A novel 27 kDa lipoprotein antigen from *Mycobacterium bovis*

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A novel *Mycobacterium bovis* antigen was identified from an expression library using sera from naturally infected cattle. The *Escherichia coli* recombinant clone expressed a 27 kDa protein, named P27. A rabbit serum against the recombinant antigen recognized a protein of 27 kDa in cellular extracts from *M. bovis* and *M. tuberculosis*. No protein was recognized in the culture supernatant. Sequence analysis indicated that P27 has a molecular mass of 24 kDa, showing a characteristic signal sequence for lipoprotein modification (a signal peptidase type II site). The gene is identical to a gene identified in the *M. tuberculosis* genome sequencing project. Cellular fractionation experiments suggested that P27 is an integral membrane protein. The antigen was recognized by individual sera and peripheral blood mononuclear cells (PBMC) from diseased cattle. PCR experiments with specific primers directed to the P27 structural gene indicated that it is only present in the *M. tuberculosis* species complex. In conclusion, a novel immunogenic lipoprotein in *M. bovis*/*M. tuberculosis* has been identified. The results presented here and elsewhere suggest that mycobacterial lipoproteins should be considered in the design of new recombinant vaccines and diagnostic methods.

Keywords: *Mycobacterium tuberculosis*, *Mycobacterium bovis*, lipoproteins, antigen, membranes

INTRODUCTION

The identification and characterization of individual antigenic proteins are essential to the understanding of the pathogenic mechanisms of mycobacteria and the immune response against them. This achievement may also contribute to understanding the pathogenesis of other intracellular bacterial infections in which cellular immunity is also involved. Such antigens are needed for the design of new diagnostic tests and subunit vaccines. However, the extremely long doubling time and the pathogenicity of mycobacteria have delayed for years the identification of antigens and virulence factors. Application of both molecular biology and immunological tools, such as gene cloning and monoclonal antibodies, has made an important contribution to our knowledge of *M. tuberculosis* and *M. leprae* antigens,

and of the cellular and humoral immune responses against them (Ivanyi *et al.*, 1985; Young *et al.*, 1985). Recent research has focused on species-specific antigens because some of the first mycobacterial antigens identified and cloned, the so-called stress proteins, are broadly present in other pathogenic bacteria (Young *et al.*, 1990).

Numerous mycobacterial antigens have been described (Young *et al.*, 1992); many of them have been expressed in *Escherichia coli* and identified by immune cells and antibodies. However, no clear immunodominant antigen covering all the stages of the disease has been found (Verbon *et al.*, 1990; Cataldi *et al.*, 1994). This fact has caused research to be directed to a mix of antigens instead of a unique protective or dominant antigen.

Mycobacteria secrete several proteins (Abou-Zeid *et al.*, 1988; Andersen *et al.*, 1991; Wiker *et al.*, 1991), many of which are antigens. Secreted proteins are thought to be essential in protective immunity, because a mix of secreted proteins may confer protection in mice against

Abbreviation: PBMC, peripheral blood mononuclear cells.

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a challenge with virulent strains (Orme, 1988; Pal & Horwitz, 1992). There is an increasing interest in these antigens to prepare new generation anti-TB vaccines (Orme 1988; Pal & Horwitz, 1992; Andersen, 1994). However, certain non-secreted proteins, such as membrane-associated, cell-wall-associated or cytoplasmic (Andersen & Brennan, 1994) proteins, have also been reported to be antigens. Some membrane protein antigens have been well studied. A 19 kDa membrane lipoglycoprotein and a 16 kDa protein or MMP (Lee *et al.*, 1991) are the most studied *M. tuberculosis* membrane antigens (Ashbridge, 1989; Collins *et al.*, 1990; Faith *et al.*, 1991; Booth *et al.*, 1993; Prestidge *et al.*, 1995).

We have identified a novel antigen that reacted with a pool of sera of *M. bovis*-infected cattle. This antigen, whose characterization we report here, is a 27 kDa hypothetical lipoprotein.

METHODS

Bacterial strains, media and cloning vectors. *M. bovis* AN5 and *M. tuberculosis* H37Rv were used to prepare whole extracts and culture supernatants. Clinical isolates were obtained from Nora Morcillo (Hospital Cetrángolo, Argentina). Mycobacteria were cultivated in Middlebrook M7H9 liquid medium (Difco). *E. coli* XL1-Blue (Stratagene) was used as the main recipient for recombinant plasmids. It was grown in LB medium (Sambrook *et al.*, 1989). pBluescript KS II (Stratagene) was used as the cloning vector.

Mycobacterial DNA preparation. DNA from *M. tuberculosis*, *M. bovis*, *M. vaccae*, *M. paratuberculosis*, *M. avium*, *M. kansasii* and *M. smegmatis* was prepared according to van Soolingen *et al.* (1991). DNA from *M. marinum*, *M. gordonae* and *M. scrofulaceum* was obtained from Philip Suffys (Fundacion Oswaldo Cruz, Rio de Janeiro, Brazil).

***M. bovis* genomic library and screening.** The construction and screening of a *M. bovis* genomic library in λ ZAP (Stratagene) has been described previously (Bigi *et al.*, 1995).

SDS-PAGE and Western blotting. Samples were boiled in loading buffer (2%, w/v, SDS, 0.125 M Tris/HCl, pH 6.8, 1%, v/v, 2-mercaptoethanol, 0.02% bromophenol blue, 10%, v/v, glycerol). Proteins (50 μ g) were separated by electrophoresis in 12% (w/v) SDS-PAGE gels by the technique of Laemmli (1970) and electrotransferred onto a nitrocellulose sheet by the semi-dry method (Kyhse-Andersen, 1984). Transfer yield was visualized by transient staining with Ponceau Rouge. Nitrocellulose membranes were incubated with the first antibody overnight at 4 °C and with an alkaline phosphatase-conjugated secondary antibody for 2 h at 37 °C. The colour reaction was developed for 30 min by the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates. When directed against *E. coli* antigens, the serum was absorbed of antibodies against *E. coli* by preincubation for 1 h at 37 °C with non-transformed *E. coli* extract (1 mg protein ml⁻¹).

Subcellular fractionation. A 4-week-old *M. bovis* 250 ml culture was used for cell fractionation. Culture supernatant and cells were separated by centrifugation at 10000 g for 20 min. Proteins in culture supernatant were precipitated with 10% (v/v) trichloroacetic acid. The precipitated material was resuspended in 1/20 of the original volume in PBS. Sonicated cell extract was obtained by resuspending the cells in 25 ml PBS. The suspension was sonicated in an ice bath for 10 min in

a Branson sonifier (model 250/450). The lysate was cleared by centrifugation at 20000 g for 15 min. The supernatant was centrifuged at 100000 g for 2.5 h. The insoluble pellet (membranes) was washed with PBS and resuspended in 2 ml distilled water. An estimate of the purity of the membrane fraction was determined by assaying for a membrane enzyme marker, lactate dehydrogenase. The specific activity of the lactate dehydrogenase in the membrane fraction was tenfold higher than in the cytosolic fraction. The membrane fraction was processed by Triton X-114 phase separation according to Bordier (1981).

T cell reactivity assay. Bovine peripheral blood mononuclear cells (PBMC) were prepared from heparinized blood by Ficoll-Paque (Pharmacia) centrifugation and washed three times in r.p.m.I1640 (Life Technologies). Cells were counted and resuspended (2×10^5 per well) in r.p.m.I1640 supplemented with 1% (w/v) penicillin/streptomycin, 2 mM L-glutamine, 20 mM HEPES and 10% (v/v) heat-inactivated calf foetal serum. *E. coli*(pMBA21) extracts were added at a concentration of 10 μ g ml⁻¹ and incubated for 24 h at 37 °C in 5% (v/v) CO₂. The culture supernatant was harvested by centrifugation and bovine interferon- γ was measured by ELISA using a commercial kit (Iddexx).

Sera. Cattle sera were obtained from cattle with macroscopical tuberculosis lesions confirmed by *M. bovis* culture as described previously (Cataldi *et al.*, 1994) and used at 1/100 dilution.

To obtain anti-27-kDa-protein-specific serum, *E. coli* recombinant extracts (100 μ g) were loaded in a large 10 cm well and developed onto a SDS-PAGE gel. The gel region where the recombinant band migrated (as determined by Western blotting) was excised, crunched, mixed with Freund's incomplete adjuvant and injected in three doses separated by 2-week intervals into one rabbit.

Sequencing. Plasmid DNA, prepared using the Wizard Mini-preps kit (Promega), was used as template. Sequencing was performed using T3, T7 and sequence-deduced primers that initiated dideoxy nucleotide chain-termination reactions (Sanger *et al.*, 1977). The Femtomol sequencing kit (Promega) and 6% polyacrylamide gels with 30% (w/v) formamide were used.

PCR. Primers 5' GACGGTCAACGCAAGATCCC 3' and 5' TGCGCTGCGACCACAAATC 3' were used in amplifications. The amplification conditions were: an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing) and 72 °C for 1 min (extension). The amplification products were detected in agarose gels.

Sequence analysis. Sequence analyses were performed using DNA Strider 1.2 software (Marck, 1988). Nucleotide sequence searches were performed using the EMBL database. Homology determinations were performed with Laser gene software (DNASTAR).

Other molecular genetics procedures. Standard procedures (Sambrook *et al.*, 1989) were used for the preparation of plasmid DNA, restriction enzyme digestions and ligations.

RESULTS

Molecular cloning of the gene encoding the 27 kDa protein

We identified a clone that reacted with a pool of sera from *M. bovis*-infected cattle by screening an *M. bovis* library constructed in λ ZAP. Details of the library

Fig. 1. Schematic representation of the pMBA21 2 kb insert.

Fig. 2. (a) P27 expression in *E. coli*. Western blot using cattle pool serum (lanes 1 and 2) or rabbit anti-P27-specific serum (lanes 3 and 4). Lanes 1 and 3, *E. coli* DH5 α (pMBA21); lanes 2 and 4, *E. coli* DH5 α . (b) Production of P27 by different mycobacteria. Western blot using rabbit anti-P27 specific sera. Lanes: 1, *M. bovis* An5; 2, *M. tuberculosis* H37Rv; 3, *M. bovis* 1372; 4, *M. tuberculosis* 124; 5, *M. tuberculosis* F15; 6, *M. bovis* 142; 7, *M. bovis* t991; 8, *M. bovis* BCG; 9, *M. microti*; 10, *M. smegmatis*; 11, *M. paratuberculosis*.

construction and screening were published previously (Bigi *et al.*, 1995). The recombinant plasmid pMBA21 was obtained from this clone by the automatic plasmid excision process of λ ZAP. It contained an insert of 2.0 kb and was characterized by restriction mapping (Fig. 1).

Cell extracts of *E. coli*(pMBA21) were prepared to study the size and level of expression of the recombinant protein. The pool of cattle sera was incubated with cell extracts of *E. coli*(pMBA21) recognizing a protein band of 27 kDa (Fig. 2a, lane 1). Another protein band of 35 kDa was also detected. Other minor bands were also recognized. The 27 kDa protein and the other proteins were not recognized in non-recombinant *E. coli*. Expression of the gene encoding the 27 kDa protein seems

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TTCAATGTCTCGGCATCACCTCGGCGGTGAATTGGCGGTGGGGCAATACATCGACGACCGTTCAGACACACGCGCTT 188
GACAGCGATCGAGTGGCGGTGGCGGTGGCGGTGGCGGTGGCGGTGGCGGTGGCGGTGGCGGTGGCGGTGGCGGTGGCGGT 265
GGGCGTCAACGCGCGGTCACTTCTCGCGGTTCCTCAACAAATTCGGGTGGCGGTGGCGGTGGCGGTGGCGGTGGCGGT 342
343 CGGACCGCGACCGCGCGGTCACTTCTCGCGGTTCCTCAACAAATTCGGGTGGCGGTGGCGGTGGCGGTGGCGGTGGCGGT 413
1 M Q G M R T P 7
414 AGA GGC CAC TGC GGT GGC ATC GCC GTC CTC GCC GGC GTT AGC ATC GGC GGC ACT GTC GTT 473
8 R R H C R R I A V L A A V S I A A T V Y 27
474 GGC GGC TGC TGC TGC GGC TGC ANG CCA AGC GGC GGA CCA CTT CCG GAC GCG AAG CCG CTG 533
28 A G C S S G S K P S G G P L P D

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Fig. 3. Main features of the of P27 coding region. The deduced amino acid sequence is shown below the nucleotide sequence. The –35 and –10 putative promoter elements are shown by plain and wavy underlining, respectively. The ribosome-binding site is shown by double underlining. Underlined amino acids 24–30 represent the consensus site for lipoprotein modification.

to be, at least partially, under the control of the *lac* promoter because there is an increase in production of the protein on the addition of IPTG (data not shown).

To obtain anti-27-kDa-protein-specific serum, the recombinant *E. coli* protein band was excised from polyacrylamide gels and the pieces of gel were injected into rabbits. The serum obtained reacted with a protein of 27 kDa in *E. coli*(pMBA21) cell extracts (Fig. 2a, lane 3). The protein was named P27. A 35 kDa protein band was also recognized by the rabbit sera. The serum recognized P27 in *M. bovis* cell extracts but not in culture supernatants. P27 is also produced by *M. bovis* BCG, and *M. tuberculosis* reference strains and clinical isolates (Fig. 2b), but not by *M. smegmatis*. The rabbit anti-P27 serum specifically precipitated P27 produced by *M. bovis* (data not shown) and solubilized by Triton X-114 (see below). Some signs of toxicity produced by P27 expression were noticed during the cloning experiments. For example, pMBA21 and derivatives expressing the full protein in *E. coli* yield lower amounts of plasmid DNA than non-expressing derivatives.

To locate the gene encoding P27 in pMBA21, shorter fragments of pMBA21 insert, obtained by restriction enzyme digestion, were subcloned in pBluescript KSII. *E. coli* cell extracts carrying the shortened pMBA21 derivatives were prepared and the presence of P27 was detected by Western blotting using anti-P27 serum. The subcloning indicated that the gene is located between the start of the insert and the *AccI* site in the insert. As a consequence, we continued to work with a shortened derivative of pMBA21 that was obtained by digestion with *AccI* and religation (plasmid pMBA23) rendering an insert of 1 kb.

Sequence analysis

The sequence of the pMBA23 insert (Fig. 3) revealed only one ORF of the required size and position. There are two probable start codons, three codons apart from each other. No clear ribosome-binding site was present upstream of the first start codon, but an AGGG motif was found 5 bases before the second ATG, favouring this codon as the true start codon. The ORF encodes a

Fig. 4. Fractionation of *M. bovis* An5 cell extract. Western blot using rabbit anti-P27-specific serum. (a) Lanes: 1, *M. bovis* An5 culture supernatant; 2, *M. bovis* An5 total cell extract; 3, 20000 g supernatant; 4, 100000 g supernatant; 5, 100000 g pellet. (b) Lanes: 1, *M. bovis* An5 total cell extract; 2, detergent phase of Triton X-114 fractionation; 3, aqueous phase of Triton X-114 fractionation.

protein of 236 aa with a molecular mass of 24.532 Da. –35 promoter elements were identified at 187–192 (TTGACA) and 242–247 (TGGTCA); a –10 element was identified at 224–231 (TAACCATC). The overall G+C content was 65%. The sequence of pMBA23 suggested that another ORF exists downstream from the gene encoding P27. This fact was further corroborated by analysing the sequence of a cosmid from the *M. tuberculosis* genome sequencing project (see below).

Analysis using the Prosite database revealed that P27 contained a characteristic motif for lipoprotein modification (a signal peptidase type II site). The consensus

Fig. 5. Recognition of *E. coli* recombinant P27 by cattle sera. Lanes 1–7, sera from infected cattle were used at 1/100 dilution.

signature, ATVVAGC, is found from aa 24 to 30. Hydrophobicity analysis, according to the Kyte & Doolittle algorithm (Kyte & Doolittle, 1982), indicated that P27 contains four hydrophobic regions (aa 10–30, 55–70, 100–120 and 140–150). This sequence showed perfect homology to a gene of a hypothetical protein detected in the *M. tuberculosis* genome sequencing project (accession no. Z80108, CDS MTCY21B4.28c). Another *M. tuberculosis* gene deduced from the *M. tuberculosis* genome sequencing project is partially homologous to P27. The putative 26.4 kDa product of this gene is postulated to be a transmembrane protein (accession no. Q11049) with four hydrophobic regions and lacking the consensus signature for a lipoprotein. Homology is stronger in some portions of these two proteins, especially from aa 141 to 152 (GLANVLN-FADA). Lesser degrees of homology were found with a copper-containing nitrite reductase from *Rhizobium hedysari* (accession no. Q60214) and a cow Tau protein (accession no. L34940).

Subcellular localization of P27

To determine the localization of P27, different subcellular fractions were prepared from *M. bovis* (Fig. 4). The protein was found in the membrane fraction. It was also found in the membrane fraction of *M. tuberculosis* (data not shown). Phase separation with Triton X-114 was used to determine the degree of P27 association with the membrane. It was found in the detergent phase, suggesting that P27 is an integral membrane protein. The membrane fraction was submitted to 2-D gel electrophoresis and anti-P27 serum recognized a single spot, indicating that it is a unique protein molecule (not shown). The isoelectric point of 6.0, determined from the 2-D gel, is in good agreement with the theoretical isoelectric point of 5.7 determined by Laser Gene software.

Immune recognition

The recognition frequency of this protein by serum of infected cattle was studied by Western blotting. The *E.*

Fig. 6. PCR to detect the gene encoding P27 in different mycobacteria. Amplification products were analysed on 0.8% agarose gels. Ten nanograms of genomic DNA was used as template. Lanes: 1, *M. smegmatis*; 2, *M. aureum*; 3, *M. goodii*; 4, *M. scrofulaceum*; 5, *M. avium*; 6, *M. marinum*; 7, *M. kansasii*; 8, *M. vaccae*; 9, *M. bovis* AN5; 10, *M. tuberculosis* H37Rv; 11, *M. bovis* 142; 12, *M. paratuberculosis*; 13, *M. bovis* BCG; 14, negative control.

coli recombinant P27 protein was recognized by 4 of 7 sera of infected cattle (Fig. 5). Cattle sera were first absorbed of anti-*E. coli* antibodies. Sera from healthy cattle showed no reaction (data not shown).

T cell recognition of P27 was assayed with PBMC from infected cattle. T cell activation was determined by measuring γ -interferon released from PBMC incubated with extracts of *E. coli*(pMBA21). Samples from three infected and three healthy animals were taken. The values (OD_{450}) were 0.39, 2.1 and 0.81 for three infected animals and 0.02, 0.05 and 0.18 for three healthy animals. On average the γ -interferon release was four times higher in assays with samples from infected cattle than in samples from healthy cattle.

Presence of P27 in other mycobacteria

To determine which mycobacterial species have the gene encoding P27, we performed PCR analysis using specific primers for this gene (Fig. 6), amplifying a 1.1 kb fragment. Only the members of the *M. tuberculosis* complex showed a strong amplification band. The *M. vaccae*, *M. paratuberculosis* and *M. avium* complex gave a faint amplification band. *M. kansasii* showed amplification bands of 1.8 and 1.0 kb. *M. smegmatis*, *M. marinum*, *M. goodii*, *M. aureum* and *M. scrofulaceum* showed no amplification.

DISCUSSION

An important effort has been directed toward the characterization of mycobacterial secreted proteins because they were reported as protective antigens (Orme, 1988; Pal & Horwitz, 1992) and as the main target of the T cell response (Andersen *et al.*, 1995). Less attention has been paid to membrane protein antigens. In this paper we describe a 27 kDa membrane protein of *M. bovis* found by molecular cloning procedures. The entire coding region of the gene encoding P27 was cloned and

sequenced. Two possible start codons could be postulated. We favour the second ATG codon because it is the only one presenting a putative ribosome-binding sequence. The amino acid sequence deduced from the gene sequence indicated that the protein has a molecular mass of 24.5 kDa. After signal sequence processing, the mature protein should have a molecular mass of 21.7 kDa. The discrepancy between the molecular mass of the sequence-deduced protein and that determined by PAGE could be due to hypothetical post-translational modifications or to the high proline content that may alter its electrophoretic behaviour. Other mycobacterial proteins show a similar irregular migration (Prestidge *et al.*, 1995).

A 35 kDa protein was also recognized by cattle and rabbit sera in recombinant *E. coli*. It is not encoded by pMBA21, because the 35 kDa protein was also detected in *E. coli* transformed with the shortened derivative pMBA23. While we have not performed experiments to clarify the nature of this protein, it may arise by the formation of a multimer between an *E. coli* protein and P27, as the 35 kDa protein is not observed in mycobacterial extracts.

Sequence analysis revealed that P27 has the consensus pattern for prokaryotic lipoprotein modification. According to studies in lipoproteins from other bacteria, a cysteine residue is the site of proteolytic processing and acylation by a type II signal peptidase (von Heijne, 1989; Mattar *et al.*, 1994). Subcellular fractionation and phase separation with Triton X-114 suggested that P27 is an integral membrane protein. This result is in agreement with the hydrophobic domains observed in the P27 amino acid sequence. It is important to note that Young & Garbe (1991) identified, without further characterization, a 27 kDa lipoprotein in *M. tuberculosis*. The 19 kDa protein (Faith *et al.*, 1991; Young & Garbe, 1991), P38 (Harboe & Wiker, 1992) and MPB83 (Hewinson *et al.*, 1996; Wiker *et al.*, 1996; Matsuo *et al.*, 1996) are examples of well-characterized mycobacterial lipoproteins. These differ from P27 in that they are also found in the culture supernatant (Fifis *et al.*, 1990; Harboe & Wiker, 1992), while we could not find P27 in culture supernatant, even when we used 8-week-old cultures (data not shown). While the function of the 19 kDa protein remains unknown, P38 is a component of the *M. tuberculosis* phosphate transport system. At the present state of our research it is difficult to speculate about the possible function of P27.

The antigenicity of P27 was demonstrated by the fact that 4 of 7 sera from cattle naturally infected with *M. bovis* recognized the recombinant protein. Preliminary experiments with bovine lymphocytes also showed T cell reactivity toward P27. The 19 kDa and the P38 lipoproteins are relevant antigens (Faith *et al.*, 1991; Young & Garbe, 1991; Harboe & Wiker, 1992). Lipoproteins may be important in directing cell-mediated immune responses. For example, acylation has been found to enhance the ability of protein antigens to induce delayed type hypersensitivity responses (Coon & Hunter, 1975) and it has been demonstrated that

synthetic lipopeptides have the ability to prime cytotoxic T lymphocytes *in vivo* (Deres *et al.*, 1989).

The gene sequence was found to be identical to that of a hypothetical 27 kDa protein gene found in the *M. tuberculosis* genome sequencing project achieved by the Sanger Centre (Cambridge, UK). According to their data (and partially ours) the gene encoding P27 and that of a protein of 55 kDa could form an operon. The 55 kDa protein is postulated to be involved in drug resistance, because it has homology with a *Streptomyces coelicolor* actinorhodin transporter. Further experiments, such as Northern blotting, will be needed to elucidate whether both genes form an operon. Interestingly, the *S. coelicolor* gene homologous to P27 has an upstream ORF encoding a putative 20 kDa protein. This fact could suggest a similar genome organization in both species. Another *M. tuberculosis* hypothetical gene, encoding a 26.4 kDa protein, also deduced from the *M. tuberculosis* genome sequencing project (but in a different cosmid from that of the gene encoding P27), showed partial homology to P27. The homology is stronger in some portions of the two proteins. The putative 26.4 kDa protein sequence lacks the consensus signature for a lipoprotein. Both proteins may form an antigenic family, but presently we do not know if our sera also recognizes the 26.4 kDa protein.

PCR experiments suggested that only *M. tuberculosis* complex strains have the gene encoding P27. The *M. vaccae*, *M. paratuberculosis* and *M. avium* complex strains gave rise to a faint amplification band, suggesting that a homologous gene exists in these species. Other mycobacteria tested showed no amplification. However, these bacteria may have genes similar to P27 with divergent sequences in the primer binding site.

In conclusion, a novel antigenic membrane protein of *M. tuberculosis*/*M. bovis* was identified. It is a putative lipoprotein. As the lipid moiety is described to enhance antigenicity, it would be interesting to study the function of lipidation in the antigenicity of P27. This study may be useful in the development of subunit vaccines against tuberculosis. We also hope that further studies on this and other membrane proteins will contribute to the comprehension of *M. bovis*/*M. tuberculosis*-host interaction.

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