P27-PPE36 (Rv2108) Mycobacterium tuberculosis Antigen – Member of PPE Protein Family with Surface Localization and Immunological Activities

Vincent Le Moigne, Wahib Mahana

Chapter Number ¶

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P27-PPE36 (Rv2108) *Mycobacterium tuberculosis* antigen; member of PPE protein family with surface localisation and immunological activities

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Le Moigne Vincent [1] and Mahana Wahib [1,2]

1, Université de Bretagne Occidentale.

2, Endotoxines, IGM, UMR 8621, Université Paris sud, Orsay.

France

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1. Introduction

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The largest and most distinctive class of mycobacteria-specific genes encode a group of 167 proteins of repetitive sequence belonging to the *pe* and *ppe* families. The uniqueness of the *ppe* genes is illustrated by the fact that these genes are restricted to mycobacteria (Cole et al., 1998; Voskuil et al., 2004 (b)). The *Rv2108* gene belongs to this family and furthermore is highly specific for the *Mycobacterium tuberculosis* (*Mtb*) complex group of mycobacterium (containing notably *Mycobacterium africanum, Mycobacterium canettii, Mycobacterium microti, Mycobacterium pinnipedi, Mycobacterium bovis* and *M. bovis* BCG strain). This gene was described by Chevrier et al., (2000) and used as a molecular probe to develop a rapid test for the detection and identification of this group of mycobacteria. *Rv2108* is a gene coding for the protein P27-PPE36, member of the PPE protein family of *Mycobacterium tuberculosis,* a group of protein thought to be of immunological significance despite the fact that the exact role of the PPE proteins stills unknown.

The P27-PPE36 protein was produced as a recombinant protein in *Escherichia coli*. The expressed protein is immunologicaly active and recognized by sera from infected patients. It was used to generate specific polyclonal and monoclonal anti-P27-PPE 36 antibodies. These antibodies were used to study the immunochemical characterization of P27-PPE36, to verify its presence in *Mycobacterium bovis* BCG and clinical *Mtb* isolates, and to characterize and localize it in a parietal position in *M. tuberculosis* cells.

Using an ELISA test we found that that the antibody immune response to P27-PPE36 in the sera of patients was dominated by an IgA antibody response accompanied by the absence of IgG response.
The immune response against the P27-PPE36 protein was investigated in mice. It was studied in the context of different pathogen associated molecular patterns (PAMPs). BALB/c mice were immunized either with the P27-PPE36 recombinant protein in Freund's adjuvant or in phosphate saline buffer (PBS), with a pcDNA3 plasmid containing the gene encoding the P27-PPE36 protein, or with the *Escherichia coli* bacteria expressing the P27-PPE36 protein genetically fused into the flagellin. We found that P27-PPE36 expressed into the flagellin led to the strongest cellular responses, where we obtained the highest production of IFN-γ and cell proliferation, an indication of specific Th1-like orientation of the immune response.

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2. Early works on Rv2108 and genetic analysis

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2.1 *Mtb* PCR-based assay detection test

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The *Rv2108* gene belongs to the *pe* and *ppe* families and furthermore is highly specific for the *Mycobacterium tuberculosis* (*Mtb*) complex group of mycobacterium. This gene was described by Chevrier et al., (2000) and was used as a molecular probe to develop a rapid test for the detection and identification of this group of mycobacteria. PCR targeting the insertion sequence IS 6110 has been considered specific for identification of *M. tuberculosis* and mycobacteria belonging to the *M. tuberculosis* complex and is frequently applied in numerous laboratories to confirm the presence of this organism directly in biological specimens (Thierry et al., 1990). However, several authors found that some *M. tuberculosis* strains failed to hybridize with the IS 6110 probe (Yuen et al., 1993; Thierry et al., 1995) and other authors found that false-positive results may be obtained for clinical samples when some methods based on IS 6110 are used (Lee et al., 1994: Kent et al., 1995). Conversely, the *Rv2108* gene was found to be highly specific for *M. tuberculosis* complex strains. In the PCR-based assay for rapid detection and identification of this mycobacterium (Chevrier et al., 2000), one pair of primers and two oligonucleotide probes were successfully used to amplify and to detect the DNA of strains belonging to the *M. tuberculosis* complex. These primers and probes did not hybridize with DNA from any of the 21 other mycobacterial species tested (*M. avium, M. intracellulare, M. gordonae, M. chelonae, M. xenopi, M. kansasii, M. peregrinum, M. fortuitum, M. marinum, M. flavescens, M. celatum, M. asiaticum, M. malmoense, M. fallax, M. simiae, M. terrae, M. interjectum, M. genavense, M. paratuberculosis, M. szulgaï* and *M. scrofulaceum*) . It is worth noting that the chosen primers and probes hybridize with DNA from the *M. tuberculosis* strain with no IS 6110, furthermore no strain without p27 was found among the 410 strains tested in the study (Chevrier et al., 2000).
Now that many mycobacterium genome have been completely sequenced, the results that *Rv2108* is specific to *Mycobacterium tuberculosis* compex have been confirmed. This name *Rv2108* is those of the gene in the *M. tuberculosis* strain H37Rv. In the *M. tuberculosis* strain CDC1551, the gene number is *MT2167* and in *Mycobacterium bovis,* this gene is called *Mb2132*. No ortholog has been identified in the genome of the closely related *Mycobacterium marinum, Mycobacterium segmatis, Mycobacterium ulcerans or Mycobacterium avium subs.*

paratuberculosis (Stinear et al., 2008) and those despite some bacteria like *M. marinum* have an higher number of PPE genes than *M. tuberculosis* (106 vs. 69) **(**Stinear et al., 2008**).**
However an other analysis found a *Rv2108* ortholog in the same strain (Agy99) of *Mycobacterium ulcerans* (Riley et al., 2008). This work presents also that *Rv2108* gene is deleted in the strain C of *Mycobacterium tuberculosis* while it is present in the stains CDC1551, F11, H37Rv and Harlem as in the two strains of *Mycobacterium bovis* tested (BCG stain Pasteur 1173 and AF2122/97*)*. According another study (Gey van Pittius et al., 2006), *Rv2108* have no orthologues in *M. smegmatis, M avium paratuberculosis, M. leprae, M. ulcerans* or *M. marinum*. This results confirm the interest of this gene in terms of diagnostic tool.
M. tuberculosis has become highly specialized for intracellular survival in a very restricted range of mammalian hosts, and several recent studies have shown that lateral gene transfer (LGT) has been a major force in the evolution of the *M. tuberculosis* complex from an environmental *Mycobacterium* (Kinsella et al. 2003; Gutierrez et al., 2005; Rosas-Magallanes et al., 2006; Becq et al., 2007). In fact, *Rv2108* appears to belong to one of the 80 regions (minimal number identified containing 360 Protein coding sequences (CDS)) that have probably been acquired by LGT in *Mtb* (Stinear et al., 2008). Whether acquired by LGT or other means, some of these *M. tuberculosis*-specific regions contain known virulence genes or code for adaptation factors making them pottentially important for bacteria belonging to *Mtb*-complex.

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2.2 Genomic organizations

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Analysis of the genomic environment of the *Rv2108* gene reveals that it is situated downstream a member of the *pe* gene family, *Rv2107,* coding for the PE22 protein (Fig. 1). These ajacent *Rv2107* and *Rv2108* genes lie in the same orientation. Occasionally, it can be noted that an insertion site IS*6110* is localized between this two genes in the strains H37Rv and CDC1551 (Beggs et al., 2000; Sampson et al., 2001). Genome analysis by the operon/gene cluster method (Strong et al., 2003; Bowers et al., 2004) suggests that the PE and PPE families are functionally linked (Gey van Pittius et al., 2006; Tekaia et al., 1999; Strong et al., 2006; Tundup et al., 2006). That is, the two genes tend to be in close chromosomal proximity on the *Mtb* genome (Strong et al., 2003; Bowers et al., 2004). Based on their short intergenic distance (56 bp) and same transcription direction, *Rv2107* and *Rv2108* were assumed to belong to the same operon (Fig. 1) and so be co-transcribed. In *Mtb* genome, these same-operon PE/PPE pairs comprise less than 10% of the total number of PE and PPE genes (14 pairs of PE and PPE genes are found adjacent — same orientation, minimal intergenic distance — in the genome) (Riley et al., 2008). Genes separated by short intergenic sequences tend to have related function and interact physically (Jacob & Monod, 1961). The structure of a complex of one PE/PPE protein pair was recently characterized (Strong et al., 2006; Tundup et al., 2006). These results indicate that there may be many other instances of interactions between PE and PPE proteins. Like the PE and PPE proteins from the gene *Rv2431c* (PE25) and *Rv2430c* (PPE41) that interact together in vitro as probably *in vivo* (Strong et al., 2006; Tundup et al., 2006), it is stongly probable that PPE36 and PE22 have the same behavior. In fact, computational methods predict that the PE22/PPE36 interaction probability is almost the strongest of all the PE/PPE possible combinations tested (Riley et al., 2008). Furthermore, according to this analysis, this putative complexe is predicted to interact specifically, that is, PPE36 do not appear to interact with PEs other than

its operon partner PE22, and vice versa (Riley et al., 2008) but this supposition would need to be experimentaly confirmed. However due to the fact that *Rv2108* is absent in *M. tuberculosis strain C* and *Rv2107* is absent in *M. tuberculosis strain F11*, it is possible that another interacting partner is able to interact with the orphaned gene, possibly restoring the PE/PPE complex's function, or introducing new complexes that help these strains survive in their environmental niches (Riley et al., 2008). A putative interaction PE22/PPE36 is probably under the form of a 1:1 heterodimeric complex (Strong et al., 2003). In their study, they found, as us (Le Moigne et al., 2005), that PPE36 is insoluble when expressed alone. The association with the relative PE protein would lead to a soluble complex: their experiments showed that proteins PE Rv2431c and PPE Rv2430 that are insosuble when expressed on their own are soluble when they are expressed together (Strong et al., 2006; Tundup et al., 2006).
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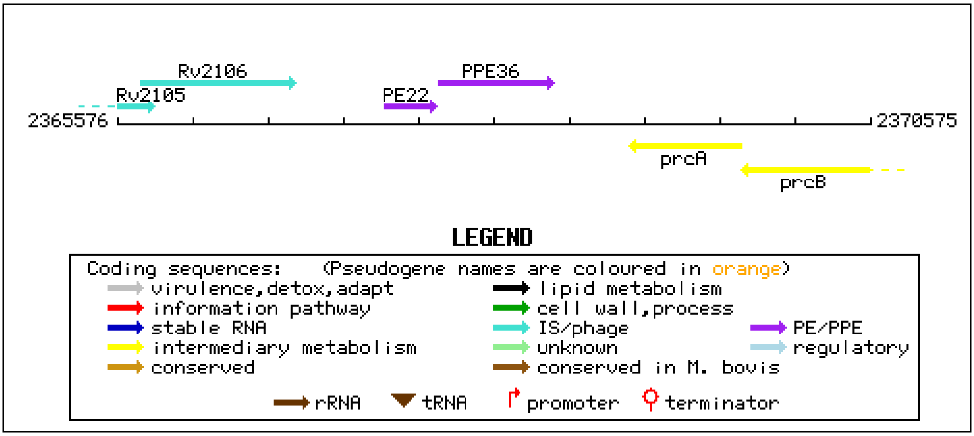

Fig. 1. Genomic environment of *Rv2108* gene.
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2.3 Regulation expression of *Rv2108*

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A fundamental step in understanding the role of *pe* and *ppe* genes is elucidating how their expression is regulated. Although various studies have demonstrated that *pe* and *ppe* genes are expressed under a range of *in vitro* and *in vivo* conditions, they have not revealed any obvious indication of global *pe* and *ppe* gene regulation (Voskuil et al., 2004 (b)). This group of Voskuil and Smith has tested a large variety of diverse conditions to analyse gene expression in *Mtb* (Manganelli et al., 2001; Sherman et al., 2001; Manganelli et al., 2002; Rodriguez et al., 2002; Schnappinger et al., 2003; Voskuil et al., 2003; Voskuil et al., 2004 (a); Voskuil et al., 2004 (b)). Among them, only two conditions induce a variation in *Rv2108* expression (at least two fold): in presence of 0.05% sodium dodecyl sulfate (SDS) 90 min, *Rv2108* expression is repressed (Manganelli et al., 2001) as after 14 days of stationnary phase culture (Voskuil et al., 2004 (a)). Other conditions (Macrophage IFNγ activated, 24 h; diethylenetriamine/nitric oxide adduct (DETA/NO or DNO) 0.5 mM, 40 min; hydrogen peroxide (H_2O_2) 10 mM, 40 min; hypoxia (oxygen from 20% to 0.20%), 2 h (Sherman et al., 2001; Rustad et al., 2008); palmitic acid 50 µm, 4h; non-replicating persistence (NRP) dormancy model 20 days; Iron high vs. low; diamide 5 mM, 1 h; potassium cyanide (KCN) 0.5 mM, 1 h; carbonyl-cyanide 3-chlorophenylhydrazone (CCCP) 0.5 mM, 1 h; ethambutol 10 µm, 24 h; nutrient starvation 24 h (Betts et al., 2002); heat shock (45°C), 30 min (Stewart et al., 2002); acid shock (pH 5.5 vs. 6.9) (Fisher et al., 2002)) do not appear to generate variation (more than 2 fold) in *Rv2108* expression. The associated *pe* gene, *Rv2107* (*pe22*), is found to be induced in macrophage culture of *Mtb* (Schnappinger et al., 2003) and in presence of 0.5 mM DETA/NO (Voskuil et al., 2003).

However, inversely, Park et al. (2003) found that *Rv2108* gene is induced by hypoxia (even if this needs confirmation since standard error deviation is elevated). However, contrarly to the majority of genes powerfully regulated by hypoxia, its induction does not requires the putative traznscription factor Rv3133/DosR.

Like the majority of other PPE gene (54 of 69), Lsr2, a small basic protein highly conserved in mycobacteria that binds DNA and is implicated in gene regulation, is able to bind Rv2108 sequence (Gordon et al., 2010). The binding of Lsr2 to the majority of *pe*/*ppe* genes suggests that this factor may negatively affect the expression of these antigenic proteins to modulate interactions with the host.

More generally, *Rv2108* has a low expression in the diverse *M. tuberculosis* strains that have been tested (Gao et al., 2005) and it does not seem that there is a difference of *Rv 2108* gene expression between *M. bovis* and *M. tuberculosis* in microarray analysis(Rehren et al., 2007).

Furthermore, a study showed, by hy density mutagenesis experiments, that *Rv2108* is not an essential gene for mycobacterial growth (Sassetti et al., 2003). In these experiments, only three of *pe* and *ppe* genes met the criteria for defining growth-attenuating mutations (*Rv1807*, *Rv3872*, and *Rv3873*). Although mutations in several other *pe* and *ppe* genes appeared to have subtle defects, the fact that such a small fraction are detected in this system suggests either that most of these genes are able to functionally complement each other, or that they are required under conditions that have not been testes. In the same study, the *Mycobacterium leprae* gene *ML0411* is presented as an orthologue of *Rv2108*. In the Sanger Institute *Mycobacterium leprae* genome project, *ML0411* is in fact described as being similar to *Rv2108*. ML0411 is coding for a protein 408-amino acid long named as a serine-rich antigen (Sra) that have been largelly described (Vega-Lopez et al., 1993; Rinke de Wit et al., 1993; Macfarlane et al., 2001; Parkash et al.,2006) *Rv2108* belongs the the 27% of genes that are not required for *in vitro* growth having *M. leprae* orthologues while the majority (78%) of the genes that they predict to be required for the optimal growth of *M. tuberculosis* have an orthologue in *M. leprae* genome. Thus, *M. leprae* appears to have selectively conserved the majority of genes that are necessary for optimal growth (Cole et al., 2001).

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3. Characterization of P27-PPE36 protein

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The *Rv2108* nucleotide sequence encoded for a 243 amino acid length protein. The P27-PPE36 antigen belongs to the PPE protein family, large family of protein present in *Mtb*, which represent ≈3% of the genome of this bacterium (Cole et al., 1998). With the related PE protein family, they account for 10% of the genome. These families 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 (Gey van Pittius et al., 2006). This asparagine or glycine-rich protein family containing 69 members has been termed PPE after the characteristic Pro-Pro-Glu motifs near the N-termini, in position 8-10. The relatively conserved N-terminal domain is about 180 amino acids lenght while C-terminal segments vary in sequence and length. According this C-terminal region, the PPE proteins are classified into four subfamilies: the first subfamily (24 members) , named PPE-SVP, has the well conserved motif Gly–X–X–Ser–Val–Pro—X–X–Trp located approximately at position 350; the second (23 members) constitutes the major polymorphic tandem repeats (MPTR) subfamily and is characterized by the presence of multiple tandem repeats of the motif Asn–X–Gly–X–Gly–Asn–X–Gly; the third subfamily (10 members), named PPE-PPW, is characterized by a highly conserved region comprising Gly–Phe–X–Gly–Thr and Pro–X–X–Pro–X–X–Trp motifs; and the last PPE subfamily (12 members) includes proteins with a low percentage of homology at the C-terminus that are unrelated other than having the PPE motif (Gordon et al., 2001; Adindla & Guruprasad, 2003; Gey van Pittius et al., 2006). P27-PPE36 belongs to this last subfamily. 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 5 sublineages which globally match the subfamilies described above (Gey van Pittius et al., 2006). *Rv2108* is classified in the sublineage III, having the most similarity with *Rv3892c*.

The role of the PPE proteins is still unknown. Firstly, they have been thought to be implicated in increasing antigenic variation and immune evasion due to the highly polymorphic nature of their C-terminal domains (Cole et al., 1998 ; Cole, 1999; Karboul et al., 2008). Concerning this, an interessant study realized by Plotkin et al. (2004) shows that PE/PPE proteins are under strong selection for amino acids substitution. They calculate volatility of codons which is the proportion of their point-mutaions neighbours that encode different amino acids. The volatility of a codon is used to quantify the chance that the most recent nucleotide mutation to that codon caused an amino-acid subtitution. According their calcul, *Rv2108* has a volatility value of 0.1029, which place it at the 594th rank of genes with the higher volatility among the 4099 genes values calculated. Furthermore, in agreement with the theory of an antigenic variation role, it has been observed that many PPE proteins present high levels of polymorphism like for exemple PPE38 (*Rv2352c*), PPE39 (*Rv2353c*) and PPE40 (*Rv2356c*) (McEvoy et al., 2009), PPE34 (*Rv1917c*) (Sampson et al., 2001(a)), PPE42 (Rv2608) (Chakhaiyar et al., 2004), PPE8 (*Rv0355c*) (Srivastava et al., 2006) or PPE18 (*Rv1196*) (Hebert et al., 2007) and sequence variation has been observed between the orthologues of the PE and PPE protein families in *in silico* analyses of the sequenced genomes of *M. tuberculosis* H37Rv, *M. tuberculosis* CDC1551 and *M. bovis* (Gordon et al., 2001; Fleischmann et al., 2002; Garnier et al., 2003). However, this variability can not be extended to all *pe/ppe* family members since some are in fact conserved across strains and species (Cubillos-Ruiz et al., 2008). It has then been suggested that the PPE proteins may play a role in the virulence of *Mtb* (Rindi et al., 1999; Li et al., 2005), in the maintenance of bacterial growth in macrophages (Camacho et al.,1999; Dubnau et al., 2002; Hou et al., 2002; Li et al., 2005; Sassetti et al., 2003) and in the regulation of bacterial iron starvation and oxidative stress responses (Rodriguez at al., 1999; Rodriguez at al., 2002). In addition, PPE might be a target for the protective immune response in experimental mouse models (Skeiky et al., 2000). It has also be emitted the hypothesis that PPE proteins, due to their abundance of asparagine, could have a possible storage function for this amino acid which is one of the preferred nitrogen sources of the tubercle bacilli (Tekaia et al., 1999). Some PPE proteins, like PPE31 (*Rv1807*) could be involved in the protection from antibiotic stress targeting the envelope and help to confer the basal level of Mtb resistance to antibacterial drugs (Provvedi et al., 2009). Many PPE proteins are also known to induce a strong T cell and B cell responses and associate with the cell wall. Following surface exposure, these PPE proteins could act as agonists to various surface receptors of APCs resulting in modulation of the host immune responses (Choudhary et al., 2003; Tundup et al., 2008; Mishra et al., 2008; Chaitra et al., 2008 (a); Chaitra et al., 2008 (b)). Recently, two PPE proteins, PPE18 (*Rv1196*) and PPE34 (*Rv1917c*), were found to specifically interact with the innate immune receptor TLR2 (Nair et al., 2009; Bansal et al., 2010).

Very little is known about the protein encoded by the *Rv2108* gene. Theoretical properties of P27-PPE36 protein are a low pH_i (4.59) and representative amino acid composition is 12% for alanine and 9% for glutamic acid. Predictive secondary structure shows that this protein would be mainly constitued of alpha-helix (58,5%) and the absence of β-feuillet. The resting amino acids (31%) would be in random coil.

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3.1 Expression and purification of the PPE36 protein

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The *Rv2108* gene was amplified, inserted into bacterial vectors, sequenced, and expressed as a recombinant protein. Either the GST (pGEX-4T-3) in *E. coli* DH5α or the pET (pET15b) in *E. coli* BL21 (DE3) plasmid were used. Induction of the PPPE36 protein by these various expression systems lead to the expression of a protein with an apparent molecular mass of 43 kDa in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 2A and B).

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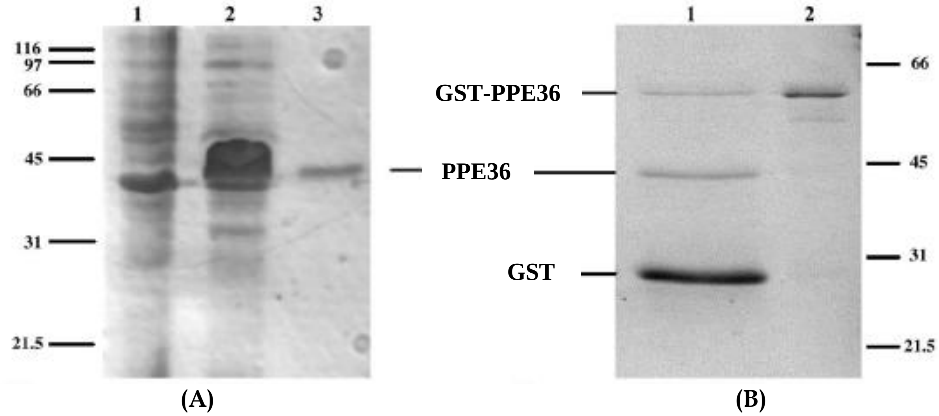

Fig. 2. Coomassie blue staining of bacterial lysates and purified recombinant PPE36 protein expressed with His-Tag (A) or with GST (B).

(A): Lanes 1 and 2: bacterial extracts of E. coli BL21 (DE3) without or with IPTG induction, respectively. Lane 3: purified PPE36 protein. (B): Lanes 1 and 2: PPE36, GST-PPE36 fusion protein, partially cleaved or not cleaved by thrombin, respectively.

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This value was higher than the theoretical mass predicted by its DNA sequence translation of 27 kDa. Mass spectrometric analysis of the expressed protein in the pET system revealed a molecule at 29 kDa, which corresponds to the P27-PPE36 putative protein estimated mass plus 2 kDa for the polyHistidine fusion Tag (Le Moigne et al., 2005). This result was confirmed by partial sequencing of the N-terminal region of the recombinant protein. The reason for this difference may be due to the nature of the P27-PPE36 protein, which belongs to a family of intrinsically unstructured proteins (IUP) with an atypical composition of amino acid sequences (Tompa, 2002). It presents notably a high proportion of Proline dimers (3 for 243 amino acids). These proteins bind less to SDS than most other proteins and their apparent molecular mass is often 1.2–1.8 times higher than the real value calculated from sequence data or measured by mass spectrometry (Dunker et al., 2001). Such a phenomen of electrophoresis abnormal migration has been observed for another protein belonging to the PE protein family of *Mtb*: the product of the gene *Rv1441c* has an apparent molecular weight of about 60 kDa instead of a theorical MW of 40,7 kDa (Banu et al., 2002).

Generally, PE and PPE proteins did not express well or expressed in insoluble or unfolded forms (Strong et al., 2006). Our attemps to express P27-PPE36 under the form of a recombinant proteins confirm this rules and lead to the obtention of an insoluble protein (Le Moigne et al., 2005), as confirmed later by an other study (Strong et al., 2006). The lack of apparent transmembrane elements is a possible explanation for their failure to express on their own is that they need protein partners to fold (Strong et al., 2006) like explained above in the *Genomic organization* paragraph.
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3.2 Physico-chemical caracteristic of the PPE36 protein

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Based on the DNA and protein sequences, the expected pI value of the P27 protein should be 4.8. To determine the PI value of the expressed p27 protein, a two-dimensional gel was applied to the cell lysates from the BCG strain. After gel transfer to a nitrocellulose membrane and blotting with the P27-PPE36-specific antibodies, only one spot with a pI between 4.5 and 5 at the same molecular mass level observed by SDS-PAGE was recognized on the membrane (Le Moigne et al., 2005).
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4. Anti-P27-PPE36 antibodies production and localization of P27-PPE36

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Very little is known about the cellular localization of the PPE protein family, a 143 kDa PPE protein encoded by the *Rv1917c* gene (PPE34) was found to be a cell-wall associated protein and probably surface exposed (Sampson et al., 2001) as well as the PPE68 protein (*Rv3873* gene) located in the cell envelope (Pym et al., 2002; Okkels et al., 2003; Demangel et al., 2004).
We have generated specific mouse monoclonal and rabbit polyclonal antibodies to P27-PPE36 and used them for the immunochemical characterization and cellular localization of this protein. Specific immunoblot analysis confirmed the presence of the P27-PPE36 antigen in *Mycobacterium bovis* BCG strain and in human clinical isolates of *M. tuberculosis* from

infected patients (Fig. 3), but not in other mycobacteria tested which does not belong to the *Mtb* complex (Le Moigne et al., 2005).
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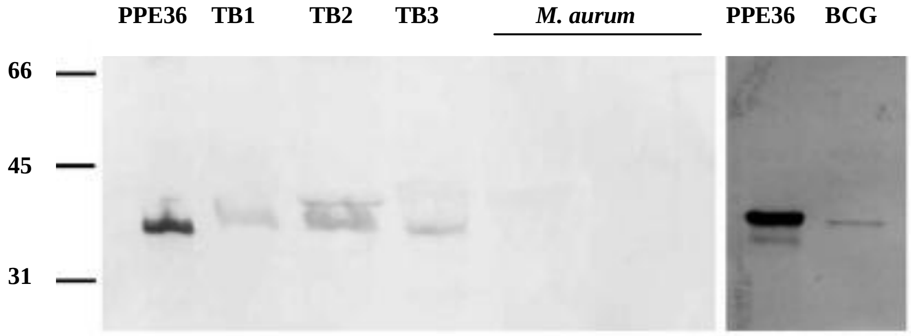

Fig. 3. Western blot analysis of bacterial lysate from different mycobacterial species. P27-PPE36 is the recombinant PPE36 protein. TB1, TB2 and TB3 are *Mycobacterium tuberculosis* clinical strains isolated from infected patients. *Mycobacterium aurum* is a fast-growing mycobacteria, and BCG is the Calmette-Guérin bacillus. The first antibody is a mouse monoclonal IgG antibody directed against PPE36.
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Then, after demonstrating that the P27-PPE36 protein was present in the *M. bovis* BCG strain and in clinical isolates of *M. tuberculosis*, we attempted to localize this PPE protein in the BCG strain. To achieve this, bacteria were washed, fixed and ultrathin sections were prepared to be analysed by electron microscopy using immunohistochemistry test with specific anti-P27-PPE36 antibodies. Results generated with monoclonal (Fig. 4 A) and polyclonal antibodies (Fig. 4 B) revealed a peripheral localization of this protein on the cell membrane. Similar results were obtained using western-blot analysis (Fig. 4 D) of the *Mtb* cell fractions with the monoclonal anti-P27-PPE36 antibody indicating that the P27-PPE36 protein is localized in the membrane of the cell (Le Moigne et al., 2005).

This protein was the third member of its family to be localized at the periphery of the cell (Sampson et al., 2001; Pym et al., 2002; Okkels et al., 2003; Demangel et al., 2004) and since, the same localization was assigned to other PPE proteins like for exemple Map3420c and Map1506 in *Mycobacterium avium* subsp. p*aratuberculosis* (Newton et al., 2009). In *Mycobacterium immunogenum*, a PPE protein (accessio no. YP_001288073) have been found to be a cell-membrane-associated antigen (Gupta et al., 2009). In a recent detailed analysis of the *Mycobacterium marinum* capsule using cryoelectron microscopy in conjunction with liquid chromatography mass spectrometry (LC-MS) demonstrated that 5 (MM1129, MM1402, MM0186, MM5047 and MM1497) of the 25 major cell surface proteins were members of the PPE familie (Sani et al., 2010). Similarly high-throughput proteomics MALDI-MS and LC-MS approaches have been utilized by Målen et al. (2010) to identify 8 PPEs in the *M. tuberculosis* envelope fractions (PPE18, PPE20, PPE26, PPE32, PPE33, PPE51, PPE60 and PPE68).
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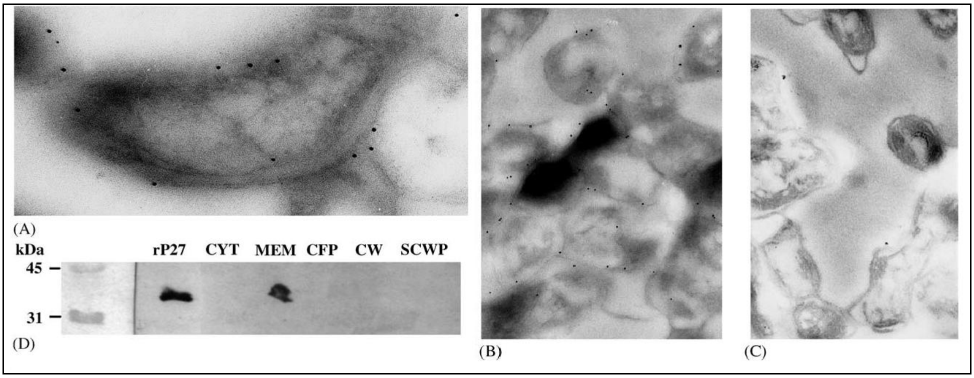

Fig. 4. Localization of P27-PPE36 antigen: Immunogold electron microscopic image (A–C) showing the peripheral localization of the P27-PPE36 protein in cryosectioned *M. bovis* BCG and western-blot on various *M. tuberculosis* cell fractions (D). Incubation was realized either with a mouse monoclonal antibody (A), or rabbit polyclonal anti-P27-PPE36 antibodies (B). Negative control was done using normal rabbit serum (C). (A: ×49 000, B and C: ×23 000). (D): Immunoblot analysis of different cell fractions of *M. tuberculosis* obtained from the Tuberculosis Research Materials and Vaccine Testing Laboratory, Colorado State University using monoclonal anti-P27-PPE36 antibody. Recombinant P27-PPE36 (rP27), cytosol fraction (CYT), cell membrane fraction (MEM), culture filtrate proteins (CFP), cell wall fraction (CW), and SDS-soluble cell wall proteins (SCWP).

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Therefore, these results suggest that cell wall/surface localization is a characteristic of several PE/PPE proteins although another PPE protein, PPE41, have been shown to be secreted by pathogenic mycobacteria (Abdallah et al., 2006). So, if for the majority of PE and PPE proteins are localize to the cell wall, some of them could be secreted into the extracellular environment.

Like explained above, P27-PPE36 should be, as a disordered protein which need a partner to fold, associated with the PE protein PE22 (Gey van Pittius et al., 2006; Strong et al., 2006). Moreover, this putative complex PPE36–PE22 could be associated with a system dedicated to the secretion of members of the potent T-cell antigen 6-kDa Early Secreted Antigenic Target (ESAT-6) family (Gey van Pittius et al., 2006). According this last computational study constructing an evolutionary history of the *pe* and *ppe* genes families, *Rv2107* and *Rv2108* genes are hypothesized to have been duplicated from the ESAT-6 (esx) gene cluster regions, as they are very homologous to their paralogues within the ESAT-6 (*esx*) gene clusters and have the same paired genomic orientation. These esx clusters encode the so-called Type VII or ESX secretion systems, of which there are 5 in *Mtb* (Gey van Pittius et al., 2001). Thus, *Rv2107* and *Rv2108* would derive from ESAT-6 gene cluster Region 2, i.e. from *Rv3893c* (PE36) and *Rv3892c* (PPE69).

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5. Serological studies

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Diverse reports point out the potential immunodominant nature of PPE proteins. Presence of antibodies against other PPE proteins have been found in mycobacterium infected human or animals: in human against the PPE17 (Rv1168c) (Khan et al., 2008), PPE41 (*Rv2340c*) (Choudhary et al., 2003), PPE42 (*Rv2608*) (Chakhaiyar et al., 2004), PPE55 (*Rv3347c*) (Singh et al., 2005), PPE57 (*Rv3425*) (Zhang et al., 2007), in human and mice against PPE68 (*Rv3873*) (Daugelat et al., 2003), in human (Rindi et al., 2007) and mice (Romano et al., 2008; Bonanni et al., 2005) against the PPE44 (*Rv2770c*), in cattle against PPE68 (*Rv3873*) (Cockle et al., 2002), and against a PPE protein of *Mycobacterium avium subsp paratuberculosis* (Newton et al., 2008). Other studies highlight the capacity of PPE proteins to induce high B cell response in TB patients like PPE41 (*Rv2340c*) (Tundup et al., 2008). Inversely, a study shows that patients with tuberculosis do not develop a strong humoral response against the PPE44 protein (Zanetti et al., 2005). In cattle, no difference is seen in the humoral response to the PPE44 (Rv2770c) between infected and TB-free animals (Molicotti et al., 2008).

The P27-PPE36 expressed protein is immunologically active, and reacts, in western-blot and ELISA, with antibodies from sera of patients infected with *Mtb* (Le Moigne et al., 2005). (Fig. 5).
¶(9pt)

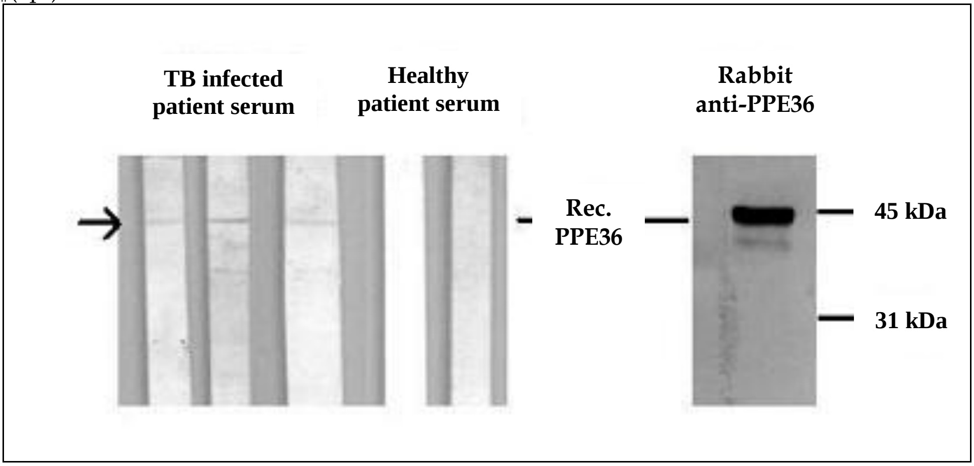

Fig. 5. Western blot analysis of the presence of anti-recombinant PPE36 antibodies in the sera of TB patients in comparison with serum from healthy donors and recombinant PPE36-hyperimmunized rabbit.
¶(9pt)
So, we then studied PPE36 specific antibody isotype distribution in sera of pulmonary tuberculosis patients and compared them to those in sera from healthy control by enzyme-linked immunosorbent assay (ELISA). Our result showed a significant increase of IgA antibody response in patient's sera, but a less important IgM response accompanied by total absence of IgG2, 3, and 4 responses and a weak IgG1 response in few patients' sera (unpublished results).
The absence of IgG response in the sera of patients allowed verifying for the presence of immune complexes that may inhibit the interaction of antibodies with our antigen on the plate. Using an immunoprecipitation test with goat anti human immunoglobulin antibodies, no immune complex containing P27-PPE36 was present in the patient's sera.

The significance of IgA and IgM is not clear. The IgA response is the more interesting and intriguing results for this protein, because this is the first study showing the presence of IgA alone and the absence of an IgG response against a peptidic antigen. The IgA response is often considered to be local (mucosa and body surface) and non systemic (sera). It has also been reported as a more specific for the non peptidic antigens comparing to the IgG response, which was more reactive (Julián et al., 2005). The IgM response is in general related to the natural auto antibodies found in the sera of healthy and infected peoples and animals, and we couldn't ascribe it a diagnostic value[6], though we note its augmentation during infection. These antibodies are in general polyspecific with weak affinity for their Ag.

The occurrence of antibodies against the PPE proteins is highly controversial; different studies highlighted the capacity of PPE proteins to induce high B cell response in TB human patients or infected animals (Tundup et al., 2008; Singh et al. 2005). Inversely, a study showed that patients with tuberculosis do not develop a strong humoral response against a PPE protein (Zanetti et al., 2005).

In comparison with other PPE proteins, P27-PPE36 proved to be less useful as a basis for the development of a TB diagnostic test. However, the presence of an IgA response in the absence of an IgG one, could be exploited as an indicator for *Mtb* diagnosis. A large number of sera should be tested to gather further information on the immune responses to this antigen.

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6. Immune response against P27-PPE36 by different immunisation ways

¶(6pt)

We have studied the immune response of mice against the *Mtb* P27-PPE36 protein.
The peripheral localization of the P27-PPE36 protein led to the belief that they might play an important immunological role either in diagnosis or in protection. So, we examined the immune response against the P27-PPE36 protein using different Pathogen associated molecular patterns (PAMPs) as adjuvants and vectors for immunization. PAMPs are expressed only by micro-organisms and are recognized by the eukaryotic cells through the pattern recognition receptors (PRRs) of the innate immune system such as the Toll-like receptors (TLRs) (Medzhitov & Janeway, 2000). The interaction of PAMPs with their corresponding TLRs helps to identify the nature of the PAMP and to guide the adequate adaptive immune response (Medzhitov & Janeway, 2000). Muramyl dipeptides, a major element of the Freund's complete adjuvant, bacterial DNA, and bacterial flagellin are three PAMPs recognized by TLR2, TLR9, and TLR5, respectively.

Different immunization protocols were used to study immunological potential of the P27-PPE36 protein. BALB/c mice were immunized either with the P27-PPE36 recombinant protein in Freund's adjuvant or in phosphate saline buffer (PBS) (classical immunization), with a pcDNA3 plasmid containing the gene encoding the P27-PPE36 protein (DNA immunization), or with the *Escherichia coli* bacteria expressing the P27-PPE36 protein genetically fused into the flagellin (flagellin immunization) (Le Moigne et al., 2008).

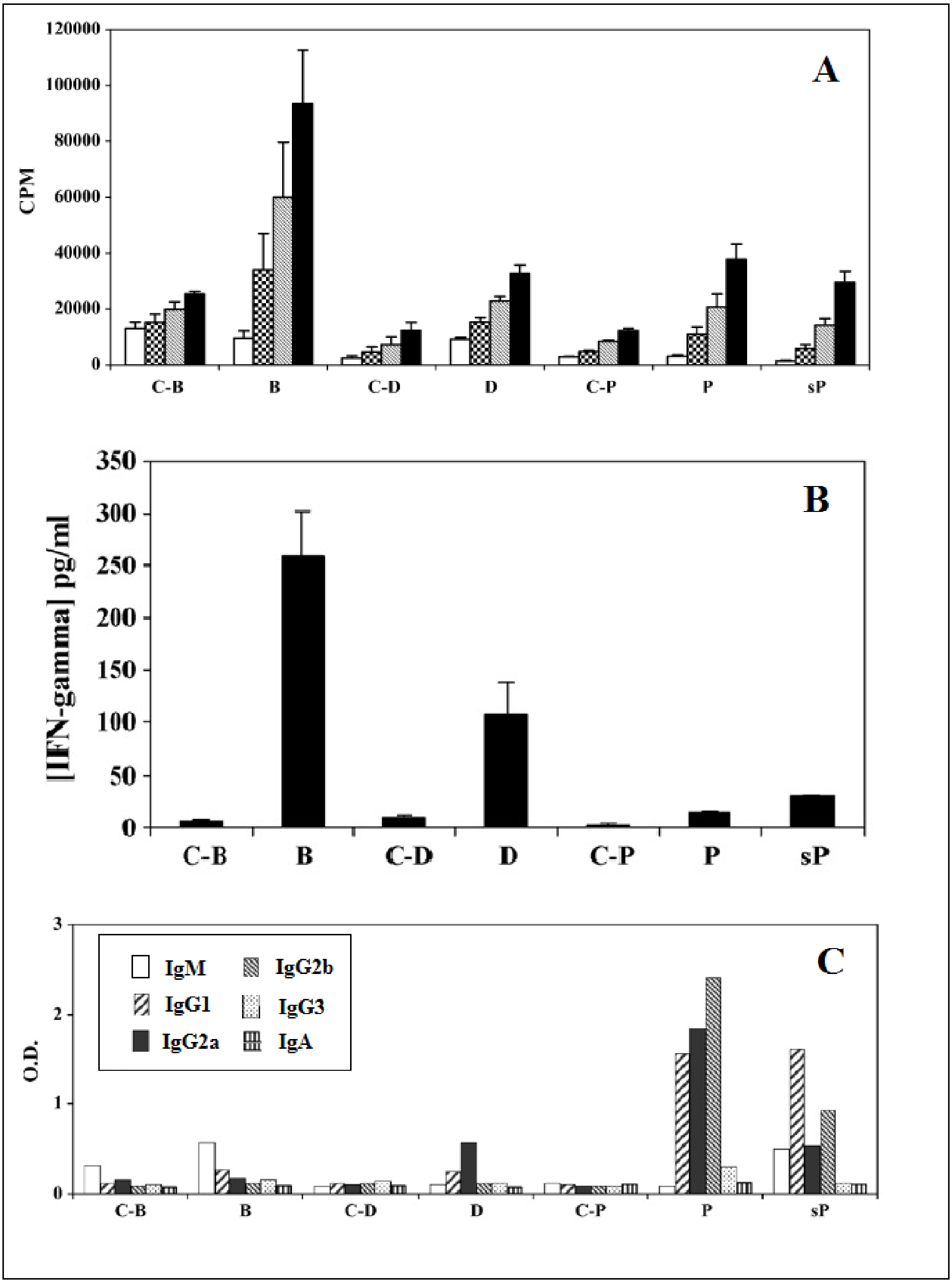

Fig. 6. Immune response generated against P27-PPE36 from mice immunized either with flagellin-modified bacteria (B), DNA plasmid containing the *Rv2108* gene (D) or with the P27-PPE36 recombinant protein associated (P) or not (sP) with Freund's adjuvant. Control

groups have been immunized with non-modified bacteria (C-B), the empty pcDNA3 plasmid (C-D) or with PBS in Freund's adjuvant (C-P).
(**A**): Proliferation of splenic cells of immunized mice after incubation *in vitro* with different concentrations (☐ 0.0 µg/ml, (◘) 1.1µg/ml, (▒) 3.3 µg/ml and (■) 10µg/ml) of purified p27 recombinant protein. The proliferation was monitored by [^{3}H] thymidine uptake at 66 h after stimulation. (**B**): Cytokine secretion by splenic cells of immunized mice. Splenic cells were stimulated *in vitro* by the recombinant P27-PPE36 protein and IFN-γ was quantified in the supernatant after one week of culture. Results are presented as mean cytokine concentrations (±standard errors) compared to a standard curve of purified cytokines.
(**C**): Specific anti-P27-PPE36 antibodies responses. Mice sera diluted at 1/500 were tested in ELISA for the presence of anti-P27-PPE36 antibodies of the different isotypes IgG1, IgG2a, IgG2b, IgG3, IgM and IgA one week after the third immunization. The results are presented as the optical density of the different isotypes.
¶(9pt)
We found that P27-PPE36 expressed into the flagellin led to the strongest cellular responses, where we obtained the highest production of IFN-γ (Fig. 6 B) and cell proliferation (Fig. 6 A), an indication of specific Th1-like orientation of the immune response. DNA immunization was less potent in the induction of such responses. We confirmed the role of flagellin in this response by using different immunization combinations (Le Moigne et al., 2008). However, the specific antibody response was weak with either method (Fig. 6 C). On the other hand, classical immunization with the recombinant protein, soluble or incorporated in Freund's adjuvant still yielded the best antibody response (Fig. 6 C). The best cellular and humoral responses were obtained in the group of mice primed with the recombinant protein and boosted by the antigen presented on the modified flagellin (Le Moigne et al., 2008). In general, the P27-PPE36 PPE antigen induced a strong proliferative response accompanied by high production of IFN-γ and low amount of IL-4 (Le Moigne et al., 2008), independently of the PAMP used. The results indicated that this antigen may be involved in the establishment of the host cellular immune responses against the *Mtb*.

Protective anti-mycobacterial immunity is primarily mediated by cellular immune responses (Flynn et al., 1992; Caruso et al., 1999). *Mtb* is rich in antigens that induce IFN-γ secretion, and the presence of such antigens has been reported in purified cell walls, the cytosolic fraction, and short-term culture filtrates (ST-CF) (Mustafa, 2001). The importance of antibodies in tuberculosis is much debated, but it has been suggested that certain antibody specificities against bacterial surface epitopes and with the correct isotype may confer protection against intracellular infections (Glatman-Freedman , 2003; Glatman-Freedman and Casadevall, 1998; Casadevall, 1995).

Other PPE proteins have been reported to be strongly immunogenic (Choudhary et al., 2003; Demangel et al., 2004, Okkels et al., 2003; Dillon et al., 1999; Skeiky et al., 2000). Antibodies against PPE41 (*Rv2430c*) are present in TB patients and not in healthy individuals (Choudhary et al, 2003); PPE68 (*Rv3873*) induces IFN-γ production from splenocytes of *M. tuberculosis*-infected mice and from peripheral blood mononuclear cells of TB patients and PPD+ healthy individuals (Demangel et al., 2004, Okkels et al., 2003) and from cattle blood cells (Cockle et al., 2002; Mustafa et al., 2002). Immune responses elicited by PPE18 (*Rv1196*) and PPE14 (*Rv0915c*) have been shown to provide some protection in mice infected with *M.*

tuberculosis (Dillon et al., 1999; Skeiky et al., 2000). Together, these studies suggest that several PPE proteins are expressed in vivo. In other mycobacteries, other PPE proteins have been shown to induce immune responses. For exemple in *M. avium subs. paratuberculosis*, two PPE proteins named Map39 and Map41 significantly elicited IFN-γ production in peripheral blood mononuclear cells from infected cattle (Nagata et al., 2005). When immunized in mice, PPE57 (*Rv3425*) and PPE46 (*Rv3018c*) induce also strong humoral and cellular responses (Wang et al., 2008; Chaitra et al., 2007)

¶(14pt)

7. Conclusion

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The P27-PPE36 protein is the third member of its family to be localized at the periphery of the cell (Sampson et al., 2001; Pym et al., 2002; Okkels et al., 2003). Now others PPE have been found to have a similar localization. This may shed some light on its role in the diagnosis and pathogenesis of Mtb.

In conclusion, the P27-PPE36 protein was found to be a specific antigen for the *Mtb* complex and was recognized by sera of tuberculosis patients and localized in the membrane of the bacterial cell.

¶(14pt)

8. Acknowledgment

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9. References

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Abdallah, A.M.; Verboom, T.; Hannes, F.; Safi, M.; Strong, M. Eisenberg, D.; Musters, R.J.; Vandenbroucke-Grauls, C.M.; Appelmelk, B.J.; Luirink, J. & Bitter, W.A. (2006). Specific secretion system mediates PPE41 transport in pathogenic mycobacteria. *Mol. Microbiol.*, **62**: 667–679.

Adindla, S. & Guruprasad, L. (2003). Sequence analysis corresponding to the PPE and PE proteins in *Mycobacterium tuberculosis* and other genomes. *J Biosci.*, **28:** 169-179.

Bansal, K.; Sinha, A.Y.; Ghorpade, D.S.; Togarsimalemath, S.K.; Patil, S.A.; Kaveri, S.V.; Balaji, K.N. & Bayry, J. (2010). Src homology 3-interacting domain of Rv1917c of *Mycobacterium tuberculosis* induces selective maturation of human dendritic cells by regulating PI3K-MAPK-NF-kappaB signaling and drives Th2 immune responses. *J. Biol. Chem.*, **285** (47): 36511-36522.

Banu, S.; Honoré, N.; Saint-Joanis, B.; Philpott, D.; Prevost, M.C. & Cole, S.T. (2002). Are the PE-PGRS proteins of *Mycobacterium tuberculosis* variable surface antigens? *Mol. Microbiol.*, **44** (1) : 9-19.

Becq, J.; Gutierrez, M.; Rosas-Magallanes, V.; Rauzier, J.; Gicquel, B.; Neyrolles, O. & Deschavanne, P. (2007). Contribution of horizontally acquired genomic islands to the evolution of the tubercle bacilli. *Mol. Biol. Evol.*, **24**: 1867–1871.

Beggs, M.L.; Eisenach, K.D. & Cave, M.D. (2000). Mapping of IS6110 insertion sites in two epidemic strains of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.*, **38** (8): 2923-2928.

Betts, J.C.; Lukey, P.T.; Robb, L.C.; McAdam, R.A. & Duncan, K. (2002). Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol.*, **43** (3): 717–731.

Bonanni, D.; Rindi, L.; Lari, N. & Garzelli, C. (2005). Immunogenicity of mycobacterial PPE44 (Rv2770c) in *Mycobacterium bovis* BCG-infected mice. *J. Med. Microbiol.*, **54** (Pt 5): 443-448.

Bowers, P.M.; Pellegrini, M.; Thompson, M.J.; Fierro, J.; Yeates, T.O. & Eisenberg, D. (2004). Prolinks: a database of protein functional linkages derived from coevolution. *Genome Biol.*, **5** (5):R35.

Camacho, L.R.; Ensergueix, D.; Perez, E.; Gicquel, B. & Guilhot, C. (1999). Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. *Mol. Microbiol.*, **34**: 257-267.

Caruso, A.M.; Serbina, N.; Klein, E.; Triebold, K.; Bloom, B.R. & Flynn, J.L. (1999). Mice deficient in CD4 T cells have only transiently diminished levels of IFN-γ, yet succumb to tuberculosis. *J. Immunol.*, **162** (9): 5407–5416.

Casadevall, A. (1995). Antibody immunity and invasive fungal infections. *Infect. Immun.*, **63**: 4211–4218.

Chaitra, M.G.; Nayak, R. & Shaila, M.S. (2007). Modulation of immune responses in mice to recombinant antigens from PE and PPE families of proteins of *Mycobacterium tuberculosis* by the Ribi adjuvant. *Vaccine*, **25** (41): 7168-7176.

(a) Chaitra, M.G.; Shaila, M.S. & Nayak, R. (2008). Detection of interferon gamma-secreting CD8+ T lymphocytes in humans specific for three PE/PPE proteins of *Mycobacterium tuberculosis*. *Microbes Infect.*, **10** (8): 858-867.

(b) Chaitra, M.G.; Shaila, M.S. & Nayak, R. (2008). Characterization of T-cell immunogenicity of two PE/PPE proteins of *Mycobacterium tuberculosis*. *J. Med. Microbiol.*, **57** (Pt 9): 1079-1086.

Chakhaiyar, P.; Nagalakshmi, Y.; Aruna, B.; Murthy, K.J.; Katoch, V.M. & Hasnain, S.E. (2004). Regions of high antigenicity within the hypothetical PPE major polymorphic tandem repeat open-reading frame, Rv2608, show a differential humoral response and a low T cell response in various categories of patients with tuberculosis. *J. Infect. Dis.*, **190** (7): 1237-1244.

Chevrier, D.; Casademont, I. & Guesdon, J.L. (2000). Cloning of a gene from *Mycobacterium tuberculosis* coding for a hypothetical 27 kDa protein and its use for the specific PCR identification of these mycobacteria. *Mol. Cell. Probes*, **14**: 241–248.

Choudhary, R.K.; Mukhopadhyay, S.; Chakhaiyar, P.; Sharma, N.; Murthy, K.J.R.; Katoch, V.M. & Hasnain S.E. (2003). PPE antigen Rv2430c of *Mycobacterium tuberculosis* induces a strong B-cell response. *Infect. Immun.*, **71**: 6338–6343.

Cockle, P.J.; Gordon, S.V.; Lalvani, A.; Buddle, B.M.; Hewinson, R.G. & Vordermeier, H.M. (2002). Identification of novel *Mycobacterium tuberculosis* antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. *Infect. Immun.*, **70** (12): 6996-7003.

Cole, S.T.; Brosch, R.; Parkhill, J.; Garnier, T.; Churcher, C.; Harris, D.; Gordon, S.V.; Eiglmeier, K.; Gas, S.; Barry, C.E. 3rd; Tekaia, F.; Badcock, K.; Basham, D.; Brown, D.; Chillingworth, T.; Connor, R.; Davies, R.; Devlin, K.; Feltwell, T.; Gentles, S.; Hamlin, N.; Holroyd, S.; Hornsby, T.; Jagels, K.; Krogh, A.; McLean, J.; Moule, S.; Murphy, L.; Oliver, K.; Osborne, J.; Quail, M.A.; Rajandream, M.A.; Rogers, J.; Rutter, S.; Seeger, K.; Skelton, J.; Squares, R.; Squares, S.; Sulston, J.E.; Taylor, K.; Whitehead, S. & Barrell, B.G. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature,* **393** (6685): 537–544.

Cole, S.T. (1999). Learning from the genome sequence of *Mycobacterium tuberculosis* H37Rv. *FEBS Lett.*, **452**: 7-10.

Cole, S.T.; Eiglmeier, K.; Parkhill, J.; James, K.D.; Thomson, N.R.; Wheeler, P.R.; Honoré, N.; Garnier, T.; Churcher, C.; Harris, D.; Mungall, K.; Basham, D.; Brown, D.; Chillingworth, T.; Connor, R.; Davies, R.M.; Devlin, K.; Duthoy, S.; Feltwell, T.; Fraser, A.; Hamlin, N.; Holroyd, S.; Hornsby, T.; Jagels, K.; Lacroix, C.; Maclean, J.; Moule, S.; Murphy, L.; Oliver, K.; Quail, M.A.; Rajandream, M.A.; Rutherford, K.M.; Rutter, S.; Seeger, K.; Simon, S.; Simmonds, M.; Skelton, J.; Squares, R.; Squares, S.; Stevens, K.; Taylor, K.; Whitehead, S.; Woodward, J.R. & Barrell B.G. (2001). Massive gene decay in the leprosy bacillus. *Nature*, **409** (6823): 1007-1011.

Cubillos-Ruiz, A.; Morales, J. & Zambrano, M.M. (2008). Analysis of the genetic variation in *Mycobacterium tuberculosis* strains by multiple genome alignments. *BMC Research Notes*. **vol. 1**: article 110.

Daugelat, S.; Kowall, J.; Mattow, J.; Bumann, D.; Winter, R.; Hurwitz, R. & Kaufmann S.H. (2003). The RD1 proteins of *Mycobacterium tuberculosis*: expression in *Mycobacterium smegmatis* and biochemical characterization. *Microbes Infect.*, **5** (12): 1082-1095.

Demangel, C. ; Brodin, P.; Cockle, P.J.; Brosch, R. ; Majlessi, L.; Leclerc, C. & Cole S.T. (2004). Cell envelope protein PPE68 contributes to *Mycobacterium tuberculosis* RD1 immunogenicity independently of a 10-kilodalton culture filtrate protein and ESAT-6. *Infect. Immun*. **72**: 2170–2176.

Dillon, D.C.; Alderson, M.R.; Day, C.H.; Lewinsohn, D.M.; Coler, R.; Bement, T.; Campos-Neto, A.; Skeiky, Y.A.W.; Orme, I.M.; Roberts, A.; Steen, S.; Dalemans, W.; Badaro, R. & Reed S.G. (1999). Molecular characterization and human T-cell responses to a member of a novel *Mycobacterium tuberculosis mtb39* gene family. *Infect. Immun.*, **67**: 2941–2950.

Dubnau, E.; Fontan, P.; Manganelli, R.; Soares-Appel, S. & Smith, I. (2002). *Mycobacterium tuberculosis* genes induced during infection of human macrophages. *Infect. Immun.*, **70**: 2787–2795.

Dunker, A.K.; Lawson, J.D.; Brown, C.J.; Williams, R.M.; Romero, P.; Oh, J.S.; Oldfield, C.J.; Campen, A.M.; Ratliff, C.M.; Hipps, K.W.; Ausio, J.; Nissen, M.S.; Reeves, R.; Kang, C.; Kissinger, C.R.; Bailey, R.W.; Griswold, M.D.; Chiu, W.; Garner, E.C. & Obradovic, Z. (2001). Intrinsically disordered protein. *J. Mol. Graph. Model.*, **19**: 26–59.

Fisher, M.A.; Plikaytis, B.B.; Shinnick, T.M. (2002). Microarray analysis of the *Mycobacterium tuberculosis* transcriptional response to the acidic conditions found in phagosomes. *J. Bacteriol.*, **184** (14): 4025-4032.

Fleischmann, R.D.; Alland, D.; Eisen, J.A.; Carpenter, L.; White, O.; Peterson, J.; DeBoy, R.; Dodson, R.; Gwinn, M.; Haft, D.; Hickey, E.; Kolonay, J.F.; Nelson, W.C.; Umayam, L.A.; Ermolaeva, M.; Salzberg, S.L.; Delcher, A.; Utterback, T.; Weidman, J.; Khouri, H.; Gill, J.; Mikula, A.; Bishai, W.; Jacobs, J.W.R. Jr.; Venter, J.C. & Fraser, C.M. (2002). Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. *J. Bacteriol.*, **184**: 5479-5490.

Flynn, J.L.; Goldstein, M.M.; Triebold, K.J.; Koller, B. & Bloom, B.R. (1992). Major histocompatibility complex class I restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. *P.N.A.S. U.S.A.*, **89** (24): 12013–12017.

Glatman-Freedman, A. (2003). Advances in antibody-mediated immunity against *Mycobacterium tuberculosis*: implications for a novel vaccine strategy. *FEMS Immunol. Med. Microbiol.*, **39**: 9–16.

Glatman-Freedman, A. & Casadevall, A. (1998). Serum therapy for tuberculosis revisited: reappraisal of the role of antibody-mediated immunity against *Mycobacterium tuberculosis*. *Clin. Microbiol. Rev.*, **11**: 514–532.

Gao, Q.; Kripke, K.E.; Saldanha, A.J.; Yan, W.; Holmes, S. & Small, P.M. (2005). Gene expression diversity among *Mycobacterium tuberculosis* clinical isolates. *Microbiology,* **151** (Pt 1): 5-14.

Gey Van Pittius, N.C.; Gamieldien, J.; Hide, W.; Brown, G.D.; Siezen, R.J. & Beyers, A.D. (2001). The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C Gram-positive bacteria. *Genome Biology*, **2** (10): RESEARCH0044.

Gey van Pittius, N.C.; Sampson, S.L.; Lee, H.; Kim, Y.; van Helden, P.D. & Warren, R.M.; (2006). Evolution and expansion of the *Mycobacterium tuberculosis* PE and PPE multigene families and their association with the duplication of the ESAT-6 (esx) gene cluster regions. *B.M.C. Evol. Biol*., **6**, 95.

Garnier, T.; Eiglmeier, K.; Camus, J.C.; Medina, N.; Mansoor, H.; Pryor, M.; Duthoy, S.; Grondin, S.; Lacroix, C.; Monsempe, C.; Simon, S.; Harris, B.; Atkin, R.; Doggett, J.; Mayes, R.; Keating, L.; Wheeler, P.R.; Parkhill, J.; Barrell, B.G.; Cole, S.T.; Gordon, S.V. & Hewinson, R.G. The complete genome sequence of *Mycobacterium bovis*. *Proc. Natl. Acad. Sci. U.S.A.*, **100**: 7877-7882.

Gordon, S.V.; Eiglmeier, K.; Garnier, T.; Brosch, R.; Parkhill, J.; Barrell, B.; Cole, S.T. & Hewinson, R.G. (2001). Genomics of *Mycobacterium bovis*. *Tuberculosis (Edinb),* **81**: 157-163

Gordon, B.R.; Li, Y.; Wang, L.; Sintsova, A.; van Bakel, H.; Tian, S.; Navarre, W.W.; Xia, B.; Liu, J. (2010). Lsr2 is a nucleoid-associated protein that targets AT-rich sequences and virulence genes in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.*, **107** (11): 5154-5159.

Gupta, M.K.; Subramanian, V. & Yadav, J.S. (2009). Immunoproteomic identification of secretory and subcellular protein antigens and functional evaluation of the secretome fraction of *Mycobacterium immunogenum* a newly recognized species of the *Mycobacterium chelonae- Mycobacterium abscessus* group. *Journal of Proteome Research*, **8** (5): 2319–2330.

Gutierrez, M.C.; Brisse, S.; Brosch, R.; Fabre, M.; Omais, B.; Marmiesse, M.; Supply, P. & Vincent, V. (2005). Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis. PloS Pathog.,* **1** (1): e5.

Hebert, A.M.; Talarico, S.; Yang, D.; Durmaz, R.; Marrs, C;F.; Zhang, L.; Foxman, B. & Yang Z. (2007). DNA polymorphisms in the pepA and PPE18 genes among clinical strains of *Mycobacterium tuberculosis*: implications for vaccine efficacy. *Infect. Immun.,* **75**: 5798-5805.

Hou, J.Y.; Graham, J.E. & Clark-Curtiss, J.E. (2002). *Mycobacterium avium* genes expressed during growth in human macrophages detected by Selective Capture of Transcribed Sequences (SCOTS). *Infect. Immun.,* **70**: 3714–3726.

Jacob, F. & Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.,* **3**: 318–356.

Julián, E.; Matas, L.; Pérez, A.; Alcaide, J.; Lanéelle, M.A. & Luquin, M. (2002). Serodiagnosis of tuberculosis: comparison of immunoglobulin A (IgA) response to sulfolipid I with IgG and IgM responses to 2,3-diacyltrehalose, 2,3,6-triacyltrehalose, and cord factor antigens. *J. Clin. Microbiol.*, **40**: 3782-3788.

Khan, N.; Alam, K.; Nair, S.; Valluri, V.L.; Murthy, K.J. & Mukhopadhyay, S. (2008). Association of strong immune responses to PPE protein Rv1168c with active tuberculosis. *Clin. Vaccine Immunol.*, **15** (6): 974-980.

Kent, L.; McHugh, T.D.; Billington, O.; Dale J.W. & Gillespie, S.H. (1995). Demonstration of homology between IS6110 of *Mycobacterium tuberculosis* and DNAs of other *Mycobacterium* spp. *J. Clin. Microbiol.*, **33** (9): 2290-2293.

Kinsella, R.J.; Fitzpatrick, D.A.; Creevey, C.J. & McInerney, J.O. (2003). Fatty acid biosynthesis in *Mycobacterium tuberculosis*: Lateral gene transfer, adaptive evolution, and gene duplication. *Proc. Natl. Acad. Sci. U.S.A.*, **100:** 10320–10325.

Karboul, A.; Mazza, A.; Gey van Pittius, N.C.; Ho, J.L.; Brousseau, R. & Mardassi, H. (2008). Frequent homologous recombination events in *Mycobacterium tuberculosis* PE/PPE multigene families: potential role in antigenic variability. *J. Bacteriol.*, **190** (23): 7838–7846.

Lee, B.W.; Tan, J.A.; Wong, S.C.; Tan C.B.; Yap, H.K.; Low, P.S.; Chia, J.N. & Tay, J.S. (1994). DNA amplification by the polymerase chain reaction for the rapid diagnosis of tuberculous meningitis. Comparison of protocols involving three mycobacterial DNA sequences, IS6110, 65 kDa antigen, and MPB64. *J. Neurol. Sci.*, **123** (1-2): 173-179.

Le Moigne, V.; Robreau, G.; Borot, C.; Guesdon, J.L. & Mahana, W. (2005). Expression, immunochemical, characterisation and localisation of the *Mycobaterium tuberculosis* protein p27. *Tuberculosis (Edinb)*, **85**: 213–219.

Le Moigne, V.; Robreau, G. & Mahana, W. (2008). Flagellin as a good carrier and potent adjuvant for Th1 response: Study of mice immune response to the p27 (Rv2108) *Mycobacterium tuberculosis* antigen. *Molecular Immunology*, **45**: 2499–2507

Li, Y.; Miltner, E.; Wu, M.; Petrofsky, M. & Bermudez, L.E. (2005). A *Mycobacterium avium* PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. *Cell. Microbiol.*, **7**: 539–548.

Macfarlane, A.; Mondragon-Gonzalez, R.; Vega-Lopez, F.; Wieles, B.; de Pena, J.; Rodriguez, O.; Suarez y de la Torre, R.; de Vries, R.R.; Ottenhoff, T.H. & Dockrell, H.M. (2001). Presence of human T-cell responses to the *Mycobacterium leprae* 45-kilodalton antigen reflects infection with or exposure to *M. leprae*. *Clin. Diagn. Lab. Immunol.*, **8** (3): 604-611.

Målen, H.; Pathak, S.; Softeland, T.; de Souza, G.A. & Wiker, H.G. (2010). Definition of novel cell envelope associated proteins in Triton X-114 extracts of *Mycobacterium tuberculosis* H37Rv. *BMC Microbiology*, **10**: 132.

Manganelli, R.; Voskuil, M.I.; Schoolnik, G.K. & Smith, I. (2001). The *Mycobacterium tuberculosis* ECF sigma factor sigmaE: role in global gene expression and survival in macrophages. *Mol. Microbiol.*, **41** (2): 423-437.

Manganelli, R.; Voskuil, M.I.; Schoolnik, G.K.; Dubnau, E.; Gomez, M. & Smith I. (2002). Role of the extracytoplasmic-function sigma factor sigma(H) in *Mycobacterium tuberculosis* global gene expression. *Mol. Microbiol.*, **45** (2): 365-374.

McEvoy, C.R.E.; van Helden, P.D.; Warren, R.M. & Gey van Pittius, N.C. (2009). Evidence for a rapid rate of molecular evolution at the hypervariable and immunogenic *Mycobacterium tuberculosis PPE38* gene region. *BMC Evolutionary Biology*, **9**: 237.

Medzhitov, R. & Janeway Jr., C. (2000). Innate immune recognition: mechanisms and pathways. *Immunol. Rev.*, **173**: 89‑97.

Mishra, K.C.; de Chastellier, C.; Narayana, Y.; Bifani, P.; Brown, A.K.; Besra, G.S.; Katoch, V.M.; Joshi, B.; Balaji, K.N. & Kremer, L. (2008). Functional role of the PE domain and immunogenicity of the *Mycobacterium tuberculosis* triacylglycerol hydrolase LipY. *Infect. Immun.,* **76** (1): 127-140.

Molicotti, P.; Bua, A.; Ortu, S.; Ladu, M.C.; Delogu, G.; Mura, A.; Sechi, L.A.; Fadda, G. & Zanetti, S. (2008). Heparin binding haemagglutin as potential diagnostic marker of *Mycobacterium bovis*. *New Microbiol*., **31** (3): 423-427.

Mustafa, A.S. (2001). Biotechnology in the development of new vaccines and diagnostic reagents against tuberculosis. *Curr. Pharm. Biotechnol*., **2 :**157–173.

Mustafa, A.S.; Cockle, P.J.; Shaban, F.; Hewinson, R.G. & Vordermeier, H.M. (2002). Immunogenicity of *Mycobacterium tuberculosis* RD1 region gene products in infected cattle. *Clin. Exp. Immunol*., **130** (1): 37-42.

Nagata, R.; Muneta, Y.; Yoshihara, K.; Yokomizo, Y. & Mori, Y. (2005). Expression cloning of gamma interferon-inducing antigens of *Mycobacterium avium* subsp. *Paratuberculosis. Infec. Immun*., **73** (6): 3778–3782

Nair, S.; Ramaswamy, P.A.; Ghosh, S.; Joshi, D.C.; Pathak, N.; Siddiqui, I.; Sharma, P.; Hasnain, S.E.; Mande, S.C. & Mukhopadhyay, S. (2009). The PPE18 of *Mycobacterium tuberculosis* interacts with TLR2 and activates IL-10 induction in macrophage. *J. Immunol*., **183** (10): 6269-6281.

Newton, V.; McKenna, S.L. & De Buck, J. (2009). Presence of PPE proteins in *Mycobacterium avium* subsp. *paratuberculosis* isolates and their immunogenicity in cattle. *Vet. Microbiol*., **135** (3-4): 394-400.

Okkels, L.M.; Brock, I.; Follmann, F.; Agger, E.M.; Arend, S.M.; Ottenhoff, T.H.M.; Oftung, F.; Rosenkrands, I. & Andersen P. (2003). PPE protein (Rv3873) from DNA segment RD1 of *Mycobacterium tuberculosis*: strong recognition of both specific T-cell epitopes and epitopes conserved within the PPE family. *Infect. Immun.,* **71**: 6116–6123.

Park, H.D.; Guinn, K.M.; Harrell, M.I.; Liao, R.; Voskuil, M.I.; Tompa, M.; Schoolnik, G.K. & Sherman, D.R. (2003). Rv3133c/dosR is a transcription factor that mediates the hypoxic response of *Mycobacterium tuberculosis*. *Mol. Microbiol*., **48** (3): 833-843.

Parkash, O.; Kumar, A.; Nigam, A.; Franken, K.L. & Ottenhoff, T.H. (2006). Evaluation of recombinant serine-rich 45-kDa antigen (ML0411) for detection of antibodies in leprosy patients. *Scand. J. Immunol*., **64** (4): 450-455.

Plotkin, J.B.; Dushoff, J. & Fraser, H.B. (2004). Detecting selection using a single genome sequence of *M. tuberculosis* and *P. falciparum*. *Nature*, **428** (6986): 942-945.

Provvedi, R.; Boldrin, F.; Falciani, F.; Palù, G.; & Manganelli, R. (2009). Global transcriptional response to vancomycin in *Mycobacterium tuberculosis.* Microbiology, **155** (Pt 4): 1093-1102.

Pym, A.S.; Brodin, P.; Brosch, R.; Huerre, M. & Cole, S.T. (2002). Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Mol. Microbiol*., **46** (3): 709-717.

Rehren, G.; Walters, S.; Fontan, P.; Smith, I. & Zárraga, A.M. (2007). Differential gene expression between *Mycobacterium bovis* and *Mycobacterium tuberculosis*. *Tuberculosis (Edinb.),* **87** (4): 347-359.

Riley, R.; Pellegrini, M. & Eisenberg, D. (2008). Identifying cognate binding pairs among a large set of paralogs: the case of PE/PPE proteins of *Mycobacterium tuberculosis*. *PLoS Comput. Biol.*, **4** (9): e1000174.

Rinke de Wit, T.F.; Clark-Curtiss, J.E.; Abebe, F.; Kolk, A.H.; Janson, A.A.; van Agterveld, M. & Thole, J.E. (1993). A *Mycobacterium leprae*-specific gene encoding an immunologically recognized 45 kDa protein. *Mol. Microbiol.*, **10** (4):829-38.

Rindi, L.; Lari, N. & Garzelli, C. (1999). Search for genes potentially involved in *Mycobacterium tuberculosis* virulence by mRNA differential display. *Biochem. Biophys. Res. Commun.,* **258**: 94–101.

Rindi, L.; Peroni, I.; Lari, N.; Bonanni, D.; Tortoli, E. & Garzelli, C. (2007). Variation of the expression of *Mycobacterium tuberculosis* ppe44 gene among clinical isolates. *FEMS Immunol. Med. Microbiol.,* **51** (2): 381-387.

Rodriguez, G.M.; Gold, B.; Gomez, M.; Dussurget, O. & Smith, I. (1999). Identification and characterization of two divergently transcribed iron regulated genes in *Mycobacterium tuberculosis*. *Tuber. Lung. Dis.,* **79**: 287–298.

Rodriguez, G.M.; Voskuil, M.I.; Gold, B.; Schoolnik, G.K. & Smith, I. (2002). IdeR, an essential gene in *Mycobacterium tuberculosis*: role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response. *Infect. Immun*., **70** (7): 3371-3381.

Romano, M.; Rindi, L.; Korf, H.; Bonanni, D.; Adnet, P.Y.; Jurion, F.; Garzelli, C. & Huygen, K. (2008). Immunogenicity and protective efficacy of tuberculosis subunit vaccines expressing PPE44 (Rv2770c). *Vaccine*, **26** (48): 6053-6063.

Rosas-Magallanes, V.; Deschavanne, P.; Quintana-Murci, L.; Brosch, R.; Gicquel, B. & Neyrolles, O. (2006). Horizontal transfer of a virulence operon to the ancestor of *Mycobacterium tuberculosis. Mol. Biol. Evol.,* **23**: 1129–1135.

Rustad, T.R.; Harrell, M.I.; Liao, R. & Sherman, D.R. (2008). The enduring hypoxic response of *Mycobacterium tuberculosis*. *PLoS One,* **3** (1): e1502.

(a) Sampson, S.L.; Lukey, P.; Warren, R.M.; van Helden, P.D.; Richardson, M. & Everett, M.J. (2001). Expression, characterization and subcellular localization of the *Mycobacterium tuberculosis* PPE gene Rv1917c. *Tuberculosis (Edinb.),* **81**: 305–317.

(b) Sampson, S.L.; Warren, R.; Richardson, M.; van der Spuy, G. & van Helden, P. (2001). IS6110 insertions in *Mycobacterium tuberculosis*: predominantly into coding regions. *J. Clin. Microbiol.*, **39** (9): 3423-3424.

Sani, M.; Houben, E.N.; Geurtsen, J.; Pierson, J.; de Punder, K.; van Zon, M.; Wever, B.; Piersma, S.R.; Jiménez, C.R.; Daffé, M.; Appelmelk, B.J.; Bitter, W.; van der Wel, N. & Peters, P.J. (2010). Direct visualization by cryo-EM of the mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. *PLoS Pathog*., **6** (3): e1000794.

Sassetti, C.M.; Boyd, D.H. & Rubin, E.J. (2003). Genes required for mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol.,* **48** (1): 77-84.

Schnappinger, D.; Ehrt, S.; Voskuil, M.I.; Liu, Y.; Mangan, J.A.; Monahan, I.M.; Dolganov, G.; Efron, B.; Butcher, P.D.; Nathan, C. & Schoolnik, G.K. (2003). Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J. Exp. Med.*, **198** (5): 693–704.

Sherman, D.R.; Voskuil, M.; Schnappinger, D.; Liao, R.; Harrell, M.I. & Schoolnik G.K. (2001). Regulation of the *Mycobacterium tuberculosis* hypoxic response gene encoding alpha -crystallin. *Proc. Natl. Acad. Sci. U.S.A.*, **98** (13): 7534-7539.

Singh, K.K.; Dong, Y.; Patibandla, S.A.; McMurray, D.N.; Arora, V.K. & Laal, S. (2005). Immunogenicity of the *Mycobacterium tuberculosis* PPE55 (Rv3347c) protein during incipient and clinical tuberculosis. *Infect. Immun.*, **73**: 5004-5014.

Skeiky, Y.A.; Ovendale, P.J.; Jen, S.; Alderson, M.R.; Dillon, D.C.; Smith, S.; Wilson, C.B., Orme, I.M.; Reed, S.G. & Campos-Neto, A. (2000). T cell expression cloning of a *Mycobacterium tuberculosis* gene encoding a protective antigen associated with the early control of infection. *J. Immunol.*, **165**: 7140–7149.

Srivastava, R.; Kumar, D.; Waskar, M.N.; Sharma, M.; Katoch, V.M. & Srivastava, B.S. (2006). Identification of a repetitive sequence belonging to a PPE gene of *Mycobacterium tuberculosis* and its use in diagnosis of tuberculosis. *J. Med. Microbiol.*, **55**: 1071-1077.

Stewart, G.R.; Wernisch, L.; Stabler, R.; Mangan, J.A.; Hinds, J.; Laing, K.G.; Young, D.B. & Butcher, P.D. (2002). Dissection of the heat-shock response in *Mycobacterium tuberculosis* using mutants and microarrays. *Microbiology*, **148** (Part 10): 3129–3138.

Stinear, T.P.; Seemann, T; Harrison, P.F.; Jenkin, G.A.; Davies, J.K.; Johnson, P.D.; Abdellah, Z.; Arrowsmith, C.; Chillingworth, T.; Churcher, C.; Clarke, K.; Cronin, A.; Davis, P.; Goodhead, I.; Holroyd, N.; Jagels, K.; Lord, A.; Moule, S.; Mungall, K.; Norbertczak, H.; Quail, M.A.; Rabbinowitsch, E.; Walker, D.; White, B.; Whitehead, S.; Small, P.L.; Brosch, R.; Ramakrishnan, L.; Fischbach, M.A.; Parkhill, J. & Cole, S.T. (2008). Insights from the complete genome sequence of *Mycobacterium marinum* on the evolution of *Mycobacterium tuberculosis*. *Genome Res*., **18** (5): 729-741.

Strong, M.; Mallick, P.; Pellegrini, M.; Thompson, M.J. & Eisenberg, D. (2003). Inference of protein function and protein linkages in *Mycobacterium tuberculosis* based on prokaryotic genome organization: a combined computational approach. *Genome Biol.*, **4** (9): R59.

Strong, M.; Sawaya, M.R.; Wang, S.; Phillips, M.; Cascio, D. & Eisenberg, D. (2006). Toward the structural genomics of complexes: crystal structure of a PE/PPE protein complex from *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.*, **103**: 8060–8065.

Tekaia, F.; Gordon, S.V.; Garnier, T.; Brosch, R.; Barrell, B.G. & Cole, S.T. (1999). Analysis of the proteome of *Mycobacterium tuberculosis* in silico. *Tuber. Lung. Dis.*, **79**: 329–342.

Thierry, D.; Cave, M.D.; Eisenach, K.D.; Crawford, J.T.; Bates, J.H.; Gicquel, B. & Guesdon, J.L. (1990). IS6110, an IS-like element of *Mycobacterium tuberculosis* complex. *Nucleic Acids Res*., **18** (1): 188.

Thierry, D.; Chavarot, P.; Marchal, G.; Le Thi, K.T.; Ho, M.L.; Nguyen, N.L.; Le, N.V.; Ledru, S.; Fumoux, F. & Guesdon, J.L. (1995). *Mycobacterium tuberculosis* strains unidentified using the IS6110 probe can be detected by oligonucleotides derived from the Mt308 sequence. *Res. Microbiol.*, **146**: 325–328.

Tompa, P. (2002). Intrinsically unstructured proteins. *Trends Biochem. Sci.*, **27**: 527–533.

Tundup, S.; Akhter, Y.; Thiagarajan, D. & Hasnain, S.E. (2006). Clusters of PE and PPE genes of *Mycobacterium tuberculosis* are organized in operons: evidence that PE Rv2431c is co-transcribed with PPE Rv2430c and their gene products interact with each other. *FEBS Lett.*, **580** (5): 1285-1293.

Tundup, S.; Pathak, N.; Ramanadham, M.; Mukhopadhyay, S.; Murthy, K.J.; Ehtesham, N.Z. & Hasnain, S.E. (2008). The co-operonic PE25/PPE41 protein complex of *Mycobacterium tuberculosis* elicits increased humoral and cell mediated immune response. *PLoS One*, **3**: e3586.

Voskuil, M.I.; Schnappinger, D.; Visconti, K.C.; Harrell, M.I.; Dolganov, G.M.; Sherman, D.R. & Schoolnik, G.K. (2003). Inhibition of respiration by nitric oxide includes a *Mycobacterium tuberculosis* dormancy program. *J. Exp. Med.*, **198** (5): 705–713.

(a)Voskuil, M.I.; Visconti, K.C. & Schoolnik, G.K. (2004). *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy. *Tuberculosis (Edinb)*, **84** (3-4): 218-227.

(b)Voskuil, M.I.; Schnappinger, D.; Rutherford, R.; Liu, Y. & Schoolnik, G.K. (2004). Regulation of the *Mycobacterium tuberculosis* PE/PPE genes. *Tuberculosis (Edinb.)*, **84** (3-4): 256-262.

Wang, J.; Qie, Y.; Zhang, H.; Zhu, B.; Xu, Y.; Liu, W.; Chen, J. & Wang H. (2008). PPE protein (Rv3425) from DNA segment RD11 of *Mycobacterium tuberculosis*: a novel immunodominant antigen of *Mycobacterium tuberculosis* induces humoral and cellular immune responses in mice. *Microbiol. Immunol.*, **52** (4): 224-230.

Yuen, L.K.; Ross, B.C.; Jackson, K.M. & Dwyer, B. (1993). Characterization of *Mycobacterium tuberculosis* strains from Vietnamese patients by Southern blot hybridization. *J. Clin. Microbiol.*, **31**: 1615–1618.

Zanetti, S.; Bua, A.; Delogu, G.; Pusceddu, C.; Mura, M.; Saba, F.; Pirina, P.; Garzelli, C.; Vertuccio, C.; Sechi, L.A. & Fadda, G. (2005). Patients with pulmonary tuberculosis develop a strong humoral response against methylated heparin-binding hemagglutinin. *Clin. Diagn. Lab. Immunol.*, **12**: 1135-1138.

Zhang, H.; Wang, J.; Lei, J.; Zhang, M.; Yang, Y.; Chen, Y. & Wang, H. (2007). PPE protein (Rv3425) from DNA segment RD11 of *Mycobacterium tuberculosis*: a potential B-cell antigen used for serological diagnosis to distinguish vaccinated controls from tuberculosis patients. *Clin. Microbiol. Infect.*, **13** (2): 139-145.