Evidence for a rapid rate of molecular evolution at the hypervariable and immunogenic *Mycobacterium tuberculosis* **PPE38** gene region

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Abstract

**Background:** **PPE38** (Rv2352c) is a member of the large **PPE** gene family of *Mycobacterium tuberculosis* and related mycobacteria. The function of **PPE** proteins is unknown but evidence suggests that many are cell-surface associated and recognised by the host immune system. Previous studies targeting other **PPE** gene members suggest that some display high levels of polymorphism and it is thought that this might represent a means of providing antigenic variation. We have analysed the genetic variability of the **PPE38** genomic region on a cohort of *M. tuberculosis* clinical isolates representing all of the major phylogenetic lineages, along with the ancestral *M. tuberculosis* complex (MTBC) member *M. canettii*, and supplemented this with analysis of publicly available whole genome sequences representing additional *M. tuberculosis* clinical isolates, other MTBC members and non tuberculous mycobacteria (NTM). Where possible we have extended this analysis to include the adjacent **plcABC** and **PPE39/40** genomic regions.

**Results:** We show that the ancestral MTBC **PPE38** region comprises 2 homologous **PPE** genes (**PPE38** and **PPE71**), separated by 2 **esat-6** (esx)-like genes and that this structure derives from an esx/esx/PPE duplication in the common ancestor of *M. tuberculosis* and *M. marinum*. We also demonstrate that this region of the genome is hypervariable due to frequent **IS6110** integration, **IS6110**-associated recombination, and homologous recombination and gene conversion events between **PPE38** and **PPE71**. These mutations result in combinations of gene deletion, gene truncation and gene disruption in the majority of clinical isolates. These mutations were generally found to be **IS6110** strain lineage-specific, although examples of additional within-lineage and even within-cluster mutations were observed. Furthermore, we provide evidence that the published *M. tuberculosis* H37Rv whole genome sequence is inaccurate regarding this region.

**Conclusion:** Our results show that this antigen-encoding region of the *M. tuberculosis* genome is hypervariable. The observation that numerous different mutations have become fixed within specific lineages demonstrates that this genomic region is undergoing rapid molecular evolution and that further lineage-specific evolutionary expansion and diversification has occurred subsequent to the lineage-defining mutational events. We predict that functional loss of these genes could aid immune evasion. Finally, we also show that the **PPE38** region of the published *M. tuberculosis* H37Rv whole genome sequence is not representative of the ATCC H37Rv reference strain.
Background

The *Mycobacterium tuberculosis* genome contains two large gene families that together comprise around 10% of its protein coding capacity [1]. These families, termed PE and PPE, appear to have originated in the fast growing mycobacterial species before undergoing extensive expansion and diversification in certain slow growing species, particularly *M. ulcerans*, *M. marinum* and members of the *M. tuberculosis* complex (MTBC) [2]. The large multi-protein families encoded by these genes are of unknown function, although reports suggest that at least some members are cell surface associated [3-6] and can be antigenic [4,5,7-9], a finding that has stimulated interest in their potential role in vaccine production, e.g. [10,11]. PPE proteins contain a proline-proline-glutamic acid (PPE) amino acid sequence at positions 7-9 in a highly conserved N-terminal domain of approximately 180 amino acids. The C-terminal domains of both PE and PPE protein families are highly variable in both size and sequence and often contain repetitive DNA sequences that differ in copy number between genes [1]. Several studies have shown that some PE and PPE genes are polymorphic and this has been interpreted as indicating strong selection pressure for antigenic variants that may aid in host immune evasion [3,7,12-17].

A recent phylogenetic analysis of the 69 PPE genes present in the *M. tuberculosis* reference strain H37Rv has uncovered their evolutionary relationships and reveals that they can be divided into several subfamilies [2] (Figure 1). PPE38 (Rv2352c) is shown to be a member of PPE sublineage IV (the SVP subfamily) and analysis of its protein sequence confirms that it encodes the SVP subfamily-defining amino acid sequence (GxxSVPxxW) at positions 309 - 317. However, along with the closely related gene PPE49 (Rv3125c), it shares a more recent common ancestor with PPE sublineage V members (the MPTR subfamily) than with any other member of the SVP sublineage (Figure 1). Although no reports are available regarding its antigenicity or other biochemical features, because of its position on the “border” of sublineages IV and V, PPE38 was included in a larger study aimed at determining the genetic variation of PE and PPE genes between various strains of *M. tuberculosis* (manuscript in preparation). Here we present our analysis of this gene and its surrounding region using a cohort of phylogenetic variants that may aid in host immune evasion [3,7,12-17].

Detailed analysis of the PPE38 region

In order to analyse the variation in this region more thoroughly, we designed additional primers (PPE38IntF/IntR, Table 1, Figure 2b) to allow PCR analysis and sequencing of the region between PPE38 and PPE71. Sequence analysis of the complete 3.4 kb product produced using the PPE38F/R primers in the H37Rv ATCC reference strain, one *M. canetti* and 4 of our clinical *M. tuberculosis* isolates species and 7 slow growing species). We show that this region is hypervariable in MTBC members and that this has resulted in a rapid rate of genetic divergence occurring between most *M. tuberculosis* strain lineages.

Results

Identification of the variable PPE38 region and the RvD7 deletion

The published *M. tuberculosis* H37Rv genome sequence [1] predicts the amplification of a 1335 bp PCR product spanning the entire PPE38 gene when using the PPE38F/R primer pair (Figure 2a, Table 1). However, our analysis of 3 *M. canetti* clinical isolates, the H37Rv and H37Ra American Type Culture Collection (ATCC) reference strains (ATCC numbers 25618 and 25177 respectively), and 40 *M. tuberculosis* clinical isolates from different IS6110 RFLP-defined strain lineages covering all three principal genetic groups (PGGs) [18], revealed that only 7 strains produced this amplicon. Most samples (including the H37Rv and H37Ra ATCC reference strains and the 3 *M. canetti*’s) produced a dominant amplicon of approximately 3.4 kb, while other samples produced amplicons of alternate sizes varying from approximately 2.5 to 5 kb, and 3 samples failed to amplify. Analysis of the H37Ra whole genome sequence revealed that the 3.4 kb amplicon (actual predicted size = 3398 bp) results from the presence of a second copy of PPE38 along with 2 esat-6 (esx)-like genes (annotated as MRA_2374 and MRA_2375 in H37Ra) in this region (Figure 2b) [19]. The second copy of PPE38 has been previously identified and designated as PPE71 in the CDC1551 whole genome sequence [20]. Its coding region is identical to PPE38 and both genes also share the same 5'-untranslated region up to position -35 bp. As previously suggested by Zheng and colleagues [19], this genomic structure suggests that the published H37Rv sequence represents the result of a homologous recombination event between PPE38 and PPE71 that has deleted one of these genes along with MRA_2374 and MRA_2375. This deletion is annotated as RvD6 in their analysis of the H37Ra whole genome sequence [19]. However, the authors did not acknowledge that the term RvD6 was appropriated in 2005 to define a specific variation between the *M. bovis* and H37Rv genomes [21]. For purposes of clarity and uniformity we therefore propose that this deletion rather be termed RvD7.
Phylogenetic reconstruction of the evolutionary relationships between members of the H37Rv M. tuberculosis PPE protein family members. The phylogenetic tree was constructed from a phylogenetic analysis done on the 180 aa N-terminal domains of the PPE proteins. Results show the division of PPE proteins into 5 sublineages with PPE38 (Rv2352c, highlighted in green) located at the border of sublineages IV (SVP subfamily) and V (MPTR subfamily). Reproduced from ref [2] with permission from the authors.
Schematic representations of the PPE38 gene region in the H37 reference strain published sequences. The PPE38 region from the published H37Rv (2a) and H37Ra (2b) sequences are shown. Colour coding as follows: PPE38 pale blue, PPE71 dark blue, MRA_2374 pale green, MRA_2375 dark green. Locations of the PPE38F/R and PPE38 IntF/R primers are shown.

**2a. H37Rv ATCC reference strain (published whole genome sequence)** The published H37Rv sequence [1] represents the RvD7 genotype. Recombination between PPE38 and PPE71 results in a single PPE38/71 gene (Rv2352c) and loss of the 2 esx-like genes MRA_2374 and MRA_2375. The PPE38F/R primers (black arrows) are predicted to produce an ampli-con of 1335 bp from the RvD7 genotype. It is impossible to determine which PPE38/71 gene has been deleted hence the mixture of colours used. The published H37Rv sequence is not representative of the H37Rv ATCC reference strain, most clinical isolates, or the H37Ra whole genome sequence [19]. This genotype is also seen in strains SAWC 2240 (CAS, F20), SAWC 1748 (Pre-Haarlem, F24), SAWC 1595 (Quebec/S), SAWC 1841 (Haarlem, F4), CPHL_A (WA-1, M. africanum), T17 (PGG1, EAI), EAS054 (PGG1, EAI), strain C (LCC, "3 bander") and Haarlem (PGG2, F4) [see additional file 1].

**2b. H37Rv ATCC reference strain (actual) and H37Ra (published whole genome sequence)** This represents the ancestral MTBC genotype that is also seen in M. canettii. It contains the 2 identical PPE38 (MRA_2373) and PPE71 (MRA_2376) genes separated by the 2 esx-like genes MRA_2374 and MRA_2375. Gene annotations are as reported for the H37Ra published sequence [19]. Locations of primers used for PCR and sequence analysis are indicated (black arrows). This is also the true genotype of the ATCC reference strain H37Rv.
confirmed its complete homology to the published sequence of H37Ra [19] apart from 3 SNPs observed in M. canettii and one SNP in isolate SAWC 1870 that are described below. The discrepancy between our H37Rv ATCC reference isolate and the published H37Rv sequence was further investigated by PCR analysis from DNA derived from three additional independent cultures of H37Rv from different sources, including one that had been newly purchased from the ATCC. In each case the H37Ra-like genotype (Figure 2b) was confirmed and not the published H37Rv RvD7 genotype (Figure 2a, data not shown). The two additional M. canettii isolates were also analysed with these PCRIs and the H37Ra-like genotype was also confirmed (data not shown). A complete list of PPE38 genotypes representing all analysed samples, comprising H37Rv, H37Ra, all 40 M. tuberculosis clinical isolates from our cohort, 3 M. canettii clinical isolates plus 15 M. tuberculosis and 8 non-M. tuberculosis members of the MTBC (analysed in silico from publicly available whole genome sequences - see below), along with group, lineage (F) and mutation details is listed in additional file 1.

**Analysis of clinical isolates displaying alternate PPE38 region genetic structures and determination of lineage specificity**

The PPE38 region of clinical isolates that produced PCR amplicons of sizes that did not correspond to the H37Ra-like genotype were analysed in more detail by sequencing PCR amplicons. In order to characterize IS6110-associated mutations, the IS5′ and XhoI primers were used (Table 1). Twelve isolates possessed IS6110-mediated mutations, with two of these also displaying indels involving presumably recombination-mediated swapping of parts of the 5′ untranslated regions of PPE38 and PPE71. One isolate revealed a 5′-untranslated region indel without an accompanying IS6110 mutation. Four isolates displayed the RvD7 genotype as defined by the H37Rv whole genome sequence (Figure 2a). The final isolate failed to produce PCR product when using any of the PPE38-associated primer pairs, although PCRs directed at other regions of the genome were successful. We conclude that this isolate possesses a large deletion in the PPE38 region. Details and figures of the characterized mutations can be found in additional files 1 and 2 (S1 - S18). Additional clinical isolates were investigated in many cases in order to determine whether specific characterized mutations were IS6110 lineage-, cluster-, or isolate-specific. Results showed that in most cases the mutation was specific to all of the different clusters analysed from within the lineage, although several instances of within-lineage and even within-cluster variation was observed. Details of this analysis can be found in additional file 2 (S1 - S18).

**In silico analysis of the PPE38 region in M. tuberculosis and other MTBC member whole genome sequences**

The results obtained from our clinical isolates encouraged us to further investigate the genomic structure of this region in isolates whose whole genome sequences are publicly available. Along with the H37Rv and H37Ra sequences previously described we also analysed the region in 13 M. tuberculosis and 6 non-M. tuberculosis MTBC members for which the whole genome sequences are publicly available. For convenience, although the daisie and oryx bacillus genomes have not been completed, we have included known information on their PPE38 regions [22,23] in this section, thus providing a total of 21 additional MTBC genomes for analysis. Surprisingly, only 4 of these genomes (H37Ra, CDC1551 and M. africanum isolates GM041182 and K85) displayed the “normal” (ancestral) H37Ra-like PPE38 genotype of 2 PPE genes separated by 2 exs-like genes (Figure 2b). Six genomes (including H37Rv) displayed the RvD7 genotype (Figure 2a). Six genomes displayed various IS6110-associated mutations that, in some cases, were associated with additional indel mutations. The remaining 7 genomes, including all of the non-human animal-associated organisms, displayed large RD5 and RD5-like [24,25] deletions that spanned the entire PPE38 region including adjacent genes. Details and figures of all the characterized muta-

### Table 1: Sequences of primers used for PCR amplification and sequencing.

| Primer name | Sequence (5′ – 3′) | Comment |
|-------------|--------------------|---------|
| PPE38F      | TTTTCGGTGTTGGATTGTCT | 3398 bp amplicon for H37Ra-like genotype, 1331 bp amplicon for RvD7 genotype. |
| PPE38R      | GCCAGGATTTCACGAGC   |         |
| PPE38IntF   | ATGTGGCCGGAAGTGGTAA | 1351 bp amplicon for H37Ra-like genotype, no product for RvD7 genotype. |
| PPE38IntR   | TTGAGCCGACGGCAAT   |         |
| 21delF      | GCCGATGATGCCGATG    | 111 bp amplicon for wild-type genotype, 90 bp amplicon for 21del genotype. |
| 21delR      | ACATGGGCGGAGGCTTG   |         |
| IS5′        | GGTACCTCCTCGATGACAC | IS6110-binding sequencing primer used to determine region upstream of IS6110. |
| XhoI        | TTCAACACATCGGCCCTCTAC | IS6110-binding sequencing primer used to determine region downstream of IS6110. |
| plcA5′      | CAAATGTCCGGGACAAGG  | Primers from the 5′ region of plcA. Used to PCR and sequence the region between plcA and PPE38 in conjunction with the PPE38IntF primer in M. canettii isolates. |
tions can be found in the additional files 1 and 2 (S19 - S32). A schematic representation of the 7 large RD5 and RD5-like deletions can be seen in Figure 3.

Analysis of micro-mutations within the PPE38/71 gene sequences

Along with the macro-mutational events described above, the PPE38 region of 15 isolates from our cohort plus the fully sequenced genomes were also examined for mutations at the micro-mutational level. Apart from the 21del mutation which is described below, only 4 isolates (M. canettii, SAWC 1870, KZN 4207 and K85) were confirmed to possess micro-mutations. These are detailed in additional file 1. These results demonstrate that micromutations within the PPE38 region are rare.

Analysis of the 21del mutation

The 21del mutation consists of an in-frame 21 bp deletion that results in the loss of amino acids 357 - 63 and was initially identified in PPE71 of the CDC1551 whole genome sequence as well as in our clinical isolate SAWC 1645 (Haarlem, F10). The M. tuberculosis strain C, which possesses the RvD7 deletion, also shows this mutation, demonstrating that PPE38, rather than PPE71, has been deleted in this case. Interestingly, while all are PGG2 members, SAWC 1645 belongs to F10 of the Haarlem lineage while CDC1551 and strain C belong to the LCC lineage (“4 banders” and “3 banders” respectively). In order to further track the presence of the 21del mutation in our clinical isolates, PCR primers were designed to distinguish between the 21del (90 bp) and wild type (111 bp) genotypes (Table 1). This PCR was initially performed on all PGG2 isolates from our cohort. Results revealed the presence of this mutation in all 4 members of the LCC (“2, 3, 4 and 5 banders”) as well as in 5 of 8 lineages representing the Haarlem, Pre-Haarlem and Haarlem-like clades (Figure 4). A simplified phylogenetic tree of PGG2 lineages in relation to their 21del genotypes is shown in Figure 5. All

| Ancestral MTBC genotype | Rv2345 | esxO | esxP | Rv2348c | picC | picB | plcA | MRA2374 | MRA2375 | PPE38 | PPE39 | PPE40 | glyS |
|-------------------------|--------|------|------|---------|------|------|------|---------|---------|-------|-------|-------|------|
| M. bovis and M. caprae | RDS    |      |      |         |      |      |      |         |         |       |       |       |      |
| M. bovis BCG            | RDS    |      |      |         |      |      |      |         |         |       |       |       |      |
| M. microti              | RDSmic |      |      |         |      |      |      |         |         |       |       |       |      |
| Oryx bacillus           | RDSoryx|      |      |         |      |      |      |         |         |       |       |       |      |
| Dassie bacillus         | RDSdas |      |      |         |      |      |      |         |         |       |       |       |      |
| T92                     | RD5-like|      |      |         |      |      |      |         |         |       |       |       |      |
| 94_M4241A               | RD5-like|      |      |         |      |      |      |         |         |       |       |       |      |

Figure 3

RD5 and RD5-like deletions seen in MTBC isolates. This region is susceptible to frequent large deletions. Here we show the genes surrounding PPE38 along with the deleted regions characterised in 5 non-M. tuberculosis MTBC members [22-25,32], along with the deletions detected in the M. tuberculosis whole genome sequences T92 and 94_M4241A. A red arrow indicates the presence and direction of IS6110 at a deletion point. Deletions caused by homologous recombination between PPE39 and PPE40 in M. bovis BCG and 94_M4241A are also shown. Numbering refers to gene nucleotide positions.
LCC members showed a "heterozygote-like" 2, WT/21del (2 genes, 1 wild type, 1 21del) genotype, suggesting the CDC1551-like structure, while results for the Haarlem lineages were more variable with "homozygote-like" signals for both the wild type and 21del genotype observed (Figures 4 and 5). In these cases PPE38F/R and PPE38IntF/IntR PCRs were used to determine the number of PPE38/71 genes present and thus distinguish between recombination (1 gene) and gene conversion (2 genes) events. Figure 5 shows that recombination and gene conversion events were observed within the F1, 2, 4, 10, 19 and 24 Haarlem lineages. Mutational analysis of the 2 Haarlem-like lineages (F6 and F7) suggests that the 3' region of PPE38, including the region corresponding to the 21del position in PPE71, has been removed by an IS6110-associated mutation [see additional file 2, S11 and S12]. The "1, 21del" genotype seen in both these lineages is therefore not due to recombination with deletion of PPE38.

We next performed the 21del PCR on additional isolates from the various LCC and Haarlem lineages in order to determine whether the observed genotypes were cluster or lineage-specific and also to investigate any additional instances of gene conversion or recombination. Four LCC "6 bander" isolates, which represent a lineage not originally used in our study, were also included. Results are shown in Table 2 and show that 90 additional isolates (including the Haarlem F4, CDC1551 and strain C whole genome sequences), representing 5 LCC and 8 Haarlem lineages (including Haarlem, Pre-Haarlem and Haarlem-like lineages) were analysed. Of these, 5 isolates (5.6%) displayed an altered genotype compared to the standard genotype observed within their lineage. Where genotypic changes were observed the PPE38F/R and PPE38IntF/IntR PCRs were again used to differentiate between recombination and gene conversion events (Table 2) No cases of within-cluster genotypic alterations were observed.

Analysis of the plcABC genes from publicly available whole genome sequences

Previous reports have revealed that the genomic region adjacent to PPE38 encompassing the three phospholipase (plc) gene loci plcA, plcB and plcC is subjected to frequent deletions and IS6110 insertions [26-30]. We therefore also examined this region in the 15 publicly available M. tuberculosis whole genome sequences and 8 non-M. tuberculosis MTBC members described above. Numerous mutations were observed. These included SNPs, micromutations that resulted in frameshifts and altered amino acid incorporation, IS6110 integration and a case of gene fusion between plcA and plcB in isolate 02_1987. Some of the observed SNPs were found to be lineage-specific. For example, a sSNP (A → C) at position 435 of plcA distinguished all "ancient" strains (TBD1+) from "modern" strains (TBD1-) [31]. Large RD5 and RD5-like deletions have been previ...
Pre-Haarlem

SAWC 1746
1 banders
F24

SAWC 716
2 banders
F19

SAWC 386
2, WT (a)
F1

2, WT/21del (a)
SAWC 1841
F4

1, WT (b)
SAWC 1645
F10

1, 21del (d)

Haarlem

SAWC 2073
2 banders
F120

SAWC 233
3 banders
F130

SAWC 861
4 banders
F140

SAWC 1162
5 banders
F150

2, WT/21del (a)

Haarlem Groups

SAWC 1127
1 banders
F6

SAWC 103
2 banders
F7

Haarlem - like

SAWC 198
1 banders
F110

SAWC 1595
Quebec/S
F28

SAWC 3656
LAM
F26

SAWC 3100
F14

SAWC 2525
F9

SAWC 2576
F15

SAWC 1815
F11

SAWC 1733
F13

SAWC 1841
2 banders
F2

2, WT/21del (a)

SAWC 1430
F3

SAWC 1430
F3

Figure 5

21del genotype results in relation to PGG2 phylogeny. A simplified phylogenetic tree of PGG2 lineages shows that the 21del mutation is seen only within the Haarlem and LCC lineages indicating that they share a recent common ancestor. Results also suggest frequent gene conversion and recombination events, particularly between the Haarlem groups. Each mutational type is shown in colour and indicates the number of PPE38/71 genes present and the genotype (WT = wildtype, ie lacking the 21del mutation). Green (a): 21 bp deletion (21del) in PPE71, both genes retained; Blue (b): Recombination between PPE38/71 with PPE71 deletion; Yellow (c): Gene conversion leading to deletion of PPE71 and duplication of PPE38; Grey (d): Recombination between PPE38/71 with PPE38 deletion. The “Haarlem-like” lineages (F6 and F7) could not be included in the analysis because the 3’ end of PPE38, including the region homologous to PPE71 21del, has been deleted due to an IS6110-associated deletion event [see additional file 2, S11 and S12].

Analysis of the PPE39 and PPE40 genes from publicly available whole genome sequences

As described above, many instances of PPE38-encompassing RD5-like deletions that span the region from plcABC to PPE39 or PPE40 have been detected in the non-M. tuberculosis MTBC members [22-25,32]. Similar deletions were also found in the T92 and 94_M4241A M. tuberculosis isolates (Figure 3, Table 4). We analysed the mutational

ously shown to affect these genes in M. bovis, M. bovis
BCG, [24,25], M. microti (where it is part of the RD5mic
deletion [32]), the dassie bacillus (where it is part of the
RD5das deletion [22]) and in the oryx bacillus (where it is
part of the RD5oryx deletion [23]) (Figure 3). Two of the M.
tuberculosis isolates (T92 and 94_M4241A) showed simi-
lar RD5-like deletions (Figure 3). A complete summary of
the results can be seen in Table 3.
Table 2: 21del analysis of lineages representing the LCC and Haarlem groups

| Group/Lineage     | Same cluster | Different clusters | Total | Standard genotype | Mutations observed |
|-------------------|--------------|--------------------|-------|-------------------|-------------------|
| LCC, 2 banders    | 4            | N.A.               | 4     | 2, WT/21del       | 0                 |
| LCC, 3 banders    | 6            | N.A.               | 6     | 2, WT/21del       | 1. (1, 21del)     |
| LCC, 4 banders    | 6            | N.A.               | 6     | 2, WT/21del       | 0                 |
| LCC, 5 banders    | 6            | N.A.               | 6     | 2, WT/21del       | 1. (1, WT)        |
| LCC, 6 banders    | 4            | N.A.               | 4     | 2, WT/21del       | 0                 |
| F6, Haarlem-like  | 3            | 3                  | 6     | 1, 21del          | 0                 |
| F7, Haarlem-like  | 3            | 5                  | 8     | 1, 21del          | 0                 |
| F1, Haarlem       | -            | 3                  | 3     | 2, WT/21del       | 0                 |
| F2, Haarlem       | 5            | 8                  | 13    | 2, WT/21del       | 1. (1, 21del)     |
| F4, Haarlem       | 4            | 6                  | 10    | 1, WT             | 0                 |
| F10, Haarlem      | 4            | 2                  | 6     | 1, 21del          | 0                 |
| F24, Pre-Haarlem  | 4            | 5                  | 9     | 1, WT             | 2. (2, WT/21del)  |
| F19, Pre-Haarlem  | 4            | 5                  | 9     | 2, WT             | 0                 |
| **Total**         | **53**       | **36**             | **90**|                   | **5**             |

N.A.: Not applicable. Because of the invariance of the IS6110 RFLP patterns LCC lineages cannot be subdivided into clusters.

*The "1, 21del" genotype observed in these lineages is due to deletion of the 3' end of PPE38 by an IS6110-mediated mechanism rather than homologous recombination with deletion of PPE38.

status of the PPE39 and PPE40 genes in all available whole genome sequences in order to determine the extent of the hypervariable region that appears to be centered around PPE38. Our analysis demonstrates that, apart from RD5-like deletions, both genes are frequently subjected to additional mutational events at both the micro- and macro-mutational scale and that in many cases the resultant protein function is predicted to be abolished or altered (Table 4). Several mutations are of particular interest. Isolates 94_M4241A and 4). Several mutations are of particular interest. Isolates 94_M4241A and isolate 02_1987 also reveals an insertion in adjoining the PPE38 gene region in non-tuberculous mycobacterial species

The extensive variability observed at the M. tuberculosis PPE38 region led us to examine its structure in more distant evolutionary time in order to gain insights into its evolutionary history. The whole genome sequences of 7 slow growing and 7 fast growing species of non-tuberculous mycobacteria (Figure 6), as well as several actinobacteria members outside the mycobacterium genus, were analysed for protein sequences showing homology to PPE38, MRA_2374, MRA_2375, plcABC and other genes found in the M. tuberculosis PPE38 region. The genomic region surrounding proteins of high homology was examined for similarities to the M. tuberculosis structure.

We first investigated the structure of this region in actinobacteria outside of the genus Mycobacterium (including members of the genera Corynebacterium, Rhodococcus, and Nocardia). In all cases glyS and an orthologue of Rv2345 (which are both located near PPE38 in M. tuberculosis, see Figures 3 and 7) could be found situated in close proximity to each other and in all cases they were separated by between 1 and 5 genes. These genes are unrelated to any genes found in the PPE38 region in the mycobacteria.

The fast-growing mycobacteria - M. smegmatis, M. sp. JLS, M. sp. MCS, M. sp. KMS, M. vanbaalenii, M. vilum, and M. abscessus

The M. smegmatis genome contains a region homologous to the M. tuberculosis esxA/esxB operon found in the RD1 region [33]. However, the single PE/PPE and esx gene pairs located within this region are the only ones present in the genome and it has previously been demonstrated that PE/PPE expansion has only occurred in certain slow growing mycobacteria and not in the fast-growers [2]. A number of fast-growing mycobacterial genomes have been...
sequenced, including *M. smegmatis*, *M*. *sp*. JLS, *M*. *sp*. MCS, *M*. *sp*. KMS, *M. vanbaalenii*, *M. gilvum*, and *M. abscessus*. Analysis of the genomes of these organisms confirmed the absence of any PE/PPE genes outside of the *esx* regions. The PPE38 surrounding region was identified in *M. smegmatis*, *M*. *sp*. JLS, *M*. *sp*. MCS, *M*. *sp*. KMS and *M. gilvum* to only contain two genes, namely *glyS* and the orthologue of *Rv2345*. This seems to represent the structure of the region before the insertion of PPE38 and the other genes found in this region in the slow-growing mycobacteria (Figure 7). The genome of *M. vanbaalenii* contains the same region with the insertion of an ortho-

### Table 3: Mutational analysis of the plcABC genes from 15 publicly available whole genome *M. tuberculosis* isolates and 8 non- *M. tuberculosis* MTBC members.

| Isolate       | plcA | plcB | plcC | Comment                                                                 |
|---------------|------|------|------|-------------------------------------------------------------------------|
| *M. bovis*    | Deleted | Deleted | Deleted | plcABC part of the RD5 region (Figure 3).                               |
| *M. bovis BCG* | Deleted | Deleted | Deleted | plcABC part of the RD5 region (Figure 3).                               |
| CPHL_A        | sSNP A → C at position 435. | +      |       | All genes predicted to be fully functional.                            |
| K85           | sSNP A → C at position 435. | +      | nsSNP C → T (Thr → Ile) at aa position 302. sSNP G → A at position 1506. | plcC function possibly impaired. |
| GM041182      | sSNP A → C at position 435. | +      |       | All genes predicted to be fully functional.                            |
| *M. microti*  | Deleted. | Deleted | 5' 867 bp deleted. | plcABC part of the RD5mic region (Figure 3).                          |
| *Oryx bacillus* | Deleted | Deleted | 5' 260 bp deleted. | plcABC part of the RD5mic region (Figure 3).                          |
| *Dassie bacillus* | Deleted | Deleted | 5' 194 bp deleted. | Major deletion results in removal of plcA, plcB and 5' region of plcC (Figure 3). |
| T17           | sSNP A → C at position 435. | IS6110 insertion at position 1307. | +      | plcB function predicted to be abolished.                               |
| EAS054        | sSNP A → C at position 435. | sSNP G → A at position 1404. | +      | All genes predicted to be fully functional.                            |
| T92           | Deleted | Deleted | sSNP T → C at position 753. | IS6110-associated recombination event has deleted plcA and 5' region of plcB (Figure 3). |
| 94_M4241A     | Deleted | 5' 793 bp deleted. | sSNP T → C at position 753. | Hybrid plcA/B gene predicted to be non-functional. Part of massive genome rearrangements seen in this isolate (Figure S23). |
| 02_1987       | Deletion of 3' end of plcA and 5' end of plcB creates hybrid plcA/B gene. Fusion point at position 145. Results in frameshift and premature protein termination. | +      | sSNP T → C at position 753. | plcA and plcC functions possibly impaired. |
| T85           | sSNP G → A at position 705. nsSNP T → A (thr → ala) at position 1336. | +      | sSNP T → C at position 753. nsSNP G → T (gly → cys) at position 1081. | plcA and plcC functions possibly impaired. |
| KZN 4207      | +      | +      | +      | Total homology to H37Rv.                                               |
| KZN 1435      | +      | +      | +      | Total homology to H37Rv.                                               |
| KZN 605       | +      | +      | +      | Total homology to H37Rv.                                               |
| F11           | +      | +      | +      | Total homology to H37Rv.                                               |
| Strain C      | T insertion at position 104. Altered reading frame and premature protein termination. | +      | +      | plcA function predicted to be abolished.                               |
| CDC1551       | A insertion at position 968. Altered reading frame and premature protein termination. | +      | +      | Total homology to H37Rv.                                               |
| Haarlem       |     | +      | +      | plcA function predicted to be impaired.                                |
| H37Rv         | +      | +      | +      | Defined as wild type sequence                                          |
| H37Ra         | +      | +      | +      | Total homology to H37Rv.                                               |

+ indicates complete homology to the H37Rv reference sequence.
‡Deletion mapping studies indicates that the plcC gene of the Oryx bacilli is present [23]. The exact sequence of the gene in this species is unknown however.
logue of Rv2248 between glyS and the orthologue of Rv2345. The other PPE, esx and plcABC genes are absent from the regions and the rest of the genomes of these organisms.

M. abscessus, which is one of the earliest mycobacterial species to diverge within the genus Mycobacterium, has an expanded region containing glyS, an aminotransferase, an 1-aminocyclopropane-1-carboxylate deaminase, an GntR family transcriptional regulator, and the orthologue of Rv2345. It is unclear whether the genes between glyS and the orthologue of Rv2345 have been inserted or whether this represents the ancient structure of the region.

M. abscessus complex (M. avium subsp. hominissuis, M. avium subsp. avium, M. avium subsp. paratuberculosis and M. intracellulare) The whole genomes of four members of the M. avium complex have been sequenced, namely M. avium subsp. hominissuis, M. avium subsp. avium, M. avium subsp. paratuberculosis and M. intracellulare. Analysis of the genomes of these four organisms revealed the presence of a region containing orthologues to the genes found in the PPE38 region. However, this region is substantially reduced and only contains orthologues of glyS, PPE38, Rv2348c and Rv2345. The other PPE, esx and plcABC genes are absent from the region and the rest of the genome.

### Table 4: Mutational analysis of the PPE39 and PPE40 genes from 15 publicly available whole genome M. tuberculosis isolates and 8 non-M. tuberculosis MTBC members.

| Isolate   | PPE39                     | PPE40                     | Comment                                                                 |
|-----------|---------------------------|---------------------------|-------------------------------------------------------------------------|
| M. bovis  | Deleted downstream from position 1358. 3 bp in-frame deletion removes aa 164 (A). |                           | PPE39 part of RDS region (Figure 3).                                    |
| M. bovis BCG | PPE39/40 gene fusion. 3 bp in-frame deletion seen in M. bovis PPE40 also present. |                           | Fused PPE39/40 (Figure 3).                                              |
| CPHL_A   |                              |                           |                                                                         |
| KB5       | +                          |                           |                                                                         |
| GM041182  | sSNP G → T position 1548   |                           |                                                                         |
|           | sSNP C → T position 1563.   |                           |                                                                         |
| M. microti | Deleted downstream of position 325. |                           |                                                                         |
| Oryx bacillus | Deleted                     | Deleted                   |                                                                         |
| Dassie bacillus | Deleted                  | Gene present.             |                                                                         |
| T17       | +                          |                           |                                                                         |
| EAS054    | 3 bp (GCG) in-frame deletion removes alanine at aa position 27. |                           |                                                                         |
| T92       | Deleted                    |                           |                                                                         |
| 94_M4241A | PPE39/40 gene fusion.      | Deleted from position 1592. |                                                                         |
| 02_1987   | Deleted                    |                           |                                                                         |
| T85       | G insertion position 830.   |                           |                                                                         |
| KZN 4207  | +                          |                           |                                                                         |
| KZN 1435  | +                          |                           |                                                                         |
| KZN 605   | IS6110 integration at position 47. 3' region of gene deleted. |                           |                                                                         |
| F11       | IS6110 integration at position 47. |                           |                                                                         |
| Strain C  | N.D.                       |                           |                                                                         |
| CDC1551   | 3 bp (GCG) in-frame deletion removes alanine at aa position 27. |                           |                                                                         |
| Haarlem   | IS6110 integration at position 47. |                           |                                                                         |
| H37Rv     | IS6110 integration at position 20. Following IS PPE39 sequence commences at position 821. |                           |                                                                         |
| H37Ra     | As for H37Rv.              |                           |                                                                         |

* indicates homology to consensus sequence.
Figure 6  
 Phylogeny of Mycobacterial species. Phylogenetic tree of 80 members of the genus *Mycobacterium* based on the 16S rRNA DNA sequence with the sequence of the species *Gordonia aichiensis* as the outgroup. Reproduced from ref [2] with permission from the authors. MTBC members analysed in this study are highlighted in yellow, while other mycobacteria analysed are highlighted in green.
From this result it seems that the ancestral region only consisted of these four genes (Figure 7).

**M. leprae**

A substantially reduced PPE38 region was identified in the genome of *M. leprae*, which contains only glyS, an IS6110 element (pseudogene - ML0827c), PPE38 (pseudogene, named PPE7 in the *M. leprae* database), plcA (pseudogene) and the orthologue of Rv2345 (pseudogene - ML0830c).

Due to the extreme reductive evolution of this organism’s genome [34], it is unclear what the original structure of this region in *M. leprae* was before genome downsizing, so
this organism was also not found to be useful for investigating the evolution of this region.

M. marinum and M. ulcerans share a recent common ancestor and both are also closely related to the MTBC (Figure 6). M. marinum has the most extensive PE/PPE gene repertoire yet discovered and contains 105 PPE genes [35]. BLAST analysis of M. marinum proteins with the PPE38 amino acid sequence identified 2 genes (MMAR_3661 and MMAR_3664) with highest homology. Two esx-like genes are located downstream from each PPE38/71 homologue suggesting that the M. tuberculosis PPE38 region evolved initially by duplication of a esx/esx/PPE sequence, to produce the structure seen in M. marinum, followed by deletion of the esx gene pair downstream of PPE38 homologue MMAR_3661 (Figure 7). The homology of this region with the M. tuberculosis PPE38 region is further confirmed by surrounding genes including the upstream genes MMAR_3665 (highest homology to PPE39), MMAR_3666 (PPE40) and MMAR_3667 (glyS).

As in M. tuberculosis, the plc region is located downstream from MMAR_3661. Unlike M. tuberculosis the PPE38/71 homologues are not identical but show 95% homology at the amino acid level. However, the esxN 4/esxN 5 (homologous to MRA_2374) and esxP 4/esxP 5 (homologous to MRA_2375) gene pairs are identical both to each other and to their M. tuberculosis counterparts. In the light of these findings we were interested to know whether M. canettii (the most ancestral MTBC member) also retained the esx gene pair located between plcA and PPE38. Using the plcAS/PPE38IntF primer pair (Table 1) we amplified the region between plcA and PPE38 in our 3 M. canettii clinical isolates. Amplicon size indicated the M. tuberculosis structure with loss of the 2 esx genes observed in M. marinum and suggests that the ancestral MTBC organism had this deletion (Figure 7). Sequence analysis of 1 amplicon confirmed that, apart from several intergenic SNPs, the structure was identical to that seen in H37Rv.

M. ulcerans

The genome sequence of M. ulcerans shows that it has recently evolved from a M. marinum-like ancestor that acquired a virulence plasmid from another actinobacterium [36]. Since their divergence M. ulcerans has undergone extensive reductive evolution that has included genome downsizing [37]. This has resulted in alterations to the PPE38 region and no region of significant homology could be found. This organism was thus not found to be useful for investigating the evolution of this region.

Discussion

Using PCR and sequencing-based analysis of clinical isolates in conjunction with data obtained from publicly available whole genome sequences of M. tuberculosis, non-M. tuberculosis MTBC members and other non-tuberculous mycobacteria, we have investigated alterations of the PPE38 gene region, along with its evolutionary history. Analysis of the M. marinum whole genome sequence shows that the MTBC PPE38 region probably arose from the duplication of an esx/esx/PPE38 gene cluster followed by the deletion of one esx/esx gene pair (Figure 7). The more ancient evolution of the region is difficult to interpret from the available mycobacterium genome sequences. Analysis of the M. avium complex suggests that the insertion of PPE38 between Rv2345 and glyS was an early event but the exact timing of the esx and plc gene appearances remains unresolved. These questions will only be answered by the sequencing of more Mycobacterial species evolutionary situated close to the M. tuberculosis and M. avium complexes (e.g. M. kansasii) as well as additional species located on different phylogenetic branches, such as M. gordonae/M. asiaticum and members of the extended helix 18 group such as M. terrae (Figure 6).

Our results demonstrate that the M. tuberculosis PPE38 region is hypervariable, adding to mounting evidence indicating that MTBC genomes are not as homogeneous as previously thought [38], and that they have undergone, and continue to undergo, considerable divergence from their most recent common ancestor. From a total of 69 MTBC isolates analysed 36 (52%) were found to contain major structural alterations. When smaller micromutations that are predicted to alter PPE38 or PPE71 protein function are included in this tally only 22 isolates (32%) remain that show the ancestral H37Ra-like structure (Figure 2b) containing the identical PPE38 and PPE71 genes. It should be noted that several of the analysed isolates were close relatives (e.g. SAWC 2576, KZN 4207, KZN 1435 and KZN 605 all belong to F15) and thus our mutation frequency may be a slight overestimate. However, countering this is the fact that genotypic analysis for many of our clinical isolates was based on PCR analysis rather than sequencing and additional micro-mutations may have gone undetected.

The hypervariability of the PPE38 region results from the combination of a high frequency of IS6110 integration events, IS6110-associated recombination/deletion events, homologous recombination and gene conversion events. The frequency and variety of IS6110-associated mutations observed was striking. At least 20 of the 69 isolates (29%) displayed IS6110-associated mutations and these ranged from direct integrations, both into genes and intergenic regions, to recombination events that resulted in partial or full gene deletions. IS6110 integrations were also found to be common in PPE39 and PPE40 (Table 4) and they are also implicated in the large RD5-like deletions observed in isolate 94_M4241A and members of the non-human animal adapted MTBC members (Figure 3). The reason...
for the high IS6110 activity within this, or any of the other previously described M. tuberculosis IS6110 hotspot regions [39-42], is unclear. The element does not display any obvious insertion site sequence specificity, although in our analysis of PPE38, PPE39 and PPE40 we documented multiple, independent, identical integration sites. A more detailed analysis of this finding has recently been accepted for publication. Also of note is the finding that of all IS6110 integrations that were found to disrupt the 4 PPE genes analysed here, all occurred in their 5’ (conserved N-terminal) regions. Apart from the obvious negative functional effects of gene deletion and disruption, IS6110 can also function as a mobile promoter and upregulate genes located downstream of its integration site [43-45]. Three of our clinical isolates revealed IS6110 integrations upstream of genes and an investigation into the transcriptional effects could be of interest. The dynamic nature of the genome in this region in relation to IS6110-associated integration and recombination is further evidence for the role of IS6110 in the generation of genome plasticity in M. tuberculosis and its influence on the organism’s evolution [46].

The finding that 10 of the 69 isolates harboured the RvD7 genotype demonstrates a high frequency of homologous recombination between PPE38 and PPE71. Additional analysis of homologous recombination and gene conversion between these genes was greatly aided by the identification of the 21del mutation. This in-frame deletion has allowed us to distinguish between the 2 genes in the PGG2 LCC and Haarlem groups. 21del analysis demonstrated a high frequency of both homologous recombination and gene conversion, particularly between the various Haarlem groups, that result in various combinations of single/double/wildtype/mutant genotypes (Figures 4 and 5, Table 2). Springer and colleagues [47] have reported that in M. smegmatis homologous recombination can only originate in regions of high (> 99%) sequence homology but, once initiated, can extend across heterologous regions with limited constraint. Termination of the event was found to require another region of high sequence similarity. This is consistent with the situation seen between PPE38 and PPE71 either with or without the 21del mutation.

The case for a high frequency of gene conversion between PPE38 and PPE71 is supported by comparisons of the M. tuberculosis and M. marinum genomes. We found that within each genome there is extreme homology between the PPE38/71 and MMAR_3661/MMAR_3664 genes (over 95% in M. marinum and generally 100% in M. tuberculosis at both the DNA and protein level), while between genomes the homology between PPE38/71 and MMAR_3661 and MMAR_3664 is only 86% at the DNA level and 37% and 36% respectively at the protein level.

This extreme intra-genomic but lower inter-genomic homology strongly suggests that both pairs of genes have diverged from a recent common ancestral sequence but have been prevented from significant intra-genome divergence by regular gene conversion events. Additional evidence is provided by the sequence of these genes in M. canettii. Here, each gene contains a non-synonymous SNP (A → C) at nucleotide position 1054. This indicates that mutation in one gene followed by gene conversion has occurred in either the M. tuberculosis or M. canettii lineages since they last shared a common ancestor. Gene conversion between PPE38 and PPE71 could thus explain the apparent paradox between a high macro-mutational frequency, suggesting non-essentiality of the genes, and low micro-mutational frequency, which would normally be an indication of gene essentiality.

These results add to accumulating evidence supporting frequent PE/PPE-associated homologous recombination and gene conversion in M. tuberculosis. Using a microarray-based methodology Karboul and colleagues [48] mapped numerous deletion mutations spanning adjacent PE and PPE genes and found that they resulted in in-frame fusion genes. Homologous recombination, using the highly conserved N-terminal gene regions as substrates, was strongly implicated in these events. Our own analysis of the PPE39/40 fusion gene observed in the M. bovis BCG and 94_M4241_A whole genome sequences provides support for this finding. Two additional reports have provided evidence for between-strain recombination in close proximity to PE and PPE genes and the authors have proposed the existence of recombination hot spots within or close to these gene family members [49,50]. Regarding gene conversion, the use of the 21del polymorphism to detect this event in 2 highly homologous proximal genes is similar to that recently reported for the PE_PGRS17 and PE_PGRS18 gene pair [51]. This study reported the presence of a 12 bp insertion associated with a set of 40 SNPs that is found in either PE_PGRS17 alone or in both genes. Analysis of this polymorphism in isolates representing a broad spectrum of M. tuberculosis lineages shows that numerous gene conversion events have occurred between these genes throughout the evolutionary history of the PGG2 and PGG3 groups. Apart from its utility in detecting PPE38/71 recombination and gene conversion events, the 21del mutation is of interest for additional reasons. Firstly, it confirms a close evolutionary relationship between the PGG2 groups, LCC and Haarlem, recently identified by our group (N. C. Gey van Pittius, unpublished results). Secondly, the mutation has become fixed in the majority of lineages and clusters from within these groups, indicating that it might provide the organism with a survival advantage that is able to override the homogenising effect of recombination/gene conversion events.
Indeed, this mutation may represent the initial stages of evolutionary divergence between these 2 genes.

Homologous recombination/gene conversion events are also presumably responsible for the indel mutations involving the exchange of PPE38/71 upstream sequence regions observed in several isolates. Typically, both genes share the same upstream sequence to position -35 before diverging. The finding that isolate SAWC 3656 contains an indel upstream of PPE38 that involves replacement of the normal sequence from position -36 to -83 with PPE71 upstream sequence indicates a gene conversion event where PPE71 has replaced PPE38 in an imperfect recombination that has included a portion of its 5’-untranslated region. The other examples, and particularly 02_1987, indicate that homologous recombination can also produce more complex results. Isolate 02_1987 is a particularly good example of the benefits of whole genome sequence analysis with respect to the large-scale mutational events described in this study. Along with the plcA/B and PPE39/40 mutations previously described (Tables 3 and 4), this genome was also found to possess numerous additional gene truncations, inversions and IS6110 insertions involving both PPE38-associated genes and others [see additional file 2, S23] and it provides an idea of the amount of genomic plasticity that can be tolerated by a M. tuberculosis isolate that has successfully infected a host and caused disease.

Because our sample cohort is well-defined in terms of evolutionary relationships we were able, in many instances, to determine mutation status at the lineage-, cluster- or isolate-specific level. Although most mutations were found to be lineage-specific, in 5 cases at least one isolate that represented a different cluster from the same lineage revealed an altered genotype. Thus, genotypic variability was often observed within RFLP-defined lineages. Variability was generally not observed within clusters although in most cases the numbers analysed were limited. However, our analysis demonstrated that within cluster alterations can occur with one lineage showing 4 distinct mutations, including 3 within the same cluster [see additional file 2, S7]. These results emphasise the hypervariability of the PPE38 region and demonstrate its rapid ongoing evolution at the within-lineage and even the within-cluster level.

Our results show that the PPE38 region’s hypervariability extends to the adjacent plcABC and PPE39/40 regions. The plcABC region has previously been reported as a preferential region for IS6110 integration [29] and our results thus extend this region from plcC to PPE40. This results in a “hot-spot region” of around 11.3 kb when using the CDC1551 sequence as a reference. The importance of the plcABC genes, along with plcD, which is located in another genomic region, has been emphasised by knockout experiments showing that triple (plcABC) or quadruple knockouts are impaired during the late phase of infection in a mouse model [52]. However, several examples of clinical isolates that possess mutations in all 4 plc genes have been reported [28,30], revealing that their functions are not always essential for the bacteria’s pathogenicity. The finding that the plcABC region is deleted in many non-M. tuberculosis MTBC members [22-25,32], is further evidence for their limited phenotypic impact (at least in their non-human hosts). Our analysis revealed large scale mutations (deletions or IS6110 insertions) in 22 of a potential 69 (23 × 3) plcABC genes analysed and indicated that 5 isolates (M. bovis, M. bovis (BCG), M. microti, Dassie bacillus and T92) had functional loss of all 3 plcABC genes (Table 3). This mutation frequency is around double that found in the extensive study of Kong and colleagues [30]. This difference might reflect the greater accuracy of whole genome sequence analysis compared to a methodological approach based on PCR and Southern analysis, along with the fact that we included non-M. tuberculosis MTBC members with known RD5 or RD5-like deletions in our analysis. Several other micromutations (nsSNPs and microinsertions) were also detected that are predicted to abolish or alter protein function (Table 3). These results confirm the frequent loss of function of these genes in clinical isolates and suggest that previous studies may have underestimated this frequency.

The susceptibility of this region to large deletions is emphasised from analysis of other MTBC members where similar, yet distinct, deletions, which may include adjacent plc and PPE39 and PPE40 genes (RD5 and RD5-like deletions), have been reported in M. bovis, M. bovis (BCG), M. caprae, M. microti, and the dassie and oryx bacilli [22-25,32] (Figure 3). RD5-like deletions were found to be less common in M. tuberculosis isolates and were observed in just 1 of our clinical isolates and 2 of the M. tuberculosis whole genome sequences (Figure 3). The relatively low frequency of RD5-like deletions in M. tuberculosis is supported by the findings of Tsolaki and colleagues [53] who identified only 1 such deletion in a total of 100 phylogenetically diverse strains. This low deletion frequency in M. tuberculosis compared to non-M. tuberculosis MTBC members may signify that the absence of this region may provide the organism with a selective advantage in non-human hosts, a hypothesis that is strengthened by the finding that the RD5mic deletion is found in vole, but not human, M. microti isolates [32].

Surprisingly, our analysis of 3 independent H37Rv samples confirmed the typical H37Ra-like structure [19], thus contradicting the published sequence [1] from which the RvD7 genotype is defined. We suggest that the hypervariability of this region may have influenced the results of
the published H37Rv whole genome sequence and conclude that the results for this genomic region are not representative of its true sequence. We propose that either a culture-specific PPE38/71 recombination/deletion occurred to produce the non-representative RvD7 genotype or, alternatively, some subclones used for the H37Rv sequencing project may have become mixed with those from other isolates. The second possibility is supported by analysis of the H37Ra sequence which, surprisingly, was found to be far more similar to the CDC1551 sequence than to H37Rv [19,20]. Whatever the explanation, we suggest that the sequence accuracy of this region for other whole genome sequences that show the RvD7 genotype be treated with caution.

The biological consequences of the described mutations are unknown but our results suggest that functional loss of PPE38/71, MRA_2374 and MRA_2375 (and possibly also plcABC, PPE39, PPE40 or combinations of all of these) do not result in a significant loss of bacterial virulence. We base this conclusion on the high frequency of independent mutations found in this region and the fact that the large number of mutations identified (at least in relation to PPE38/71, MRA_2374 and MRA_2375) were mostly lineage specific, indicating that the original mutated organism had successfully caused disease, transmitted to new hosts and undergone further evolutionary expansion and divergence. The best example of this is that of the typical Beijing’s (F29) where IS6110-associated recombination/deletion events have resulted in the complete loss of functional PPE38, PPE71, MRA_2374 and MRA_2375 [see additional file 2, S4]. Despite this, Beijing F29 represents the dominant M. tuberculosis lineage throughout much of Asia and its incidence continues to rise rapidly in many countries and regions throughout the world [54,55]. Beijing F29 is also known to have diverged into many distinct sub-lineages [56,57]. The apparent absence of a deleterious phenotypic effect from mutations in the PPE38 region is supported by the transposon site hybridisation studies of Sassetti and colleagues who found that none of the genes analysed in our study were essential for growth either in vitro [58] or in an in vivo mouse model of infection [59]. In addition to these studies, which relate to M. tuberculosis in growth phase, these genes also do not undergo significant differential regulation during dormancy phase [60,61]. The plc, PPE and esx genes are all members of multi-gene families with numerous members within the M. tuberculosis genome and it is possible that genetic redundancy is responsible for the observations of Sassetti et al. Whether the loss of expression of these genes can, in some cases, be beneficial to the organism remains unclear but many examples of potential "virulence suppressor" genes have been documented [62]. PldA, PPE and esx genes have all been shown to produce antigenic proteins [7-9,63-65] and it is conceivable that the loss of such potentially potent antigens could aid in immune escape. A recent study [66] has characterised the cellular immune response to 167 peptides representing 8 ORFs (Rv2346c - Rv2353c) within the RD5 region (referred to in this study by the Behr et al [24] annotation, RD7) that is absent in M. bovis, M. caprae and M. bovis BCG compared to M. tuberculosis. A high secretion ratio of IFN-γ to IL-10 was observed in response to this peptide pool suggesting that expression of genes within RD5 might produce a protective effect. Loss of genes within this region could therefore result in increased pathogenesis and disease virulence. Finally, our work provides a cautionary note regarding vaccine development studies (which often utilise PE and PPE proteins or peptides) by indicating that at least some PPE gene family members are able to undergo rapid evolutionary change.

Conclusion
This study presents a detailed analysis of mutations at the PPE38 genomic region in a variety of M. tuberculosis isolates representing all major evolutionary lineages, along with analysis of this region from other MTBC and nontubercle mycobacterial species, in order to ascertain its evolutionary history. We conclude that this region is hypervariable due to frequent IS6110 integrations, IS6110-associated recombination/deletion events, and gene conversion and recombination between PPE38 and PPE71. Gene conversion was implicated in the low levels of variation observed at the micro-mutational scale between PPE38 and PPE71. Furthermore, mutational analysis of numerous additional isolates at the lineage and cluster levels has provided insights into the molecular evolution of this region. We describe multiple instances of fixation of PPE38-associated mutations at the lineage level, along with examples of within-lineage and even within-cluster variation, indicating rapid and extensive evolution of the region. Because these mutations generally result in the functional loss of genes we conclude that they do not result in a significant loss of fitness and that, since they have been shown to be highly antigenic, they may in fact aid in the organism’s survival.

Methods

DNA sample collection and determination of strain/lineage/cluster
M. tuberculosis isolates from patients residing in an epidemiological field site near Cape Town, South Africa, were genotyped according to the internationally standardized IS6110 DNA fingerprinting method [67]. DNA fingerprints were analyzed with GelCompar software, using the unweighted-pair group method using average linkages and Dice coefficients [68]. Isolates with an IS6110 similarity index of ≥ 65% were grouped into strain lineages [69]. Spoligotyping was also done to further classify lineages into clades [70].
PCR and sequencing

All primer sequences are listed in Table 1. PCRs using the PPE38F/R and PPE38IntF/IntR primer pairs were done in a reaction mixture containing 0.1 μg template DNA, 3 μl GC-rich solution, 1.5 μl 10× buffer containing MgCl₂, 2.4 μl 10 mM dNTP's, 0.6 μl each primer (50 pmol/μl) and 0.12 μl FastStart Taq (Roche, Germany) made up to 15 μl with H₂O. Amplification comprised an initial 6 min template denaturation followed by 35 cycles of 94°C for 30 s, 55°C 30 s and 72°C 2 min. After the final cycle samples were incubated at 72°C for 7 min. For the 21del analysis samples were subjected to PCR amplification in a reaction mixture containing 0.1 μg DNA template, 1.5 μl 10× Buffer, 1.2 μl 25 mM MgCl₂, 2.4 μl 10 mM dNTP's, 0.6 μl of each primer (50 pmol/μl), 0.075 μl HotStarTaq DNA polymerase (Qiagen, Germany) and made up to 15 μl with H₂O. Amplification was initiated by incubation at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 55°C 30 s and 72°C for 5 s. After the final cycle, the samples were incubated at 72°C for 7 min. For sequencing analysis PCR product was electrophoresed through a 1.5% low melting point agarose gel. The amplicon was then cut from the gel and purified using a Promega Wizard SV Gel and PCR Clean-up System (Madison, USA). Sequencing was performed using an ABI 3100 automated DNA sequencer.

In silico whole genome sequence analysis

The following M. tuberculosis and M. afric anus whole genome sequences are available from the Broad Institute Microbial Sequencing Center Databases [71]: M. tuberculosis C strain, Haarlem, F11, KZN 4207, KZN 1435, KZN 605, 02_1987, T85, T92, T17, 94_M4241A, EAS054, CPHL_A and K85. The CDC1551 whole genome sequence is available at The Institute for Genomes Research (TIGR) [72]. Analysis of the H37Rv whole genome sequence was performed using the TubercuList website [73]. Analysis of the H37Ra genomic clone along with M. avium subspecies paratuberculosis strain K-10, M. intracellularare strain 13950, M. smegmatis strain MC2155, M. abscessus, M. gilvum strain PYR-GCK, M. sp. JLS, M. sp. MCS, M. sp. KMS, M. vanbaalenii strain PYR-1, Nocardia farcinica strain IFM-10152, Rhodococcus jostii strain RHA 1, Rhodococcus erythropolis strain PR4, Corynebacterium glutamicum strain 13032 and Corynebacterium diphtheria strain NCTC 13129 was done using the NCBI genomic BLAST website [74]. Whole genome analysis of other bacterium species was done using the following websites: M. bovis strain AF2122/97 - BovLiSt [75]. M. bovis BCG strain Pasteur 1173P2 - BCGList [76]. M. africanaum strain GM041182 and M. microti strain OV254 - Sanger Centre [77]. M. marinum strain M - MarinoList [78]. M. ulcerans strain Agy99 - BuruLiSt [79]. M. leprae strain TN - Leproma [80]. Gene sequence alignments were performed using the CLUSTALW multiple sequence alignment programme [81].

Abbreviations

CAS: Central Asian clade; EAI: East African-Indian clade; ESAT-6: 6 kDa Early Secreted Antigenic Target (esx); F: family/lineage; indel: insertion/deletion where one DNA segment has been deleted and replaced by another; LAM: Latin American and Mediterranean clade; LCC: Low IS6110 copy clade; MTBC: Mycobacterium tuberculosis complex; PE: protein family characterised by Proline-Glutamic Acid motif; PGG: principle genetic group; PPE: protein family characterised by Proline-Proline-Glutamic Acid motif; PGRS: "polymorphic GC-rich repetitive sequence" subfamily of the PE family; SAWC: South African Western Cape.

Authors' contributions

CREM, NCGvP, PDvH and RMW conceived and designed the study. CREM carried out all PCR and sequence analysis. CREM and NCGvP carried out bioinformatic analysis. CREM, NCGvP, PDvH and RMW carried out interpretation of the data. CREM drafted the manuscript with assistance from NCGvP, RMW and PDvH. All authors read and approved the final manuscript.

Additional material

Additional file 1

Tabulated results of PPE38 region analysis. Summary of the PPE38 region genetic structures seen in all 69 samples analysed in this study. Click here for file

[http://www.biomedcentral.com/content/supplementary/1471-2148-9-237-S1.DOC](http://www.biomedcentral.com/content/supplementary/1471-2148-9-237-S1.DOC)

Additional file 2

Detailed structures of variable PPE38 regions. All isolates, including those from whole genome sequence analysis, that did not display the ancestral H37Ra-like genotype are described and, where appropriate, a figure is included below the text. Click here for file

[http://www.biomedcentral.com/content/supplementary/1471-2148-9-237-S2.DOC](http://www.biomedcentral.com/content/supplementary/1471-2148-9-237-S2.DOC)

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