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Molecular Cloning, Purification, and Serological Characterization of MPT63, a Novel Antigen Secreted by *Mycobacterium tuberculosis*

CLAUDIA MANCA,[1] KONSTANTIN LYASHCHENKO,[1] HARALD GOTTEN WIKER,[2] DONATELLA USAI,[2]† ROBERTO COLANGELI,[1] AND MARIA LAURA GENNARO[1]*

Public Health Research Institute, New York, New York 10016,[1] and Institute of Immunology and Rheumatology, University of Oslo, N-0172 Oslo 1, Norway[2]

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Proteins that are actively secreted by *Mycobacterium tuberculosis* generate immune responses in the infected host. This has prompted the characterization of protein components of mycobacterial culture filtrates to develop subunit vaccines and immunodiagnostic reagents. Fractionation of filtrates of *M. tuberculosis* cultures has yielded an abundant protein called MPT63, which has an apparent molecular mass of 18 kDa. We report the molecular cloning and nucleotide sequence of the *mpt63* gene, purification of recombinant MPT63 antigen from *Escherichia coli* cells, and serological characterization of MPT63. Nucleotide sequence analysis of *mpt63* identified an open reading frame encoding a protein of 159 amino acids (aa) consisting of a 29-aa secretion signal peptide and a 130-aa mature MPT63 protein. Recombinant MPT63 protein, purified from *E. coli* cells, and native MPT63, purified from *M. tuberculosis* culture filtrates, were indistinguishable in serological assays. Thus, the recombinant protein constitutes a valuable reagent for immunological studies. MPT63 evoked humoral immune responses in guinea pigs infected with virulent *M. tuberculosis* by the aerosol route. The *mpt63* gene is found only in species of the *M. tuberculosis* complex, as shown by DNA hybridization experiments. Moreover, polyclonal antibody against MPT63 does not cross-react with proteins of a common environmental mycobacterial species, *Mycobacterium avium*. The absence of cross-reactive epitopes makes MPT63 an attractive candidate as an *M. tuberculosis* complex-specific diagnostic reagent. In particular, evaluation of MPT63 as an *M. tuberculosis* complex-specific reagent for diagnostic skin testing is under way.

One of the important goals of research on *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), is the identification of mycobacterial antigens that induce cellular and/or humoral immune responses during TB and would therefore constitute reagents for developing improved TB vaccines and/or diagnostic assays. Only live, dividing mycobacteria efficiently induce protective immunity (9, 24). The immunogenicity of proteins that are actively secreted by *M. tuberculosis* during growth may provide a partial explanation for this, since many of these proteins have been shown to generate protective immune responses in animal models of TB (2, 3, 17, 18, 25, 27). Moreover, secreted proteins of *M. tuberculosis* induce delayed-type hypersensitivity responses in guinea pigs (15) and antibody production in TB patients (5, 16). Thus, secreted proteins of *M. tuberculosis* have attracted considerable attention both in vaccine research and as potential tools for diagnostic skin test and serological diagnosis of TB.

Of the ~100 proteins present in filtrates from short-term (4- to 5-day) cultures (1a), approximately 10 have been characterized by gene cloning and nucleotide sequencing (1, 4, 8, 11, 19–21, 23, 30, 33). Most of the sequenced culture filtrate proteins induce immune responses (34); however, strong cellular and humoral immune responses to culture filtrate fractions in TB patients involve as-yet-uncharacterized antigens (5, 6, 10, 30). Thus, it is important to identify additional components of *M. tuberculosis* culture filtrates and to define their role in inducing immune responses during the course of tubercular infection.

Work by Nagai and his collaborators in the early 1990s led to the purification and partial characterization (including determination of molecular weight, isoelectric point measurement, and NH_2-terminal sequencing) of several proteins of the *M. tuberculosis* culture filtrate (22). Here we report cloning and sequencing of the gene encoding one of those proteins, a ~16-kDa protein named MPT63. The *mpt63* gene of *M. tuberculosis* was isolated by immunoscreening a λ phage expression library of *M. tuberculosis* H_{37}Rv DNA. It encodes a mature protein of 130 amino acid (aa) residues (M_r, 13,655) preceded by a secretion signal peptide. The MPT63 protein elicits humoral immune responses in guinea pigs infected with *M. tuberculosis*. The *mpt63* gene is found only in mycobacteria of the *M. tuberculosis* complex, and there is no serological cross-reactivity between the MPT63 antigen and proteins of a common atypical mycobacterial species, *Mycobacterium avium*. The specificity of MPT63 for the *M. tuberculosis* complex makes this antigen a candidate for *M. tuberculosis* complex-specific diagnostic assays.

MATERIALS AND METHODS

General methods. *Escherichia coli* strains were grown with standard liquid and solid media (28). Restriction endonuclease analysis and DNA cloning were performed by standard procedures (28). Restriction DNA fragments were purified from agarose gels by the Qiaex extraction procedure (Qiagen). DNA fragments were generated by PCR using 25 cycles of amplification (94°C for 1 min, 55°C for 2 min, 72°C for 3 min) in a Perkin-Elmer thermocycler. PCR products were cloned in the pCRII plasmid vector with the TA cloning system (Invitrogen) and then transferred into the pQE30 plasmid vector (Qiagen) by manufacturer's protocols. Nucleotide sequencing was done by the chain termination method (29) using single-stranded DNA from recombinant pBluescript (Stratagene) plasmids as a template and oligonucleotides derived from vector and insert DNA sequences as primers. Synthetic oligonucleotides for PCR and nucleotide sequencing were obtained from Integrated DNA Technologies, Inc.

Construction and immunoscreening of an expression library of *M. tuberculosis* H_{37}Rv DNA. A genomic DNA library was constructed in bacteriophage λ ZAPII (Stratagene), with DNA extracted from *M. tuberculosis* H_{37}Rv cultures grown on 7H11 Middlebrook agar. In the λ ZAPII vector, DNA inserts in the correct

* Corresponding author. Phone: (212) 578-0844. Fax: (212) 578-0804. E-mail: gennaro@phri.nyu.edu.

† Present address: Department of Microbiology and Virology, School of Medicine, University of Sassari, Sassari, Italy.

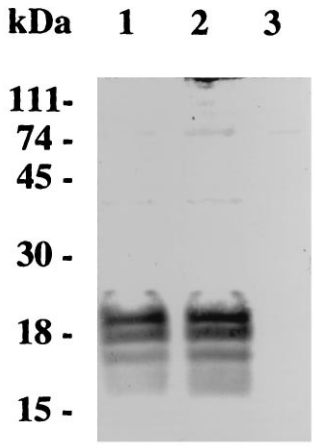

FIG. 1. Immunoblot analysis of the MPT63 protein produced by the pEOS2 clone and derived subclones. Crude cell extracts were obtained from *E. coli* cells containing recombinant pBluescript plasmids (as indicated below), separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and reacted with anti-MPT63 antibodies (see Materials and Methods). Molecular masses are indicated on the left. Lanes: 1, pEOS2 (pBluescript carrying a 6.5-kb insert of *M. tuberculosis* DNA); 2, pCM1B, a subclone containing a 1.6-kb *Xba*I-*Pst*I fragment of the pEOS2 DNA insert; 3, pCM3, a subclone containing a 4-kb *Pst*I fragment of the pEOS2 DNA insert. pEOS2 and pCM1B, but not pCM3, express the ~17- to 20-kDa protein doublet reacting with anti-MPT63 antibodies. A third, fast-migrating band visible in this figure is a gel loading artifact and does not appear in other experiments.

orientation and reading frame are expressed as β-galactosidase fusion proteins after induction of cultures with isopropyl-thio-β-D-galactopyranoside (IPTG). By use of a protocol described by the manufacturer, plaques were screened with a 1:1,000 dilution of rabbit monospecific anti-MPT63 antibodies (32) to identify clones expressing the MPT63 antigen. Positive plaques were visualized by a reaction with alkaline phosphatase-conjugated protein G (Zymed Laboratories) diluted 1:250 and the substrate 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (Kirkegaard & Perry Laboratories). Positive plaques were purified by two rounds of picking, plating, and testing for antibody binding. Recombinant pBluescript plasmids were excised in vivo from positive plaques as described in a manufacturer's protocol.

Screening of a cosmid library of *M. tuberculosis* H_{37}Rv by DNA hybridization. To isolate clones that contained the 5′ end of the gene expressing MPT63, a cosmid library of *M. tuberculosis* H_{37}Rv DNA (constructed in W. R. Jacob's laboratory and kindly provided by J. Dubnau) was screened by DNA hybridization using a 1.5-kb DNA probe containing sequences encoding the mature MPT63 protein. Nonradioactive labelling of DNA, colony lifts, and hybridization were performed with enhanced chemiluminescence labelling and detection systems (Amersham) and protocols recommended by the manufacturer.

Southern blot analysis. Mycobacterial chromosomal DNAs were digested with *Pvu*II, and the digestion products were separated on 1% agarose gels and transferred to Hybond-N membranes (Amersham) with an LKB Vacugene XL transfer unit (Pharmacia) by use of the standard protocol (28). Transferred DNA was fixed to membranes by UV cross-linking. The DNA probe was a 378-bp *Nru*I fragment internal to the cloned *mpt63* gene. Nonradioactive DNA labelling with horseradish peroxidase, hybridization, membrane washing, and chemiluminescence detection were performed with protocols and solutions provided in the enhanced chemiluminescence direct nucleic acid labelling and detection systems (Amersham). Specifically, prehybridization and hybridization with 100 ng of labelled probe were performed at 42°C for 30 min and 16 h, respectively. Membranes were washed at 42°C twice in 0.5× SSC–0.4% sodium dodecyl sulfate (SDS)–6 M urea for 15 min and twice in 2× SSC for 20 min (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Preparation of *E. coli* crude extracts and immunoblotting. Overnight cultures of *E. coli* XL1-Blue MRF′ harboring the recombinant plasmid pMPT63 were diluted 1:100 in fresh Luria broth containing 50 μg of ampicillin per ml and grown with vigorous shaking to an optical density at 600 nm of 0.5. IPTG was added to the culture to a final concentration of 0.4 to 1 mM, and the induced culture was grown for an additional 3 h. Crude cell extracts were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by washing cells with distilled water, resuspending them in SDS-sample buffer, and heating at 95°C for 5 min. Protein was separated by SDS-PAGE and transferred onto nitrocellulose membranes with a Miniblotter 16 (Immunetics) and by use of a standard protocol (14). Anti-MPT63 antibodies and the color development system were added as described for immunoscreening of the expression library.

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1    GTTCCTATCGAATCTGAGTTAGCAGCGGGTCATTTGCGGCTTAAGGTAATGACGTCGGCG   60

                                   SD
61   AGGTTCGAACCAGGTAATCGCCCCCAACAAGTAGTGGAGGTAGGGACCAATGAAGCTCACC  120
                                                    M  K  L  T

121  ACAATGATCAAGACGGCAGTAGCGGTCGTGGCCATGGCGGCCATCGCGACCTTTGCGGCA  180
      T  M  I  K  T  A  V  A  V  V  A  M* A  A  I  A  T  F  A  A

                          ↓
181  CCGGTCGCGTTGGCTGCCTATCCCATCACCGGAAAACTTGGCAGTGAGCTAACGATGACC  240
      P  V  A  L  A  A  Y  P  I  T  G  K  L  G  S  E  L  T  M  T

241  GACACCGTTGGCCAAGTCGTGCTCGGCTGGAAGGTCAGTGATCTCAAATCCAGCACGGCA  300
      D  T  V  G  Q  V  V  L  G  W  K  V  S  D  L  K  S  S  T  A

301  GTCATCCCCGGCTATCCGGTGGCCGGCCAGGTCTGGGAGGCCACTGCCACGGTCAATGCG  360
      V  I  P  G  Y  P  V  A  G  Q  V  W  E  A  T  A  T  V  N  A

361  ATTCGCGGCAGCGTCACGCCCGCGGTCTCGCAGTTCAATGCCCGCACCGCCGACGGCATC  420
      I  R  G  S  V  T  P  A  V  S  Q  F  N  A  R  T  A  D  G  I

421  AACTACCGGGTGCTGTGGCAAGCCGCGGGCCCCGACACCATTAGCGGAGCCACTATCCCC  480
      N  Y  R  V  L  W  Q  A  A  G  P  D  T  I  S  G  A  T  I  P

481  CAAGGCGAACAATCGACCGGCAAAATCTACTTCGATGTCACCGGCCCATCGCCAACCATC  540
      Q  G  E  Q  S  T  G  K  I  Y  F  D  V  T  G  P  S  P  T  I

541  GTCGCGATGAACAACGGCATGGAGGATCTGCTGATTTGGGAGCCGTAGATCGTAGCTAAT  600
      V  A  M  N  N  G  M  E  D  L  L  I  W  E  P  *

601  GCACGCCCAGGCGACCGCTGAGGTATTGGGCGCGGCAGGCTGGCGAGCCAGCTTCCCGCT
     GGTGGTGCGTGGAATGGCGCCG                                        643
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FIG. 2. Nucleotide sequence of the gene encoding the MPT63 protein of *M. tuberculosis*. The nucleotide sequence shown in this figure contains an ORF of 477 nucleotides that we call *mpt63*. The initiation codon (ATG), which is shown in bold, is preceded by a putative ribosomal binding site (GGAGG) at a suitable distance (9 bp) upstream. The *mpt63* gene encodes a full-length protein of 159 aa, whose deduced amino acid sequence is also shown. The start of the extracellular, mature form of the MPT63 protein (130 aa; M_r, 13,655) is marked by a pentapeptide (AYPIT) that is underlined in the figure. The 29-aa stretch preceding the mature protein constitutes the hydrophobic secretion signal peptide. The cleavage site is indicated by an arrow. M* indicates the first amino acid residue encoded by the *M. tuberculosis* DNA insert in the pEOS2 clone.

Purification of the polyhistidine-tagged recombinant MPT63. Growth of cultures of *E. coli* XL1-Blue MRF′ harboring the recombinant plasmid pMPT63 and purification of polyhistidine-tagged recombinant MPT63 (recMPT63) by nickel-affinity chromatography using nondenaturing conditions were essentially as recommended by Qiagen. Mid-log-phase *E. coli* cultures were obtained as described above and induced with 1 to 2 mM IPTG for 5 h. Cells were harvested by centrifugation at 4,000 × *g* for 20 min and resuspended in 2 to 5 ml of sonication buffer (50 mM Na phosphate [pH 8.0], 300 mM NaCl) per g of wet cells. Cells were subjected to one cycle of freezing-thawing and then lysed by sonication. Cell lysates were centrifuged at 15,000 × *g* for 20 min, and supernatants were mixed with equal volumes of a 50% slurry of Ni-nitrilotriacetic acid resin (Qiagen), previously equilibrated in sonication buffer, and stirred at 4°C for 1 h. The resin was then loaded into a column and washed with 10 to 20 volumes of sonication buffer followed by 10 to 20 volumes of wash buffer (50 mM Na phosphate [pH 6.0], 300 mM NaCl, 1 mM imidazole, 10% glycerol). Protein was eluted in the same buffer with a gradient of 1 to 500 mM imidazole. Fractions were analyzed by SDS-PAGE, and those fractions containing recMPT63 were dialyzed overnight in ResourceQ start buffer (30 mM Tris-HCl [pH 8.7]) and then applied to a ResourceQ column preequilibrated in the same buffer. The column was washed with 10 to 20 volumes of start buffer, and the protein was eluted with a 0 to 0.5 M NaCl gradient. Fractions containing recMPT63 were pooled and dialyzed in phosphate-buffered saline. Typically, 10 to 15 mg of purified MPT63 was obtained per liter of starting culture.

Double immunodiffusion. Double immunodiffusion in agarose gels was performed as described previously (26) with recMPT63 purified from *E. coli* cells, native MPT63 (nMPT63) purified from *M. tuberculosis* H_{37}Rv culture filtrates, and a rabbit monospecific antiserum against nMPT63 (22). Purified nMPT63 was kindly provided by S. Nagai, Osaka Medical School, Osaka, Japan.

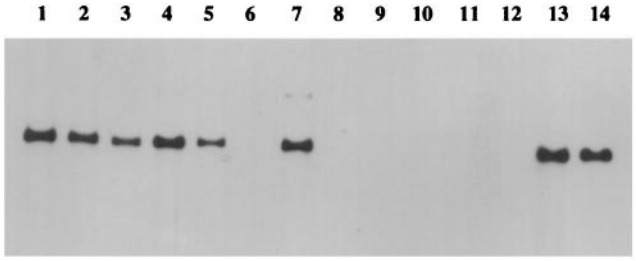

FIG. 3. Genomic analysis of the *mpt63* gene by Southern hybridization. Genomic DNAs were digested with *Pvu*II and electrophoresed on 1% agarose gels. The separated digestion products were transferred to nylon membranes and hybridized with a nonradioactively labelled DNA fragment internal to the *mpt63* gene. After chemiluminescence detection, a single 5-kb band was observed in all *M. tuberculosis* and *M. bovis* (including *M. bovis* BCG) DNAs tested but not in DNAs from nontubercular mycobacteria. Lanes: 1, *M. tuberculosis* W (7) (clinical isolate); 2, *M. tuberculosis* H_{37}Rv; 3, *M. tuberculosis* Erdman; 4, *M. tuberculosis* H_{37}Ra; 5, *M. bovis* Ravenel; 6, *M. avium*; 7, *M. bovis* BCG Pasteur; 8, *M. microti*; 9, *M. avium*; 10, *M. hemophilus*; 11, *M. smegmatis*; 12, *M. kansasii*; 13 and 14, *M. tuberculosis* (clinical isolates).

Enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter 96-well plates (Costar) were coated overnight with nMPT63, recMPT63, recombinant antigen 85B (Ag85B), and bovine serum albumin (BSA; Sigma) at 0.1 μg/well and with *M. tuberculosis* H_{37}Rv culture filtrate proteins (kindly supplied by J. Belisle, Colorado State University, Fort Collins) and purified protein derivatives (PPD) from *Mycobacterium bovis* and *M. avium* (Kursk Biofactory, Kursk, Russia), each at 0.5 μg/well. Plates were blocked with 2% BSA in Tris-buffered saline (TBS; pH 7.5) for 2 h and washed extensively with TBS. One-tenth milliliter of serum (diluted 1:500 to 1:16,000 in TBS plus 0.05% Tween 20 [TBST]) was added to antigen-coated wells in duplicate, and the plates were incubated for 90 min at room temperature. The plates were washed extensively with TBST, 0.1 ml of the appropriate alkaline phosphatase-conjugated antibody (anti-rabbit immunoglobulin G from Bio-Rad or anti-guinea-pig immunoglobulin G from Sigma; 1:1,000 dilution in TBST) was added to the wells, and the plates were incubated for 60 min at room temperature. After washing with TBST, alkaline phosphatase activity was assayed with 0.1 ml of *p*-nitrophenylphosphate (Bio-Rad) per well and stopped after 30 min by adding 0.1 ml of 0.4 M NaOH per well. Optical density was measured at 405 nm with a Spectra Shell microplate reader (Tecan).

In a competitive ELISA, rabbit monospecific immune sera against MPT63 and against Ag85B were diluted 1:4,000 in TBST and preincubated with increasing concentrations (0.1 to 3 mg/ml) of *M. bovis* PPD, *M. avium* PPD, or BSA for 2 h at 37°C. When specified, antibody binding to the recMPT63 and to nMPT63 was measured in the presence of increasing concentrations (0.01 to 3 M) of the mild chaotropic agent KSCN (13).

Nucleotide sequence accession number. The GenBank accession number of the nucleotide sequence of the gene encoding the MPT63 protein is U27119.

RESULTS

Construction and immunoscreening of an *M. tuberculosis* H_{37}Rv expression library. An expression library of *M. tuberculosis* H_{37}Rv DNA was constructed in the bacteriophage λ ZAPII vector and screened with polyclonal rabbit antisera raised against the MPT63 antigen (32) to identify clones expressing MPT63. In a screen of approximately 1.5×10^5 plaques, one positive phage clone was obtained (data not shown). The corresponding plasmid, pEOS2, was excised in vivo for further analysis.

Characterization of recombinant pEOS2. We analyzed the production of MPT63 protein in *E. coli* transformed with pEOS2 by SDS-PAGE and immunoblotting with anti-MPT63 antibodies. As shown in Fig. 1 (lane 1), multiple proteins in the pEOS2-containing *E. coli* extracts displayed strong reactivity with the anti-MPT63 antibodies. The apparent molecular masses (17 to 20 kDa) of these proteins were consistent with those of bona fide MPT63 protein. Expression of the MPT63 antigenic activity as multiple bands by the mycobacterial DNA insert in pEOS2 in *E. coli* is presumably due to partial proteolysis. To locate the *mpt63* gene within the 6.5-kb mycobacterial DNA insert in pEOS2, we subcloned overlapping fragments of the DNA insert in the pBluescript (Stratagene) plasmid vector and tested the resulting plasmids for MPT63 production. Western blot (immunoblot) analysis indicated that the ~17- to 20-kDa protein was expressed by a subclone, pCM1B, that contains a 1.6-kb *Xba*I-*Pst*I fragment of mycobacterial DNA (Fig. 1, lane 2).

Characterization of the *mpt63* gene. pCM1B was used for nucleotide sequence analysis. The sequence contained an open reading frame (ORF) of 432 nucleotides coding for a protein of 144 aa residues (Fig. 2). The deduced amino acid sequence contained a pentapeptide (AYPIT) identical to the NH_2-terminal sequence of the MPT63 protein purified from *M. tuberculosis* culture filtrates (22). The presence of the AYPIT sequence identifies the ORF as the gene encoding MPT63. Further, the AYPIT pentapeptide defined the NH_2 terminus of the mature, extracellular form of MPT63, a polypeptide of 130 aa having a calculated M_r of 13,655. The pI of the deduced polypeptide sequence, calculated with a program by the Genetics Computer Group, is 4.4. This value is in excellent agreement with that of 4.2 to 4.8 determined experimentally for the MPT63 protein purified from *M. tuberculosis* culture filtrates (22). Comparison of nucleotide and deduced amino acid sequences with sequences in the database revealed no homology with known genes and proteins, indicating that the gene designated *mpt63* that we have isolated in the present study is a novel gene.

The AYPIT sequence of the mature protein was preceded by a 14-aa stretch (starting with M* in Fig. 2) that showed properties resembling those of a secretion signal peptide (a helix-breaking residue, P, at position −5, and two residues with small, uncharged side chains, A, at positions −3 and −1 relative to the cleavage site) (31). However, the polypeptide was shorter than a typical signal peptide (22 to 42 aa in *M. tuberculosis*), and it lacked positively charged residues at its NH_2 terminus (31). The first amino acid of the putative signal peptide (M*, at position −14) was the first residue encoded by the *M. tuberculosis* DNA insert in pCM1B; thus, we considered it likely that the 5′ end of the complete mycobacterial gene was missing from the clone. Since *mpt63* was not in frame with *lacZ* of the vector and was therefore not expressed as a gene fusion, transcription and translation of the mycobacterial gene must

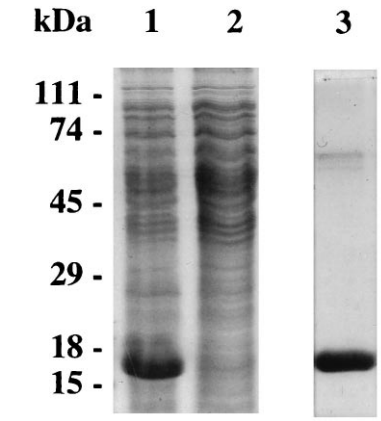

FIG. 4. Purification of the mature MPT63 protein carrying an NH_2-terminal polyhistidine tag. recMPT63 expressed from *E. coli* cells containing the recombinant pQE30::*mpt63* plasmid was purified by nickel-affinity and anion-exchange chromatographies as described in Materials and Methods. Crude extracts of the *E. coli* cells carrying the recombinant pQE30::*mpt63* plasmid were grown in the presence (lane 1) and absence (lane 2) of IPTG (1 to 2 mM final concentration) for 5 h. Purified recMPT63 obtained after high-salt elution of an anion-exchange column is also shown (lane 3).

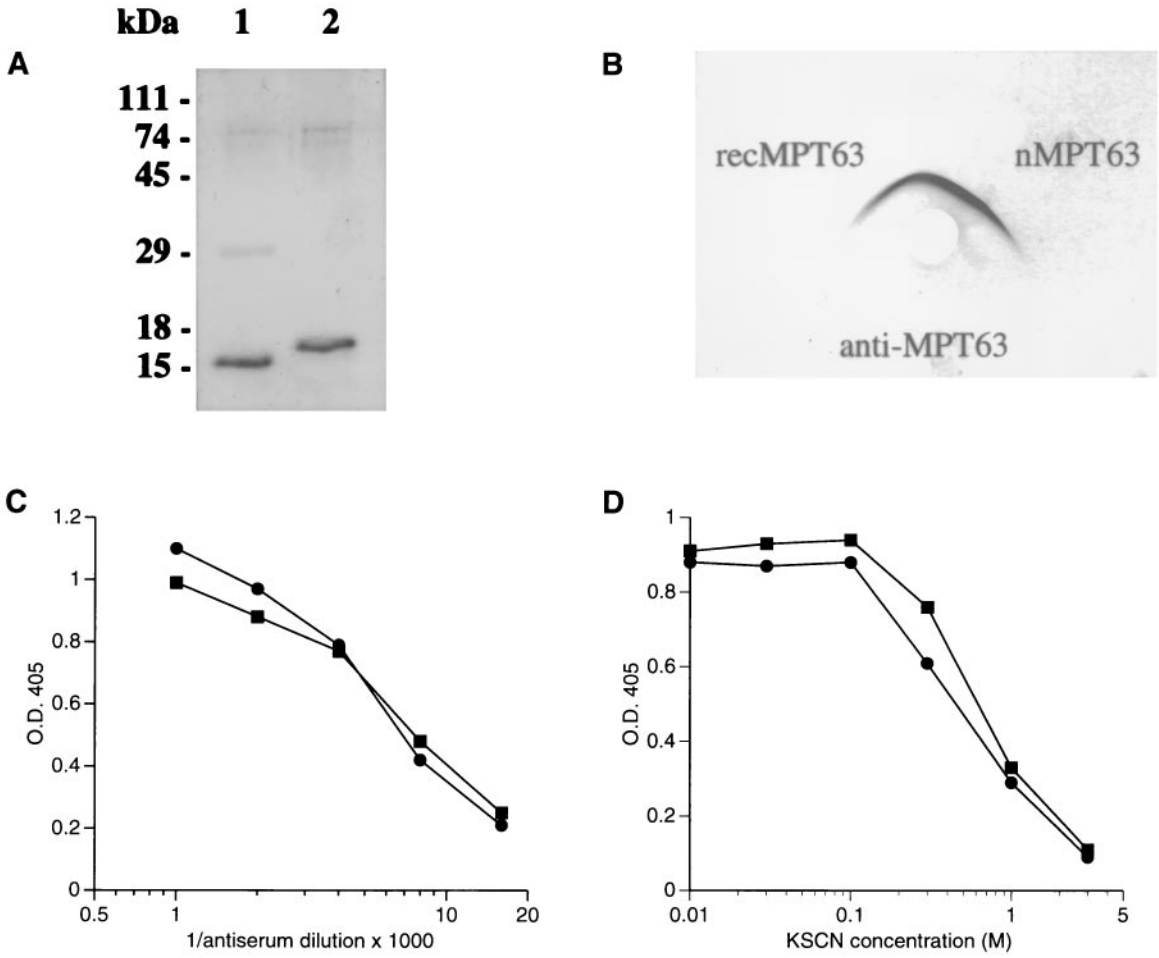

FIG. 5. Comparative analyses of recMPT63 and nMPT63. Polyhistidine-tagged MPT63 protein (recMPT63) was purified from *E. coli* cell extracts as described in Materials and Methods. MPT63 purified from *M. tuberculosis* culture filtrates (nMPT63) was provided by S. Nagai. (A) SDS-PAGE analysis. nMPT63 (lane 1) and recMPT63 (lane 2) were analyzed in 12.5% polyacrylamide gels containing SDS. (B) Immunodiffusion in gel. recMPT63 and nMPT63 were tested with anti-MPT63 antibodies by double diffusion in the gel. The two antigens yielded a reaction of identity. (C and D) Binding of rabbit anti-nMPT63 polyclonal antibody to nMPT63 (■) and recMPT63 (●) proteins in an ELISA using different antiserum dilutions (C) and in the presence of increasing concentrations of KSCN (D). O.D. 405, optical density at 405 nm.

have initiated from fortuitous *E. coli* signals and used the ATG codon in the signal peptide region (encoding the M* residue in Fig. 2) as the initiation codon.

To obtain DNA containing the 5′ end of the *mpt63* gene, a cosmid library of *M. tuberculosis* H_{37}Rv DNA was screened by Southern transfer and hybridization using a 1.5-kb DNA probe containing the sequence coding for the mature MPT63 protein. One of three positive clones obtained in a screen of approximately 200 cosmid-containing colonies was selected for further characterization. A hybridization-positive, 5.5-kb *Bam*HI fragment from the selected clone was subcloned in a pBluescript plasmid and sequenced. As expected, nucleotide sequence analysis of the subclone showed that the ORF encoding MPT63 extended toward the 5′ direction to include an ATG sequence located at positions 109 to 111 (Fig. 2). We propose that $ATG_{(109–111)}$, rather than $ATG_{(124–126)}$ or $ATG_{(154–156)}$ in the signal peptide, is the translation start site of the full-length *mpt63* gene for two reasons. First, translation starting at $ATG_{(109–111)}$, but not at either of the downstream ATG codons, generates a secretion signal peptide of typical length (29 aa) containing two positively charged residues (K) at its NH_2 terminus (positions −28 and −22 relative to the cleavage site) (Fig. 2). Second, only $ATG_{(109–111)}$ is preceded by a typical *E. coli* ribosome-binding sequence (GGAGG) at a suitable distance (9 bp) from the initiation codon.

Genomic analysis of the *mpt63* gene. To detect the *mpt63* gene in the *M. tuberculosis* genome, Southern transfer and hybridization were performed with *Pvu*II-digested DNA from *M. tuberculosis* H_{37}Rv and a nonradioactively labelled 378-bp *Nru*I fragment internal to *mpt63*. A single band of approximately 5 kb was visualized after hybridization and chemiluminescence detection (Fig. 3, lane 2), indicating that the *mpt63* gene is present as a single copy in the bacterial chromosome. The same positive signal was detected with DNAs extracted from other reference strains (H_{37}Ra and Erdman) and over 30 clinical isolates of *M. tuberculosis* as well as from isolates of *M. bovis* (Ravenel and Branch) and *M. bovis* BCG (substrains Montreal, Connaugh, and Pasteur). Some of this data is shown in Fig. 3. No restriction fragment length polymorphism was observed in DNAs that tested positive. In contrast, no hybridization signal was detected with DNAs extracted from unrelated mycobacterial species (*Mycobacterium kansasii*, *Mycobacterium smegmatis*, *Mycobacterium haemophilus*, and *M. avium*) (Fig. 3). These hybridization results suggest that *mpt63* is con-

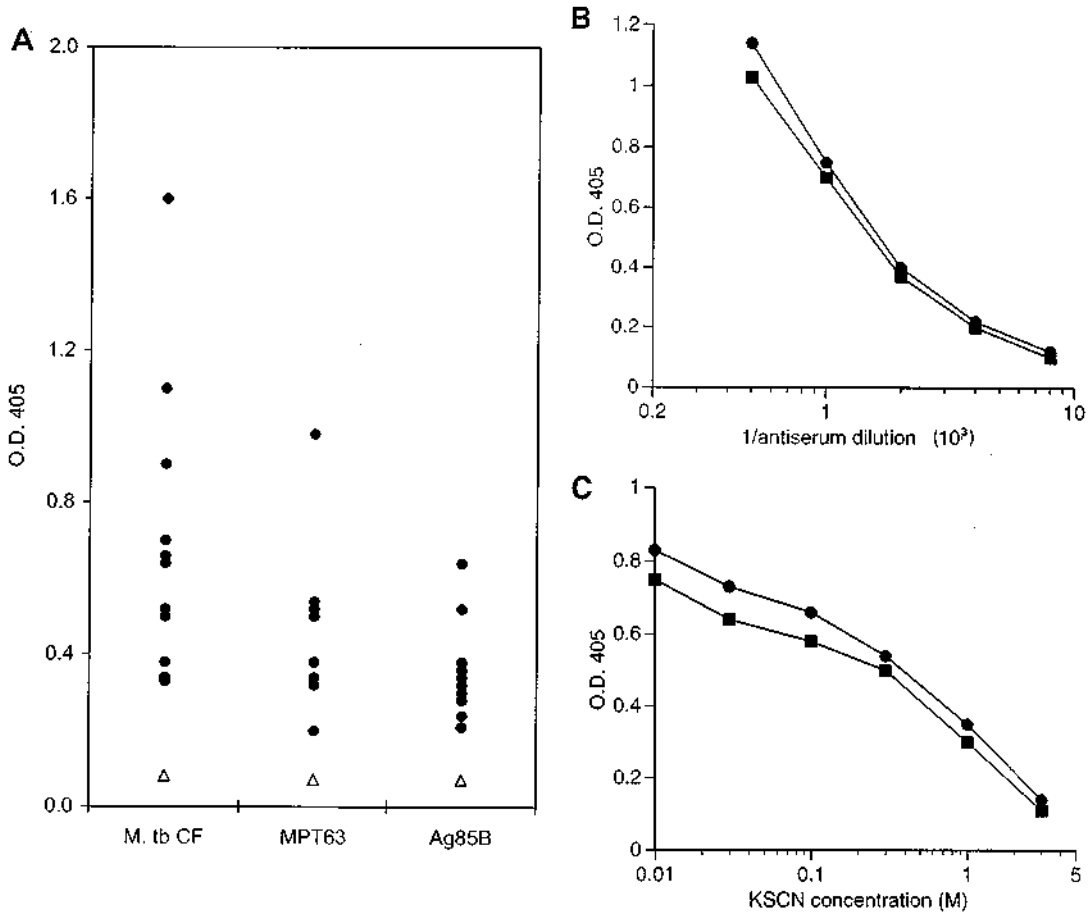

FIG. 6. Antibody responses to MPT63 in guinea pigs aerosol infected with *M. tuberculosis* $H_{37}Rv$. (A) Reactivity of sera from 11 *M. tuberculosis*-infected guinea pigs to *M. tuberculosis* culture filtrate proteins (M. tb CF), recMPT63, and recombinant Ag85B in an ELISA. Ag85B, nickel-affinity-purified, His-tagged recombinant Ag85B obtained by use of the same *E. coli* vectors and purification protocols described for recMPT63. Antibody levels to each antigen in each infected guinea pig (●) and in a control, noninfected guinea pig (△) are indicated. (B and C) Binding of serum from an *M. tuberculosis*-infected guinea pig to native (■) and recombinant (●) MPT63 proteins in an ELISA using different antisera dilutions (B) and in the presence of increasing concentrations of KSCN (C). O.D. 405, optical density at 405 nm.

served in mycobacterial species of the *M. tuberculosis* complex but absent in more distantly related mycobacteria. Thus, *mpt63* gene could be used as an *M. tuberculosis* complex-specific DNA probe.

High-level expression and purification of the MPT63 antigen. To obtain pure MPT63 protein in large amounts for immunological and immunochemical characterization, the sequence encoding the mature MPT63 protein (extending from nucleotides 196 to 588) (Fig. 2) was subcloned in the *E. coli* plasmid pQE-30 (Qiagene) as a fusion protein bearing a short, 6-histidine tract at its NH_2 terminus. The tagged protein, which had an apparent molecular mass of 17.5 kDa (Fig. 4, lane 1), was purified to near-homogeneity by nickel-affinity chromatography under nondenaturing conditions, followed by anion-exchange chromatography (Fig. 4, lane 3).

Comparative analysis of recMPT63 versus nMPT63. Purified recMPT63 was compared with nMPT63 purified from *M. tuberculosis* culture filtrates. In SDS-PAGE, nMPT63 displayed a slightly faster mobility than did recMPT63 (16 kDa versus 17.5 kDa apparent molecular masses) (Fig. 5A), most likely due to the presence of the polyhistidine tag on the recombinant protein. In Western blot analysis, both proteins reacted with rabbit polyclonal anti-nMPT63 antibodies (data not shown). In double immunodiffusion in a gel with the same polyclonal antibodies, recMPT63 and nMPT63 showed a reaction of identity (Fig. 5B), implying that the polyhistidine tag in recMPT63 causes no apparent alterations in antibody recognition. Antibody binding to nMPT63 and to recMPT63 was measured by an ELISA at different antibody concentrations (Fig. 5C) and in the presence of KSCN, a mild chaotropic agent that prevents antibody binding to the plate-coating antigen in a dose-dependent fashion (13) (Fig. 5D). Thus, the effect of increasing concentrations of KSCN on antibody binding to MPT63 on the plates can be taken as an indirect parameter of antibody affinity. In both sets of experiments, the kinetics of antibody binding to recMPT63 and nMPT63 showed no significant differences.

Taken together, immunodiffusion in gel and antibody binding analysis by ELISA indicate that the native and the recombinant forms of MPT63 are serologically indistinguishable. These results provide strong evidence that the recombinant product can be utilized to measure immune responses to MPT63 during the course of TB without the limitations imposed by purification of the protein from *M. tuberculosis* culture filtrates.

Antibody responses to MPT63 in guinea pigs infected with virulent *M. tuberculosis*. Sera from 15 guinea pigs infected with *M. tuberculosis* $H_{37}Rv$ by the aerosol route were obtained 15

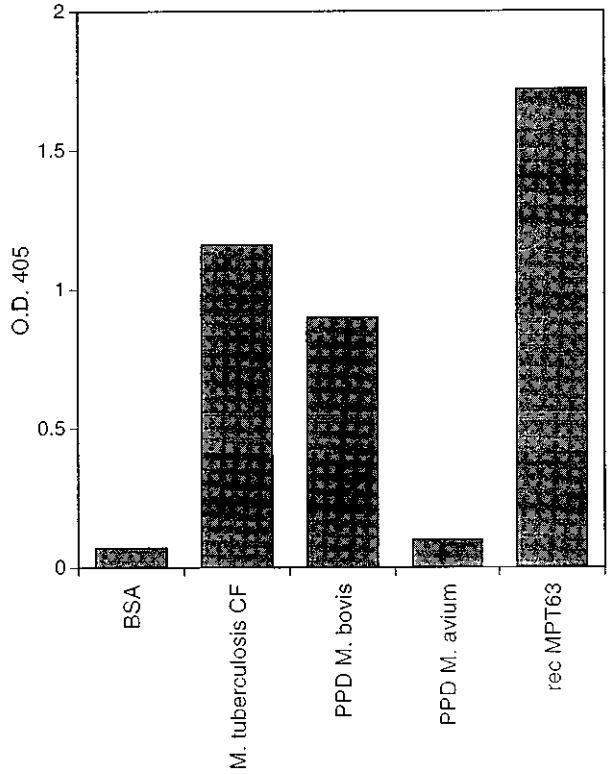

FIG. 7. Reactivity of rabbit anti-MPT63 antiserum to mycobacterial antigens in an ELISA. Concentrations of plate-coating antigens and the dilution of rabbit polyclonal anti-MPT63 antiserum used in this experiment are indicated in Materials and Methods. CF, culture filtrate; O.D. 405, optical density at 405 nm.

weeks after infection (kindly given by D. McMurray, Texas A&M University, College Station). Four infected animals did not display measurable levels of serum antibodies against *M. tuberculosis* culture filtrate proteins and were not analyzed further. Of the 11 responders, all produced antibodies against MPT63 (Fig. 6A). By using serum from the strongest responder among the infected guinea pigs, we compared the levels of antibody binding to recMPT63 and to nMPT63 in an ELISA at different serum dilutions (Fig. 6B) and in the presence of KSCN (Fig. 6C). As observed with sera from hyperimmune rabbits (Fig. 5C and D), the antiserum from the *M. tuberculosis*-infected guinea pig did not distinguish between nMPT63 and recMPT63 proteins, thus confirming that recMPT63 can be used for immunological studies.

Species specificity of the MPT63 antigen. The lack of the *mpt63* gene in mycobacterial species that do not belong to the *M. tuberculosis* complex (shown in Fig. 3) indicated the possibility that MPT63 is an *M. tuberculosis* complex-specific antigen. To test this hypothesis, we investigated the presence of cross-reactive epitopes between MPT63 and proteins of *M. avium* by an ELISA. *M. avium* was chosen because it is a common atypical mycobacterial species that is often associated with false positivity in TB skin testing and with opportunistic infections in immunocompromised individuals. Our results showed that anti-MPT63 antibodies reacted with purified MPT63 and with protein mixtures from *M. tuberculosis* (culture filtrates) and *M. bovis* PPD but not with *M. avium* PPD (Fig. 7). The lack of cross-reactive epitopes was further demonstrated by competitive ELISA. In this type of analysis, binding to MPT63 by anti-MPT63 antibodies was significantly inhibited by *M. bovis* PPD but not by *M. avium* PPD or by an unrelated, control antigen such as BSA (Fig. 8A). In contrast, the binding of monospecific, polyclonal antibodies to a cross-reactive component of the *M. tuberculosis* culture filtrate, such as Ag85B (11), was inhibited equally well by *M. bovis* PPD and *M. avium* PPD but not by control BSA (Fig. 8B).

Taken together, results presented in this section show that MPT63 contains no epitopes that cross-react with a common environmental mycobacterial species, such as *M. avium*. Thus, MPT63 represents a potentially useful *M. tuberculosis* complex-specific reagent for protein-based immunodiagnostic assays.

DISCUSSION

We report the molecular cloning of the gene encoding MPT63, a ~16-kDa extracellular protein of *M. tuberculosis*, by immunoscreening an expression library of *M. tuberculosis* $H_{37}Rv$ DNA with polyclonal antibodies directed against MPT63. Nucleotide sequence analysis of the mycobacterial

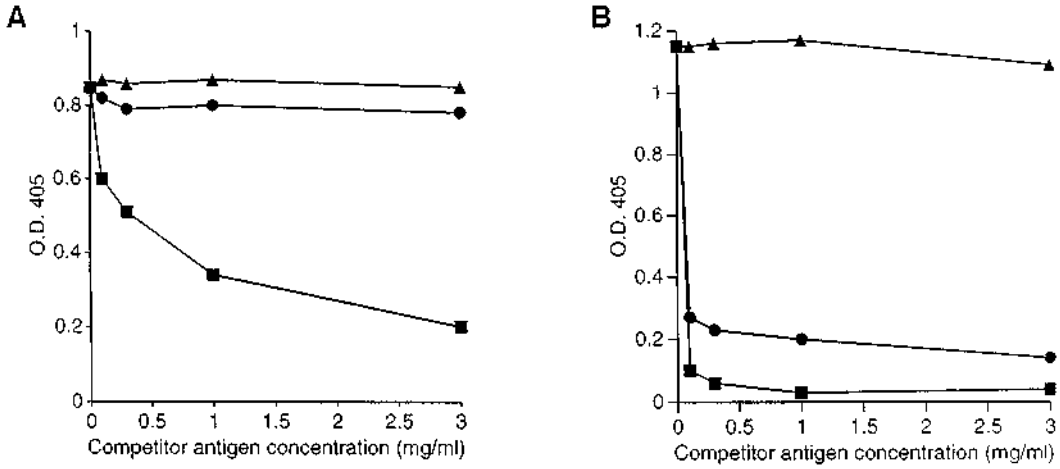

FIG. 8. Binding of monospecific rabbit antisera to MPT63 (A) and Ag85B (B) of *M. tuberculosis* in the presence of increasing concentrations of *M. bovis* PPD (■), *M. avium* PPD (●) and BSA (▲) in a competitive ELISA. Concentrations of competitor antigens are as indicated. Serum dilutions and concentrations of plate-coating antigens are indicated in Materials and Methods. Anti-Ag85B antiserum was obtained from a rabbit immunized with *M. tuberculosis* Ag85B (gift of S. Nagai). O.D. 405, optical density at 405 nm.

DNA insert of a positive clone revealed an ORF encoding a protein of 159 aa. The pentapeptide AYPIT, which is found at the NH_2 terminus of the MPT63 protein purified from *M. tuberculosis* culture filtrates (22), confirms the identity of the ORF as the *mpt63* gene and defines the mature, extracytoplasmatic form of MPT63 as a polypeptide of 130 aa (M_r, 13,655). The deduced amino acid sequence of mature MPT63 is also identical to the 20-aa NH_2-terminal sequence (NH_2-AYPIT GKLGSELTMTDTVGQ) of a 16-kDa extracellular protein of *M. tuberculosis* purified from *M. tuberculosis* culture filtrates and used in guinea pig protection studies by Horwitz et al. (17). The mature MPT63 protein is preceded by a 29-aa stretch having properties that are typical of secretion signal peptides (31), including peptide length and predominantly hydrophobic composition, the NH_2-terminal location of basic residues, a helix-disrupting P residue at −5, and small side chain residues (A) at −3 and −1 with respect to the cleavage site.

The presence of a signal peptide defines MPT63 as a protein that is actively secreted by *M. tuberculosis*. This conclusion is consistent with findings by Nagai et al. (22) showing that (i) probing of subcellular fractions of *M. tuberculosis* with anti-MPT63 antibodies found MPT63 protein almost exclusively in culture filtrates rather than in association with bacterial cells and (ii) guinea pigs sensitized with heat-killed, washed *M. tuberculosis* cells lacked skin test reactivity to MPT63.

The nucleotide sequence presented in this paper was not found in the MycDB database, indicating that *mpt63* is a novel gene of *M. tuberculosis*. Further, the MPT63 protein shows no significant homologies with proteins of other organisms, thus leaving the physiological role of MPT63 to be established, possibly by genetic analysis of *mpt63*-deleted mutants or by biochemical characterization of the MPT63 protein. A lack of knowledge of their physiological role is still common to the majority of the *M. tuberculosis*-secreted proteins, even when their involvement in immune responses to TB is well established, as is the case for ESAT-6, MPB/T64, MPB/T70, to cite only a few. Notable exceptions, however, include the well-characterized 38-kDa (PhoS) antigen and the 30-kDa antigen (Ag85B), whose enzymatic activities have been defined (1, 6a).

In this work, MPT63 was expressed in *E. coli* as a recombinant protein having a polyhistidine purification tag and purified to near-homogeneity by nickel-affinity and anion-exchange chromatographies. Purified MPT63 tested free of *E. coli* lipopolysaccharide (data not shown), as determined by chemiluminescence in diluted blood (31a). Thus, the level of purity achieved is suitable for serological as well as for cell-based immunological assays.

One concern is often voiced in the case of recombinant antigens with respect to potential structural (and, therefore, functional) differences between recombinant and native (i.e., in this context, produced from *M. tuberculosis* cells) proteins. These differences may include posttranslational modifications occurring in *M. tuberculosis* but not in *E. coli* that may affect the suitability of a recombinant protein as a reagent in immunological assays. We observed no size differences in SDS-PAGE between recMPT63 and nMPT63 that cannot be accounted for by the presence of 12 aa residues (including 6 histidines) that are added to the NH_2 terminus of recMPT63. More importantly, by using several different assays and antibodies from hyperimmunized rabbits as well as from *M. tuberculosis*-infected guinea pigs, we have observed no differences between recMPT63 and nMPT63 in terms of their immunochemical properties and kinetics of antibody recognition of the two proteins. Thus, while further investigations are required to formally rule out posttranslational modification of MPT63 in *M. tuberculosis*, our data provide strong evidence that recMPT63 from *E. coli* is a suitable reagent for immunological studies.

It is widely accepted that proteins that are secreted by *M. tuberculosis* are involved in immune responses during TB (recently reviewed in reference 12). Thus, with the purified recMPT63 at hand, it is important to determine whether MPT63 evokes immune responses in TB. In the present studies, we have shown that guinea pigs that have been aerosol infected with virulent *M. tuberculosis* produce high-level antibodies against MPT63. Further, guinea pigs injected with MPT63 purified from *M. tuberculosis* culture filtrates mount delayed-type hypersensitivity responses in guinea pigs (17). Taken together, these results indicate that MPT63 induces immune responses during TB. Further immunological characterization will be undertaken to establish T-cell reactivity to MPT63 during TB.

We have shown that the *mpt63* gene is present only in *M. tuberculosis* and *M. bovis* strains and not in mycobacterial species that do not belong to the TB complex, most notably, a common environmental species such as *M. avium*. This finding supports the possibility that the *mpt63* DNA be used as an *M. tuberculosis* complex-specific probe in DNA-based diagnostic assays. Of perhaps even greater consequence are our observations that MPT63 lacks epitopes that cross-react serologically with *M. avium* antigens, as shown by indirect and competitive ELISAs. The diagnostic value of PPD skin testing is severely hampered by the inability of this test to distinguish between infection with *M. tuberculosis* or *M. bovis* and exposure to saprophytic mycobacteria often present in the environment. The specificity of *mpt63* for the *M. tuberculosis* complex and the lack of cross-reactivity between MPT63 and *M. avium* proteins strongly encourage further evaluation of MPT63 in terms of specific skin test reagent. Skin test experiments in guinea pigs are in progress.

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