyzed on SDS-PAGE. Purified protein was dialyzed against PBS, pH 7.5, quantified and stored at -20°C in aliquots.

**Protein Expression and Purification of *Esat6***

*E. coli* BL21 (DE3) cells harbouring flagesat6pET28a were grown till mid-log phase and induced with 1 mM IPTG for 3 hours with constant shaking at 37°C. Cells were harvested and the pellet was washed with PBS. Cell pellet was resuspended in lysis buffer (8 M urea in 40 mM phosphate buffer, pH 7.5) and sonicated till a clear lysate was obtained. The lysate was centrifuged and the supernatant was transferred to a fresh tube. The supernatant was mixed with DEAE-sepharose (Amersham) and incubated at room temperature for 1.5 hours with constant shaking. Beads were washed thrice with lysis buffer and the resin bound proteins were eluted using a gradient of NaCl from 0 M to 1 M, prepared in lysis buffer. The gradient elution was performed using AKTA FPLC (Amersham) system and the eluted fractions were analyzed on SDS-PAGE. For purification of His-tagged Esat6, log phase culture of *E. coli* TOPO10 (Invitrogen) cells containing esat6pBADHisAnn were induced with 0.02% L-arabinose for 3 hours at 37°C. Cells were pelleted and washed with PBS. Pellet was resuspended in lysis buffer (100 mM NaH₂PO₄, 10 mM tris-Cl, pH 8.0 and sonicated. Lysate was cleared by centrifugation and pellet thus obtained was solubilized in lysis buffer containing 8 M urea, 10 mM imidazole, and 1% Triton X-100. Solution was centrifuged and supernatant was loaded on a Ni-NTA agarose (Qiagen) column. Beads were washed once with buffer B and thrice with buffer C (buffer B containing 20 mM imidazole). Finally, protein was eluted using elution buffer (100 mM NaH₂PO₄, 10 mM tris-Cl, 8 M urea, 350 mM imidazole, pH 8.0). Purified protein was refolded by sequentially and slowly dialyzing it against PBS with decreasing concentration of urea. Protein was concentrated using Amicon® Ultra (Millipore) column, quantified and stored at -20°C in aliquots.

**Protein Expression and Purification of *Cfp10***

*E. coli* TOP10 cells harbouring cfp10pBADHisAnn were grown till mid-log phase and induced with 0.02% L-arabinose for 3 hours at 37°C. Cells were pelleted and resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole, pH 8.0). Lysozyme was added to a final concentration of 1 mg/ml and tubes were incubated at 4°C for 30 minutes with shaking. Samples were sonicated till a clear lysate was obtained. Triton X-100 was added to a final concentration of 1% and incubated at 4°C for 30 minutes with shaking. Lysate was centrifuged and the supernatant was loaded on a Ni-NTA agarose column. Beads were washed once with lysis buffer and thrice with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole, pH 8.0). Protein was eluted using elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 300 mM imidazole, pH 8.0), dialyzed against PBS, quantified and stored at -20°C.

**In Vitro Protein-Protein Interaction**

**Esat6 and Cfp10.** Ni-NTA agarose beads were mixed with 10 µg of purified Cfp10-His protein and incubated at 4°C with shaking for 1.5 hours. Beads were washed twice with 50 mM phosphate buffer containing 500 mM NaCl (wash buffer 1) and once with PBS containing 0.1% Tween-20 and 14 mM β-mercaptoethanol (wash buffer 2). Beads were then blocked with

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**Anti-Mycobacterial Peptides**

[Further content follows the image.]
1% polyvinyl pyrrolidone (PVP) for 1 hour. To this, 10 µg of purified FLAG-Esat6 protein was added and incubated at 4°C for 1.5 hours. In a control experiment, Ni-NTA agarose beads were blocked with 1% PVP and incubated with 10 µg of purified FLAG-Esat6 protein at 4°C for 1.5 hours. Finally, beads were extensively washed with wash buffer 1. Resin bound proteins were eluted by boiling the beads in 2X SDS loading dye (20% glycerol, 4% SDS, 125 mM tris-Cl, pH 6.8, and 4% β-mercaptoethanol) and resolved on 16.5% tricine-SDS polyacrylamide gel.

**Esat6 and Hc1.** Purified His-tagged Esat6 protein (10 µg) was incubated with Ni-NTA agarose beads for 1.5 hours at 4°C. Beads were washed twice with buffer B (100 mM NaH₂PO₄, 10 mM tris-Cl, 20 mM imidazole, pH 7.4) and finally one wash with buffer C (30 mM imidazole in PBS, pH 7.4). To the beads, 10 µg purified Hc1 protein was added and kept for binding at 4°C for 1.5 hours. In a control experiment, 10 µg purified Hc1 protein was added to Esat6 bound Ni-NTA agarose beads. Beads were finally washed twice with buffer C and proteins bound were eluted by boiling the beads with 2X SDS loading dye and resolved on 16.5% tricine-SDS polyacrylamide gel.

**Construction of Expression Vectors**

The original pVV16 vector was modified to have a unique SnoBI site in its MCS for blunt-end cloning. Forward (5’-CCC CAT GGC ATA TGT ACG TAA TGA TTT ATG AGG CTC TCT TCG A-3’) and reverse (5’-CCC TCG AGA AGC TTT AGC TAG ACC TTC AGC AGG TCG CCT TC-3’) primers were designed to amplify a 599 bp fragment from pfrpBT1. Amplified PCR product was digested with NdeI/HinIII and cloned in pVV16 SnoBI vector. The corresponding clone was purified as described above and cloned in SnoBI-cut gfppVV16 to generate hcl1gfppVV16.

**Expression of Hc1 in M. tuberculosis**

Mycobacteria harbouring either hcl1gfppVV16 or pVV16 were grown at 37°C with constant shaking till mid-log phase in Middlebrook 7H9 broth (Difco™, BD), supplemented with 10% ADG (BBL™, BD), 0.05% Tween-80, 0.2% glycerol, and 25 µg/ml kanamycin. Cells were harvested and fixed with 2% paraformaldehyde solution for 1 hour at 4°C [28]. Fixed cells were allowed to adsorb on a 300 mesh copper grid and air dried. Samples were then stained with 1% uranyl acetate for 30 seconds and photographed using a FEI Tecnai 12 Electron Microscope.

**Transmission Electron Microscopy**

M. tuberculosis H37Rv cells harbouring either hcl1gfppVV16 or pVV16 were grown at 37°C with constant shaking till mid-log phase in Middlebrook 7H9 broth (Difco™, BD), supplemented with 10% ADG (BBL™, BD), 0.05% Tween-80, 0.2% glycerol, and 25 µg/ml kanamycin. Cells were harvested, washed with PBS and fixed by treating with 2% paraformaldehyde solution for 1 hour at 4°C [28]. Fixed cells were allowed to adsorb on a 300 mesh copper grid and air dried. Samples were then stained with 1% uranyl acetate for 30 seconds and photographed using a FEI Tecnai 12 Electron Microscope.

**Total RNA Isolation from M. tuberculosis**

Total RNA was isolated as described previously [8] with some modifications. Cells were harvested from exponentially growing mycobacteria harbouring either hcl1gfppVV16 (Mtb/Hcl1-treated sample) or pVV16 (Mtb/pVV16-control sample) and washed with 0.1% Tween 20. Pellet was resuspended in 400 µl TES buffer (50 mM Tris-Cl, pH 7.4, 10 mM EDTA, and 1% SDS). To this an equal volume of water-saturated phenol was added. Approximately an equal volume of acid-washed glass beads was added for efficient cell disruption. Tubes were vigorously vortexed and incubated at 65°C for 1 hour with intermittent vortexing after every 15 minutes. Finally, the tubes were chilled on ice for 5 minutes and centrifuged at 13,000 rpm for 15 minutes at 4°C. Aqueous phase was transferred to fresh tubes and extracted thrice with chloroform: isoamylalcohol (24:1). RNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volume of chilled ethanol. For efficient precipitation of RNA, tubes were incubated at −80°C for 30 minutes and centrifuged at 13,000 rpm for 30 minutes at 4°C. RNA pellet was washed with 70% ethanol, dried and resuspended in 30 µl of DEPC-treated water. RNA was checked for quality on 1% denaturing agarose gel and purified using RNeasy purification kit (Qiagen) followed by on-column DNase (0.7 U/µl) (Ambion) treatment for 30 minutes at room temperature, as described by the manufacturer. RNA was checked for quantity and quality on a NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific).

**Labeling of cDNA with Cy Dyes**

Equal amounts of RNA (9.0 µg) from both control (Mtb/ pVV16) and treated (Mtb/Hc1) samples were used for preparing labeled cDNA for microarray experiment. Total RNA was labeled
with Cy3/Cy5-dUTP using CyScribe First-Strand cDNA labeling kit (Amersham) as described by the manufacturer. Labeled product was treated with RNAse A (0.03 U/μl) (Fermentas) and RNAse H (0.05 U/μl) (Fermentas) for 20 minutes at 37 °C to remove the RNA. Labeled cDNA was purified and eluted using MinElute PCR Purification kit (Qiagen) and analyzed on a NanoDrop™ spectrophotometer for quantity and quality. Incorporation of Cy3 and Cy5 dye was determined by measuring the absorbance at 550 nm and 650 nm, respectively. Labeled cDNA was vacuum dried and stored at −20°C till further use.

**Microarray Slides Hybridization**

*M. tuberculosis* H37Rv microarray slides (Version 4) were obtained from Pathogen Functional Genomics Resource Center (PFGRC) [1]. Craig Venter Institute (JCVI). The hybridization protocol used here is as described by The Institute for Genomic Research, SOP#8 (http://pfgrc.jcvi.org/index.php/microarray/protocols.html) with some minor modifications. The slides were incubated in pre-warmed (42°C) pre-hybridization buffer (5X SSC, 0.1% SDS, 1% BSA) and incubated at 42°C for 1 hour in 50 ml Falcon tubes. The slides were thoroughly rinsed with distilled water followed by isopropanol and quickly dried by centrifugation at 1,500 rpm for 15 minutes. The labeled cDNA probe was resuspended in 50 μl hybridization buffer (5X SSC, 40% formamide, 0.1% SDS, 0.1 mM DTT, 0.6 μg/ml salmon sperm DNA). This probe mixture was vortexed and heated at 95°C for 5 minutes, vortexed and then again heated at 95°C for another 5 minutes. The denatured probe mixture was then added to the slides and covered with a coverslip. The hybridization cassette (Corning) was sealed and submerged in a water bath set at 42°C for 20 hours. Post hybridization, coverslips were removed and slides were washed 5 times with pre-warmed (at 55°C) Low Stringency Wash buffer (2X SSC, 0.1% SDS, 0.1 mM DTT). Slides were then washed 5 times with Medium Stringency Wash buffer (0.1X SSC, 0.1% SDS, 0.1 mM DTT). Finally, slides were washed five times with High Stringency Wash buffer (0.1X SSC, 0.1 mM DTT) and thoroughly rinsed with distilled water. Slides were dried by centrifugation at 1,500 rpm for 15 minutes and stored under dark and dry conditions.

**Microarray Data Analysis**

Slides were scanned using a dual laser ScanArray Gx Scanner (PerkinElmer) at best signal and least noise PMT gain. ScanArray Express (PerkinElmer) software was used to convert the hybridization signals into raw intensity values, which were then transferred to Microsoft Excel spreadsheet for further analysis. Data analysis was done using Z-score method described previously [29]. Raw intensity data for Cy3/Cy5 dyes (for both Mtb/pVV16-control as well as Mtb/Hcl1-treated samples) as ‘mean-background’ was extracted from six arrays and used for normalization. Raw intensity values were log_{20} transformed and average and standard deviation calculated for all the genes in each replicate and across all the arrays. Z-score values were calculated by subtracting the overall average intensity from log_{20} transformed raw intensity and dividing by measured standard deviation in a single experiment and for all replicates. Average of all the replicates for both treated (Mtb/Hcl1) and control (Mtb/pVV16) samples were used to calculate Z-Score values.

The hybridization cassette (Corning) was sealed and submerged in a water bath set at 42°C for 20 hours. Post hybridization, coverslips were removed and slides were washed 5 times with pre-warmed (at 55°C) Low Stringency Wash buffer (2X SSC, 0.1% SDS, 0.1 mM DTT). Slides were then washed 5 times with Medium Stringency Wash buffer (0.1X SSC, 0.1% SDS, 0.1 mM DTT). Finally, slides were washed five times with High Stringency Wash buffer (0.1X SSC, 0.1 mM DTT) and thoroughly rinsed with distilled water. Slides were dried by centrifugation at 1,500 rpm for 15 minutes and stored under dark and dry conditions.

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**Supporting Information**

**Table S1** Differentially regulated genes in microarray experiments.

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**Author Contributions**

Conceived and designed the experiments: KN YRS AR. Performed the experiments: KK MT SG AN JAK RP AG SKS SK KB DG. Analyzed the data: KK MT SG GR KN YRS AR. Contributed reagents/materials/analysis tools: KK MT. Wrote the paper: KK AR.
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