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

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1. Introduction

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 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.

2. Early works on Rv2108 and genetic analysis

2.1 *Mtb* PCR-based assay detection test

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 strains 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*. These 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.

2.2 Genomic organizations

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 adjacent *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).

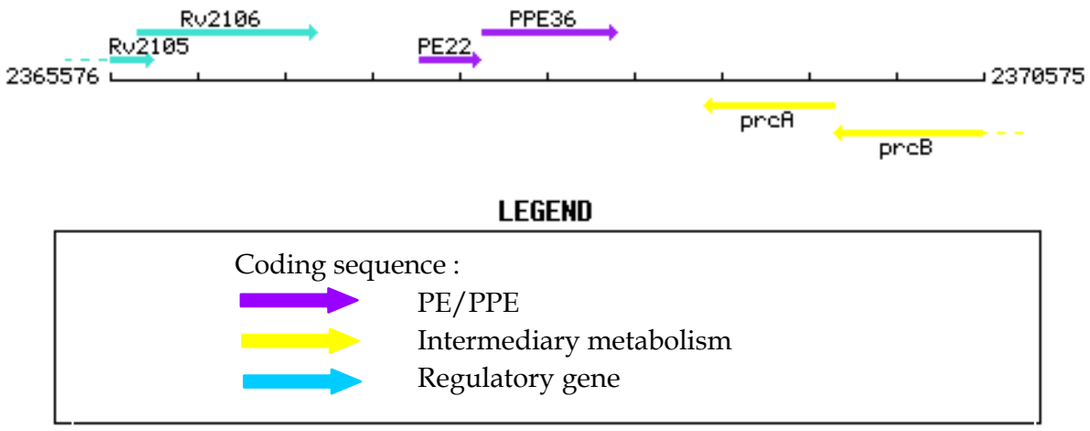

Fig. 1. Genomic environment of *Rv2108* gene. (adapted from TubercuList, http://genolist.pasteur.fr/TubercuList/)

2.3 Regulation expression of *Rv2108*

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 require the putative transcription 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 *Rv2108* gene expression between *M. bovis* and *M. tuberculosis* in microarray analysis (Rehren et al., 2007).

Furthermore, a study showed, by high 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).

3. Characterization of P27-PPE36 protein

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 to 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 stills 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.

3.1 Expression and purification of the PPE36 protein

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).

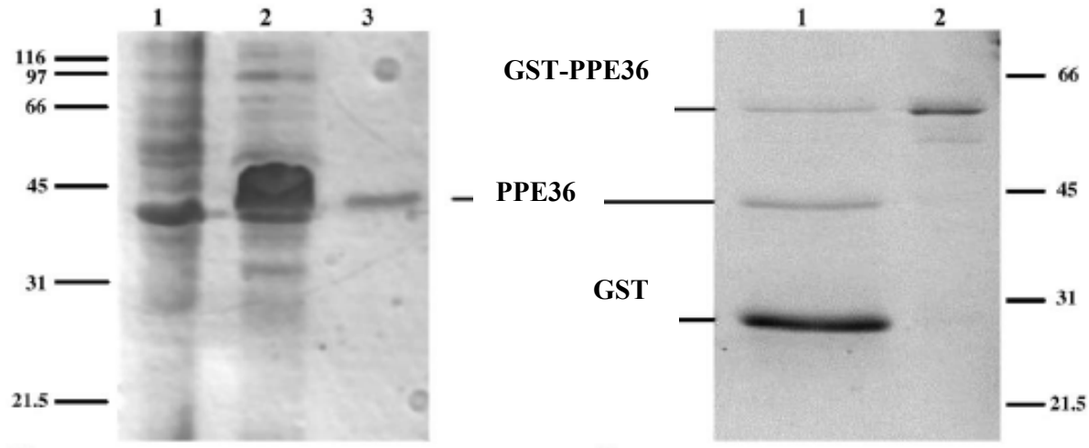

Fig. 2. Coomassie blue staining of bacterial lysates and purified recombinant PPE36 protein expressed with His-Tag (A) or with GST (B).
(A): Lanes 1 and 2: bacterial extracts of E. coli BL21 (DE3) without or with IPTG induction, respectively. Lane 3: purified PPE36 protein. (B): Lanes 1 and 2: PPE36,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

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.

3.2 Physico-chemical caracteristic of the PPE36 protein

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).

4. Anti-P27-PPE36 antibodies production and localization of P27-PPE36

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).

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).

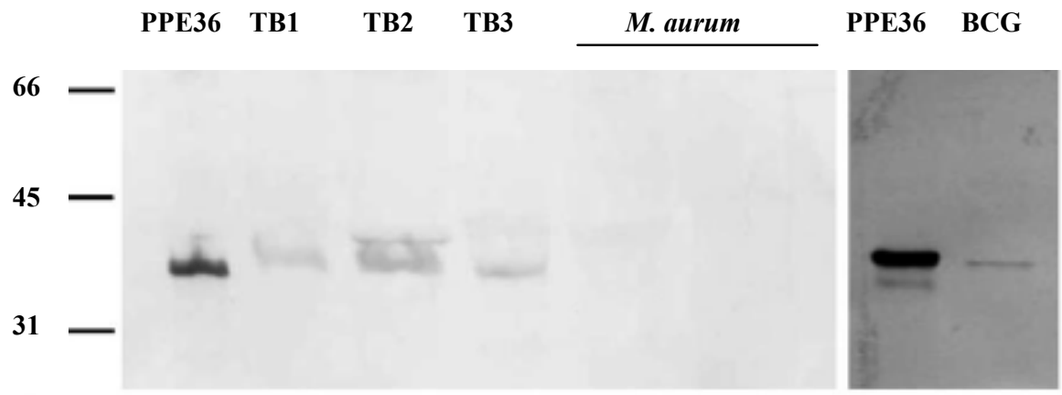

Fig. 3. Western blot analysis of bacterial lysate from different mycobacterial species. P27-PPE36 is the recombinant PPE36 protein. TB1, TB2 and TB3 are *Mycobacterium tuberculosis* clinical strains isolated from infected patients. *Mycobacterium aurum* is a fast-growing mycobacteria, and BCG is the Calmette-Guérin bacillus. The first antibody is a mouse monoclonal IgG antibody directed against PPE36.

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).

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).

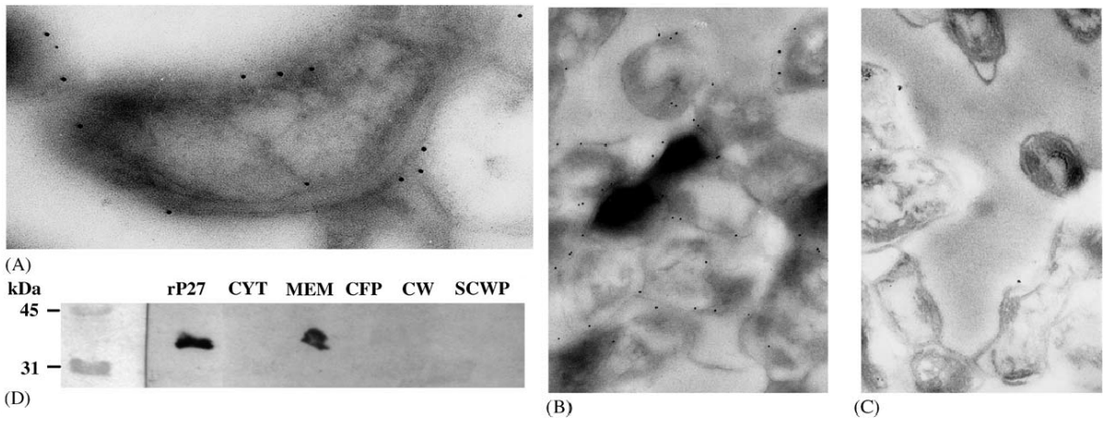

Fig. 4. Localization of P27-PPE36 antigen: Immunogold electron microscopic image (A–C) showing the peripheral localization of the P27-PPE36 protein in cryosectioned *M. bovis* BCG and western-blot on various *M. tuberculosis* cell fractions (D). Incubation was realized either with a mouse monoclonal 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).

5. Serological studies

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).

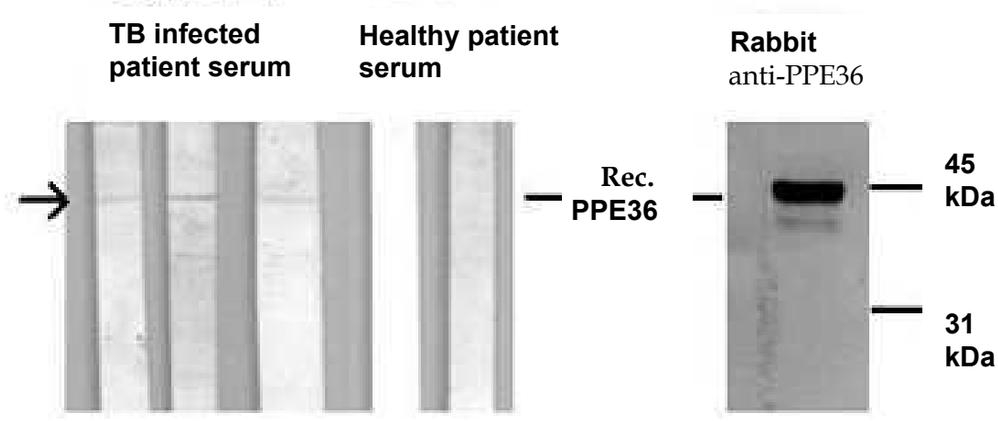

Fig. 5. Western blot analysis of the presence of anti-recombinant PPE36 antibodies in the sera of TB patients in comparison with serum from healthy donors and recombinant PPE36-hyperimmunized rabbit.

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, though we note its augmentation during infection. These antibodies are in general polyspecific with weak affinity for their antigens.

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.

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

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).

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).

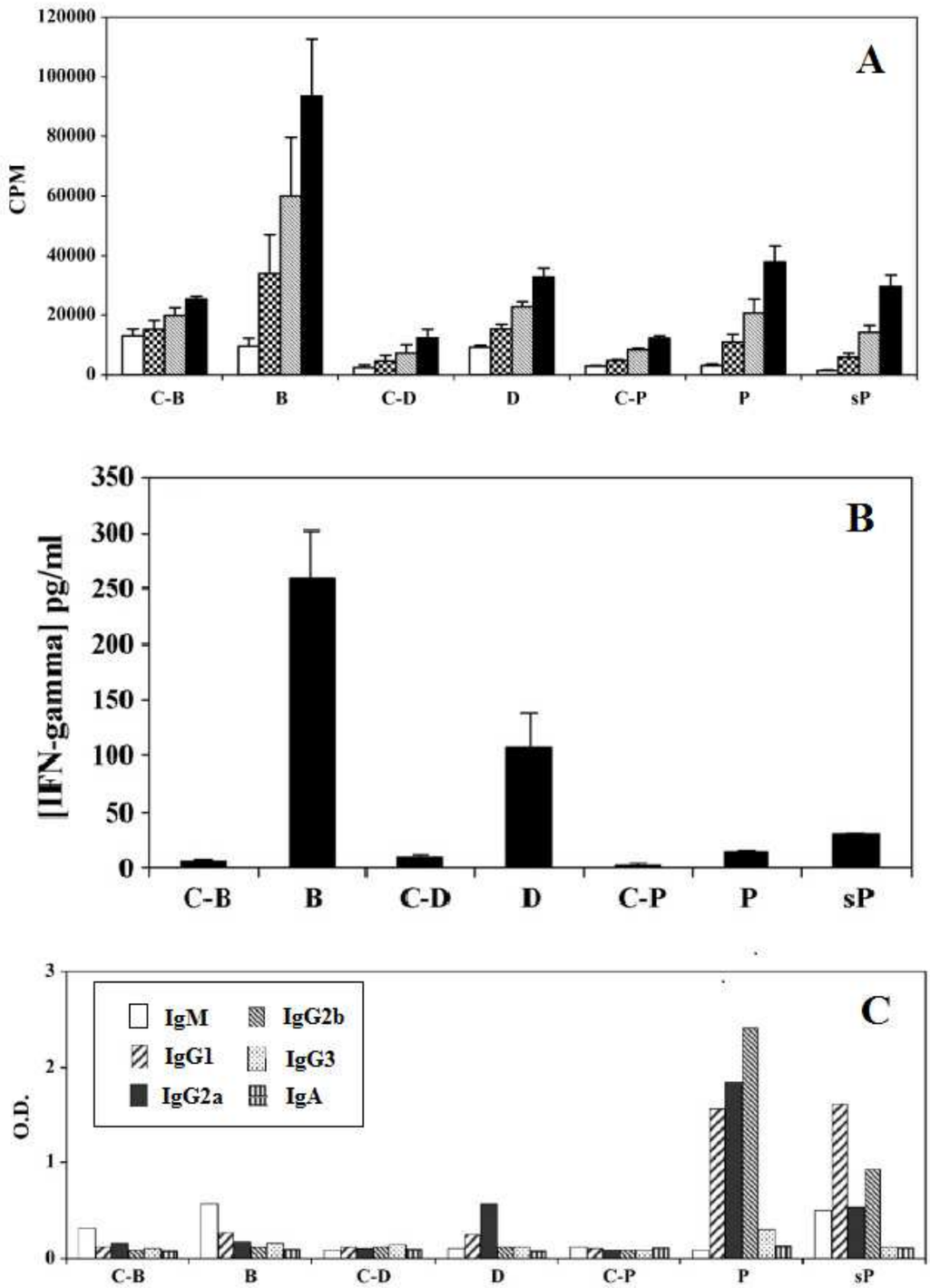

Fig. 6. Immune response generated against P27-PPE36 from mice immunized either with flagellin-modified bacteria (B), DNA plasmid containing the *Rv2108* gene (D) or with the P27-PPE36 recombinant protein associated (P) or not (sP) with Freund's adjuvant. Control groups have been immunized with non-modified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

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)

7. Conclusion

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.

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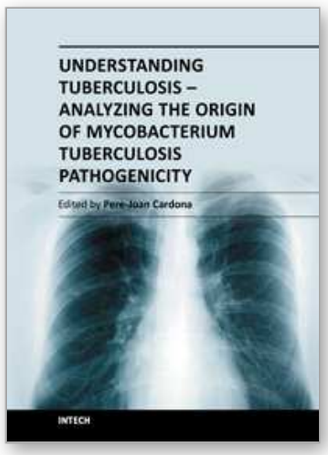

Understanding Tuberculosis - Analyzing the Origin of Mycobacterium Tuberculosis Pathogenicity

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Mycobacterium tuberculosis in an attempt to understand the extent to which the bacilli has adapted itself to the host and to its final target. On the other hand, there is a section in which other specialists discuss how to manipulate this immune response to obtain innovative prophylactic and therapeutic approaches to truncate the intimal co-evolution between Mycobacterium tuberculosis and the Homo sapiens.

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