INFECTION AND IMMUNITY, Dec. 1997, p. 4951–4957 Vol. 65, No. 12
0019-9567/97/$04.00+0

MTC28, a Novel 28-Kilodalton Proline-Rich Secreted Antigen Specific for the *Mycobacterium tuberculosis* Complex

CLAUDIA MANCA, KONSTANTIN LYASHCHENKO, ROBERTO COLANGELI, AND MARIA LAURA GENNARO*

Public Health Research Institute, New York, New York 10016

Received 3 April 1997/Returned for modification 31 May 1997/Accepted 16 September 1997

Proteins that are actively secreted by *Mycobacterium tuberculosis* serve as major targets of immune responses in the infected host. To identify and purify novel proteins in the filtrates of *M. tuberculosis* cultures, a bacteriophage λ library of *M. tuberculosis* H_{37}Rv DNA was immunoscreened by using an anti-culture filtrate rabbit antiserum. Of 20 positive clones isolated, 6 were analyzed and found to express the genes for two known components of the early culture filtrate, the secreted 45/47-kDa antigen complex and the KatG protein, and two novel genes. Here we report the molecular cloning and nucleotide sequence of one of the new genes encoding a culture filtrate protein of 310 amino acid (aa) residues. We called this gene *mtc28*. The deduced polypeptide sequence contained an NH_2-terminal, highly hydrophobic 32-aa region having properties of a secretion signal peptide. The putative 278-aa mature MTC28 protein was characterized at its NH_2 and COOH termini by a high content of proline and alanine residues organized in an $(AP)_n$ motif. Thus, MTC28 is a new member of a group of proline-rich antigens found in *M. tuberculosis* and *Mycobacterium leprae*. As shown by DNA hybridization experiments, the *mtc28* gene was present only in species of the *M. tuberculosis* complex. Purified recombinant MTC28 antigen evoked strong delayed-type hypersensitivity and antibody responses in guinea pigs immunized with *Mycobacterium bovis* BCG, but not in guinea pigs immunized with *Mycobacterium avium*. The strong immunological activity of MTC28 and the absence of B- and T-cell epitopes cross-reactive with a common environmental mycobacterial species, such as *M. avium*, make this novel antigen an attractive reagent for immunodiagnosis of tuberculosis.

Tuberculosis (TB) accounts for approximately 8 million new cases of disease and 3 million deaths a year in the world (10). If current trends continue, at least 30 million people will die from TB in the next 10 years (38). Implementation of control measures for TB requires the development of TB vaccines affording higher protection than the currently used bacillus Calmette-Guérin (*Mycobacterium bovis* BCG) vaccine and of new, sensitive immunodiagnostic agents that distinguish TB from other mycobacterial infections. To achieve these goals, it is necessary to develop a knowledge both of the antigens of *Mycobacterium tuberculosis* that are involved in protective immunity against TB and of antigens that allow measurement of *M. tuberculosis*-specific immune responses. Protein antigens present in the culture filtrate of *M. tuberculosis* have attracted considerable attention because of their ability to induce strong immune responses in TB (reviewed in references 1, 14, and 40). Further, there is a large body of evidence that culture filtrate proteins elicit protective immunity in animal models of TB (3, 21, 22, 32). Thus, culture filtrate proteins constitute prime candidates for the development of subunit vaccines against TB and as potential tools for diagnostic skin test and serological diagnosis of TB.

Fewer than 20 of the ~100 proteins present in filtrates from short-term (4- to 5-day) cultures (2a) have been characterized by gene cloning and nucleotide sequencing (2, 4, 9, 13, 24, 25, 27, 28, 31, 34, 40). Most of the sequenced proteins are potent antigens (40); however, yet-uncharacterized antigens contribute to the strong cellular and humoral immune responses against culture filtrate fractions in TB patients (5, 6, 11, 34). Thus, it is essential to identify other novel components of *M. tuberculosis* culture filtrates and to define their role in immune responses during tubercular infection.

To identify novel secreted antigens of *M. tuberculosis*, we have undertaken screening of a λ phage expression library of *M. tuberculosis* H_{37}Rv DNA with rabbit antisera raised against an early culture filtrate or purified components thereof. Using this immunoscreening approach, we have recently cloned and sequenced the gene encoding the secreted protein MPT63, purified this protein from *Escherichia coli* cells, and shown that MPT63 is an *M. tuberculosis* complex-specific antigen (27). Here we report cloning and sequencing of another novel *M. tuberculosis* complex-specific gene identified by library immunoscreening. This gene, which we named *mtc28*, encodes a mature protein of 278 amino acid (aa) residues (M_r, 28,660) preceded by a putative 32-aa secretion signal peptide. The mature MTC28 protein, which is found in the culture filtrate of *M. tuberculosis*, is characterized by proline- and alanine-rich NH_2 and COOH termini. The *mtc28* gene is present only in mycobacteria of the *M. tuberculosis* complex, and the MTC28 antigen shares no T- or B-cell epitopes with proteins of *Mycobacterium avium*, a common environmental species.

MATERIALS AND METHODS

General methods. Restriction endonuclease analysis and DNA cloning were performed by standard procedures (33). Restriction DNA fragments were purified from agarose gels by the Qiaex extraction procedure (Qiagen). DNA fragments were generated by PCR with 25 cycles of amplification (94°C for 1 min, 55°C for 2 min, and 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 manufacturers' protocols. Nucleotide sequencing was done with an Applied Biosystems Model 373 DNA sequencer with double-stranded plasmid DNA 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. Determination of nucleotide

* Corresponding author. Mailing address: Public Health Research Institute, 455 First Ave., New York, NY 10016. Phone: (212) 578-0844. Fax: (212) 578-0804. E-mail: gennaro@phri.nyu.edu.

and amino acid sequence homology was performed with the on-line database of the National Center for Biotechnology Information, Bethesda, Md.

Bacterial strains and products. *E. coli* strains were grown with standard liquid and solid media (33). *M. bovis* BCG Japanese ATCC 35737 and *M. avium* ATCC 25291 were obtained from the American Type Culture Collection. BCG Japanese was chosen because it produces the MPB64 antigen (26), whose *M. tuberculosis* homolog, MPT64, was used in some of the present studies. Mycobacteria were grown at 37°C in rotating bottles in 7H9 medium enriched with 0.05% Tween 80 and standard albumin-dextrose additive. *M. tuberculosis* H_{37}Rv lipoarabinomannan-free culture filtrates and cells killed by gamma irradiation were kindly provided by J. Belisle, Colorado State University, Fort Collins. Tuberculin purified protein derivative (PPD) from *M. tuberculosis* PPD-CT-68 was purchased from Connaught Laboratories Inc. (Swiftwater, Pa.).

Rabbit antibodies. Antiserum against an early culture filtrate of *M. tuberculosis* H_{37}Rv was kindly given to us by H. G. Wiker (University of Oslo, Oslo, Norway). The culture filtrate used for rabbit immunization tested negative by Western blot analysis for the heat shock 65-kDa antigen, taken as a marker of cytosolic contamination (37). To obtain anti-MTC28 polyclonal antibody, three female New Zealand White rabbits (Pocono Rabbit Farm and Laboratory, Inc., Canadensis, Pa.) were injected biweekly with 50 to 200 μg of recombinant MTC28 protein in incomplete Freund's adjuvant by the subcutaneous and intradermal routes. Animals were bled 1 week after the fourth immunization.

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, Inc.), by using DNA extracted from *M. tuberculosis* H_{37}Rv cultures grown on 7H11 Middlebrook agar as described previously (27). By a protocol of the manufacturer, plaques were screened with a 1:1,000 dilution of rabbit anti-culture filtrate antiserum to identify clones expressing culture filtrate proteins. Positive plaques were visualized by a reaction with alkaline-phosphatase-conjugated antibody against rabbit immunoglobulin G (Bio-Rad) diluted 1:1,000 and the substrate 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (Kirkegaard and 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, according to the manufacturer's protocol.

Screening of a cosmid library of *M. tuberculosis* H_{37}Rv by DNA hybridization. To isolate clones that contained DNA upstream of the gene expressing MTC28, a cosmid library of *M. tuberculosis* H_{37}Rv DNA (constructed in W. R. Jacobs's laboratory and kindly provided by J. Dubnau) was screened by DNA hybridization with as probe a 2.4-kb *Pst*I fragment containing the *mtc28* gene. Nonradioactive labelling of DNA, colony lifts, and hybridization were performed with the ECL 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), according to the standard protocol (33). Transferred DNA was fixed to membranes by UV cross-linking. The DNA probe was a 2.4-kb *Pst*I fragment containing the *mtc28* gene. Nonradioactive DNA labelling with horseradish peroxidase, hybridization, membrane washing, and chemiluminescence detection were performed with protocols and solutions provided in the ECL direct nucleic acid labelling and detection systems (Amersham). In our experiments, 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 (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.4% sodium dodecyl sulfate (SDS)–6 M urea for 15 min and twice in 2× SSC for 20 min.

Preparation of *E. coli* crude extracts and immunoblotting. Overnight cultures of *E. coli* XL1-Blue MRF′ harboring the recombinant plasmid pMTC28 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 (OD_{600}) of 0.5. IPTG (isopropyl-β-D-thiogalactopyranoside) 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 by washing cells with distilled water, resuspending them in SDS sample buffer, and heating them at 95°C for 5 min. Protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes by a standard protocol (17). Rabbit antisera and the color development system were added as described for immunoscreening of the expression library.

Purification of the polyhistidine-tagged recombinant MTC28. Mid-log-phase cultures of *E. coli* XL1-Blue MRF′ harboring the recombinant plasmid pMTC28 were obtained as described above and induced with 1 to 2 mM IPTG for 5 h. Cells were washed in 10 mM phosphate buffer, pH 7.4, and then collected by centrifugation at 20,000 × *g* for 30 min. Cells were resuspended in 10 mM phosphate buffer, pH 7.4, to a density of 50 mg of cell paste/ml and subjected to two 1,300-lb/in cycles of a French press (American Instrument Company). Cell lysates were clarified by ultracentrifugation at 25,000 rpm in a Ti-50 rotor for 30 min. Polyhistidine-tagged MTC28 protein was purified by a three-step protocol, which is described elsewhere (12), consisting of sequential chromatography with metal chelate affinity, size exclusion, and anion-exchange columns. Purified protein was aliquoted and stored at −70°C. Lipopolysaccharide (LPS) was measured in purified protein preparations by a lucigenin-induced chemiluminescence assay in 1% blood (23) (courtesy of Jerrold Weiss, New York University Medical Center).

Guinea pig skin testing. Three groups of six guinea pigs, weighing approximately 300 g each, were immunized by intradermal injection in the abdomen with 10^7 live *M. bovis* BCG Japanese or *M. avium* cells in 0.1 ml of phosphate-buffered saline (PBS) or by intramuscular injection with 1 mg of gamma-irradiated *M. tuberculosis* H_{37}Rv mixed with incomplete Freund's adjuvant (1:1) in 0.2 ml of PBS.

Five weeks after immunization, the animals were shaved on the back and injected intradermally with 2 μg of each purified recombinant antigen in 0.1 ml of PBS and with 10 tuberculin units (TU) of PPD. Local skin reactions were measured 24 h after antigen injection.

Enzyme-linked immunosorbent assay (ELISA). Serum samples from immunized guinea pigs were collected 8 weeks after immunization and tested for anti-MTC28 antibodies by ELISA. Polystyrene microtiter 96-well plates (Costar) were coated with 0.1 μg of recombinant protein per well in 0.05 M carbonate buffer (pH 9.6) at 4°C overnight followed by incubation with 3% nonfat milk (Sigma) in PBS. A total of 0.1 ml of serum diluted 1:200 in PBS plus 0.05% Tween 20 (PBS-T) and 1% nonfat milk were added to the antigen-coated wells in duplicate. After 90 min of incubation at room temperature, plates were extensively washed with PBS-T and then incubated for 1 h with alkaline-phosphatase-conjugated antibody against guinea pig immunoglobulin G (Sigma) diluted 1:1,000 in PBS-T. Plates were washed with PBS-T, and alkaline phosphatase activity was assayed with *p*-nitrophenyl-phosphatase (Bio-Rad). OD_{405} was measured on a Spectra Shell automatic microplate reader (Tecan).

Nucleotide sequence accession number. The GenBank accession number for the *mtc28* gene is U75271.

RESULTS

Immunoscreening of an *M. tuberculosis* H_{37}Rv expression library. An expression library of *M. tuberculosis* H_{37}Rv DNA in the bacteriophage λ ZAPII vector was screened with a rabbit antiserum raised against an *M. tuberculosis* early culture filtrate to identify clones expressing novel culture filtrate proteins. In a screen of approximately 1.5×10^5 plaques, 20 positive clones were isolated (data not shown). Of these, six were characterized by in vivo excision of the corresponding plasmids, analysis of IPTG induction of recombinant gene expression, subcloning, and partial nucleotide sequence analysis. Two of the clones expressed novel genes. Characterization of one of these novel genes, carried by a clone named pC5, is the subject of the present work. Of the other four positive clones, two encoded the secreted 45/47-kDa antigen (24) and two encoded the KatG protein (19) (data not shown). It is noteworthy that both the 45/47-kDa antigen complex and KatG are found in early culture filtrates of *M. tuberculosis* (2a, 25), thus confirming our immunoscreen to be highly specific for culture filtrate proteins.

The pC5 clone (whose map is sketched in Fig. 1A) contained a 7.4-kb mycobacterial DNA insert and expressed under *lac* control a protein of ~42 kDa that reacted with the anti-culture filtrate antiserum used for library immunoscreening (data not shown). Subcloning of a 2.4-kb *Pst*I fragment of pC5 (Fig. 1) in a pBluescript (Stratagene) vector yielded a plasmid, pCM5, which also produced the ~42-kDa protein (Fig. 1B).

Characterization of the *mtc28* gene. Nucleotide sequence analysis of the pCM5 subclone identified an open reading frame (ORF) of 1,362 bp resulting from a gene fusion between the vector's *lacZ* and mycobacterial DNA. We determined that this 1,362-bp ORF encoded the ~42-kDa protein shown in Fig. 1B by demonstrating that a frameshift mutation introduced at the junction between vector and insert DNAs abolished production of this antigen (data not shown). Nucleotide sequence analysis of the 1,362-bp ORF also revealed the presence of a shorter, internal ORF of 930 bp. This 930-bp ORF started with an ATG triplet that was preceded by a putative ribosomal binding site (GGAGG) at a suitable distance (11 bp) (Fig. 2). Manual and computer-assisted analysis of the amino acid sequence deduced from the 930-bp ORF indicated that the NH_2-terminal 32-aa region had properties typical of a bacterial

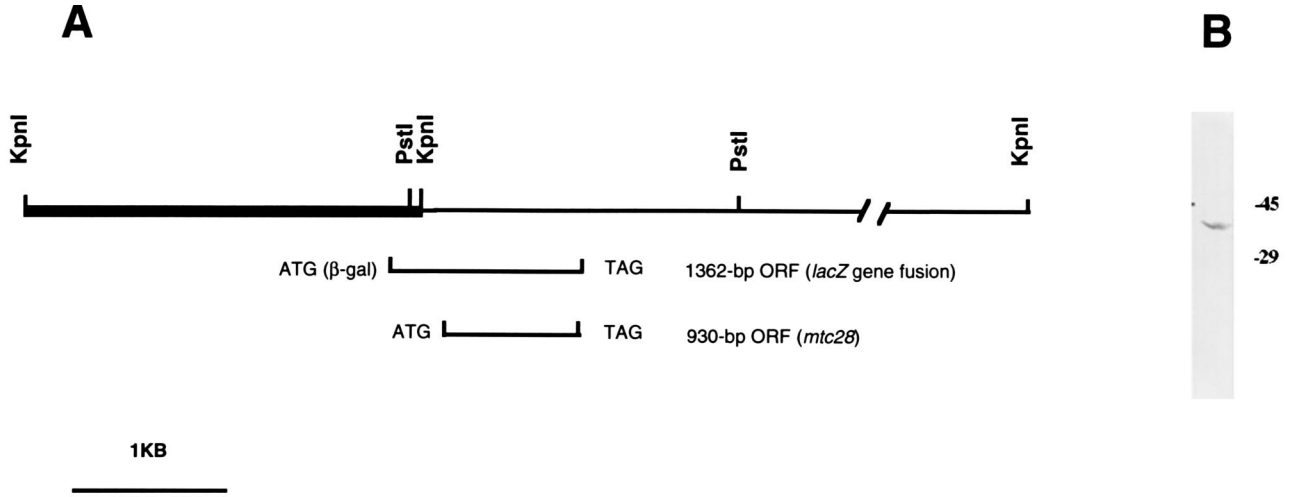

FIG. 1. The recombinant pC5 clone. (A) This schematic map of the pC5 clone shows the pBluescript plasmid vector (thick lines) and the 7.4-kb insert of *M. tuberculosis* DNA (thin lines). Below the map are shown the positions of the longer 1,362-bp ORF (derived from a fusion of the vector's *lacZ* and mycobacterial DNA) and the shorter, internal 930-bp ORF that we call *mtc28*. The 2.4-kb *Pst*I fragment shown in the map was used to generate the pCM5 subclone. (B) Crude cell extracts were obtained from *E. coli* cells containing the pCM5 plasmid, separated by SDS–10% PAGE, transferred to nitrocellulose membranes, and reacted with a rabbit anti-culture filtrate antiserum used for immunoscreening of our *M. tuberculosis* H_{37}Rv DNA library. The pCM5 subclone expressed a ~42-kDa protein reacting with the anti-culture filtrate antiserum. Molecular mass (in kilodaltons) is indicated on the right.

secretion signal peptide (36) including peptide length (22 to 42 aa in *M. tuberculosis*) and hydrophobic composition, the NH_2-terminal location of basic residues (R) at −27 and −24, a helix-breaking G residue at −5, and small side chain residues (A) at −3 and −1 with respect to the putative cleavage site. We also analyzed sequences upstream of the DNA insert in pC5 contained in an overlapping clone isolated from a cosmid library of *M. tuberculosis* H_{37}Rv DNA and failed to identify suitable translational signals (appropriately spaced start codons and ribosome binding sites) for the larger ORF expressed as a 1,362-bp *lacZ* fusion in pC5 (data not shown).

Based on these observations, we propose that the novel gene of *M. tuberculosis* identified by our analyses contains a 930-bp ORF encoding a 32-aa secretion signal peptide and a mature 278-aa protein (M_r, 28,660) that we call MTC28. The same 930-bp ORF has been identified in cosmid SCY21D4 and named gene MTCY21D4.03c of unknown function (*M. tuberculosis* Genome Sequencing Project, Sanger Centre, Cambridge, United Kingdom).

Analysis of the amino acid composition of MTC28 showed a high overall content of proline (16%) and alanine (13%) residues. The majority of proline residues were localized at the NH_2-terminal (24 of 70 [34%]) and at the COOH-terminal (12 of 40 [30%]) regions of the protein in association with several poly(AP) stretches.

Genomic analysis of the *mtc28* gene. We analyzed the presence of the *mtc28* gene in laboratory strains and clinical isolates of *M. tuberculosis*, in mycobacterial species of the *M. tuberculosis* complex, and in mycobacteria other than *M. tuberculosis*. Genomic analysis of *mtc28* was performed by Southern blotting and hybridization, with *Pvu*II-digested chromosomal DNAs and the *mtc28* gene as probe. Results of these experiments are shown in Fig. 3. The *mtc28* gene was found in all *M. tuberculosis* isolates and in mycobacteria of the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum*). *mtc28* was associated with a polymorphism: a small band of 0.9 kb was visible in all hybridization-positive strains with the exception of *M. africanum*, a variable band in the 4- to 5-kb region was visible in all *M. tuberculosis* and *M. bovis* BCG strains, and two different bands in the same 4- to 5-kb region were seen in *M. microti*. No hybridization signal was detected with DNAs extracted from nontubercular mycobacterial species (*M. avium*, *Mycobacterium smegmatis*, *Mycobacterium intracellulare*, and *Mycobacterium fortuitum*), indicating that *mtc28* is specific to the *M. tuberculosis* complex.

Presence of MTC28 in the culture filtrate of *M. tuberculosis*. The presence of a putative secretion signal peptide in the product of the *mtc28* gene constitutes strong evidence that MTC28 is a secreted protein of *M. tuberculosis*. To confirm this view, we purified MTC28 protein from *E. coli* cells, raised antibodies against the purified recombinant protein, and probed by Western blotting the *M. tuberculosis* culture filtrate for the presence of MTC28. To obtain pure MTC28 protein, we subcloned the sequence encoding the mature form of MTC28 (extending from nucleotide 369 to 1205 [Fig. 2]) in the *E. coli* plasmid pQE30 (Qiagen) as a fusion protein having a polyhistidine tag at its NH_2 terminus. The tagged protein was purified by three sequential chromatographic steps (a metal-chelating column, a gel filtration column, and an anion-exchange column). Purification of MTC28 and other *M. tuberculosis* culture filtrate proteins from *E. coli* cells as polyhistidine-tagged fusions is described elsewhere (12). After the final anion-exchange column, the LPS content of the protein preparation was $<<0.01$ ng of LPS per μg of protein, as determined by using a lucinogenin-based chemiluminescence assay in 1% blood (23) (data not shown).

Pure recombinant MTC28 protein was used for rabbit immunization to obtain anti-MTC28 polyclonal antibodies. SDS-PAGE and Western blot analysis of purified recombinant MTC28 and culture filtrate proteins of *M. tuberculosis* H_{37}Rv showed that (i) antibodies raised against recombinant MTC28 reacted with a single protein band in the culture filtrate that migrated with an apparent molecular mass of 49 kDa (Fig. 4, lane 1) and (ii) pure recombinant MTC28 (28 kDa) migrated as a single band with an apparent molecular mass of ~51 kDa (Fig. 4, lanes 2 and 3). The difference in the apparent molecular weights of the native and recombinant proteins is fully accounted for by the polyhistidine purification tag on the recombinant product. The abnormally low mobility observed

```
                                                    GGTACCGTGGCACGTCGGAGTCCGCGTC  28
GTCGGCACGGGGCACGCCGCCAGGCCCAGCGGTTGGCGATTCGGTCACGCCCAACAGGGT  88
ATAAGGGTGGCCCGGGAACCTCCGGGGCCGCGCTACCGGCCACGGGTTGGTCTCGGTTCC 148
GTTGCACCACGATCAGAGGTTCATTCCAGCTGCATTTCAAGCCTGTGCACTGCCATGGAG 208
                                                        SD
CGCTGGTTACATTCAGCCTCGACGACGGGCACCGTCGCCCGGCCATTCGGAGGGACCGAC 268
GCAAATGATCCAGATCGCGCGCACCTGGCGGGTCTTCGCAGGCGGCATGGCCACCGGTTT 328
      M  I  Q  I  A  R  T  W  R  V  F  A  G  G  M  A  T  G  F

CATCGGCGTGGTGCTGGTCACCGCCGGGAAGGCCTCAGCGGATCCCCTGCTGCCACCGCC 388
  I  G  V  V  L  V  T  A  G  K  A  S  A  D  P  L  L  P  P  P
                                        ↑
GCCTATCCCTGCCCCAGTCTCGGCGCCGGCAACAGTCCCGCCCGTGCAGAACCTCACGGC 448
  P  I  P  A  P  V  S  A  P  A  T  V  P  P  V  Q  N  L  T  A

GCTTCCGGGCGGGAGCAGCAACAGGTTCTCACCGGCGCCAGCACCCGCACCGATCGCGTC 508
  L  P  G  G  S  S  N  R  F  S  P  A  P  A  P  A  P  I  A  S

GCCGATTCCGGTCGGAGCACCCGGGTCCACCGCTGTGCCCCCGCTGCCGCCGCCAGTGAC 568
  P  I  P  V  G  A  P  G  S  T  A  V  P  P  L  P  P  P  V  T

TCCCGCGATCAGCGGCACACTTCGGGACCACCTCCGGGAGAAGGGCGTCAAGCTGGAGGC 628
  P  A  I  S  G  T  L  R  D  H  L  R  E  K  G  V  K  L  E  A

ACAGCGACCGCACGGATTCAAGGCGCTCGACATCACACTGCCCATGCCGCCGCGCTGGAC 688
  Q  R  P  H  G  F  K  A  L  D  I  T  L  P  M  P  P  R  W  T

TCAGGTGCCCGACCCCAACGTGCCCGACGCGTTCGTGGTGATCGCCGACCGGTTGGGCAA 748
  Q  V  P  D  P  N  V  P  D  A  F  V  V  I  A  D  R  L  G  N

CAGCGTCTACACGTCGAATGCGCAGCTGGTGGTGTATAGGCTGATCGGTGACTTCGATCC 808
  S  V  Y  T  S  N  A  Q  L  V  V  Y  R  L  I  G  D  F  D  P

CGCTGAGGCCATCACACACGGCTACATTGACAGCCAGAAATTGCTCGCATGGCAGACCAC 868
  A  E  A  I  T  H  G  Y  I  D  S  Q  K  L  L  A  W  Q  T  T

AAACGCCTCGATGGCCAATTTCGACGGCTTTCCGTCATCAATCATCGAGGGCACCTACCG 928
  N  A  S  M  A  N  F  D  G  F  P  S  S  I  I  E  G  T  Y  R

CGAAAACGACATGACCCTCAACACCTCCCGGCGCCACGTCATCGCCACCTCCGGAGCCGA 988
  E  N  D  M  T  L  N  T  S  R  R  H  V  I  A  T  S  G  A  D

CAAGTACCTGGTTTCGCTGTCGGTGACCACCGCGCTGTCGCAGGCGGTCACCGACGGGCC 1048
  K  Y  L  V  S  L  S  V  T  T  A  L  S  Q  A  V  T  D  G  P

GGCCACCGATGCGATTGTCAACGGATTCCAAGTGGTTGCGCATGCGGCGCCCGCTCAGGC 1108
  A  T  D  A  I  V  N  G  F  Q  V  V  A  H  A  A  P  A  Q  A

GCCTGCCCCGGCACCCGGTTCGGCACCGGTGGGACTACCCGGGCAGGCGCCTGGGTATCC 1168
  P  A  P  A  P  G  S  A  P  V  G  L  P  G  Q  A  P  G  Y  P

GCCCGCGGGCACCCTGACACCAGTCCCGCCGCGCTAGGTCGCGATGAGGCCGAGCAGAAA 1228
  P  A  G  T  L  T  P  V  P  P  R  *

CACGGGCCCGCATGGAGCTCGGTGAGCGGATTCGTCGGCGGCCTCGTATGGTGAACGAAT 1288
GTTCCTCGCGGGTGTGCTGTGCATGTGTGCGGCGGCGGCGTCCGCCCTGTTCGGGAGCTG 1348
GTC 1351
```

FIG. 2. Nucleotide sequence of the gene encoding the MTC28 protein of *M. tuberculosis*. The nucleotide sequence shown in this figure contains an ORF of 930 nucleotides that we call *mtc28*. The initiation codon (ATG), which is shown in boldface, is preceded by a putative ribosomal binding site (GGAGG) at a suitable distance (11 bp). The *mtc28* gene encodes a full-length protein of 310 aa, whose deduced amino acid sequence is also shown. Manual and computer-assisted (Psort v.6.3 for prediction of protein localization) analysis of the deduced amino acid sequence indicates that the NH_2-terminal 32 aa residues, which are underlined in the figure, have properties that are typical of secretion signal peptides. The putative cleavage site is indicated by an arrow. SD, Shine-Dalgarno sequence.

with MTC28 (28 kDa) in SDS-PAGE is also a property of other mycobacterial proteins having proline-rich termini, such as the secreted 45/47-kDa antigen complex of *M. tuberculosis* (see Discussion).

Immunological activity and species specificity of MTC28. To investigate both immunological activity and species specificity of the MTC28 antigen, we measured delayed-type hypersensitivity (DTH) and antibody responses in guinea pigs immunized with live *M. bovis* BCG, with live *M. avium*, and with killed (gamma-irradiated) *M. tuberculosis* $H_{37}Rv$. Animals were skin tested with 10 TU of PPD and with 2 μg each of recombinant proteins (MTC28, MPT64, and the 45/47-kDa antigen) obtained as polyhistidine-tagged fusion proteins and purified as described for MTC28. Skin reactivity to MTC28 was strong in BCG-immunized guinea pigs (Fig. 5, solid bars) and very low in animals immunized with *M. avium* (Fig. 5, striped bars). The

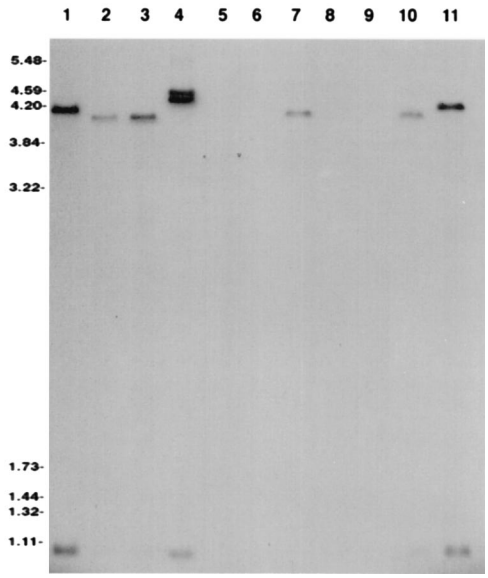

FIG. 3. Genomic analysis of the *mtc28* gene by Southern hybridization. Genomic DNAs were digested with *Pvu*II and electrophoresed on 1% agarose gels. The separated digestion products were transferred to nylon membranes and hybridized with a nonradioactively labelled DNA fragment internal to the *mtc28* gene. After chemiluminescence detection, a positive hybridization signal was observed only in species of the *M. tuberculosis* complex, but not with DNAs extracted from nontubercular mycobacteria. Lanes: 1, *M. tuberculosis* W (clinical isolate) (8); 2, *M. bovis* BCG Pasteur; 3, *M. bovis* BCG Montreal; 4, *M. microti* ATCC 19422; 5, *M. smegmatis*; 6, *M. avium*; 7, *M. africanum* ATCC 35711; 8, *M. fortuitum*; 9, *M. intracellulare*; 10, *M. tuberculosis* $H_{37}Rv$; 11, *M. tuberculosis* W. It is noted that a small, 0.9-kb band is detectable, even though weakly, in all hybridization-positive strains, with the exception of *M. africanum* (lane 7), and did not reproduce well in the figure. It is possible that the high-molecular-weight doublet visible with *M. microti* DNA (lane 4) is due to incomplete *Pvu*II digestion. Molecular size markers (in kilobases) are indicated on the left.

DTH responses induced by MTC28 compared well both in strength and in specificity for the *M. tuberculosis* complex with those elicited by the *M. tuberculosis* complex-specific skin test reagent MPT64 (30).

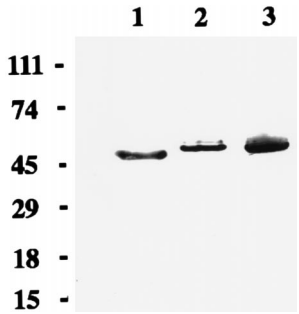

FIG. 4. Presence of MTC28 protein in the culture filtrate of *M. tuberculosis*. The mature MTC28 protein expressed from *E. coli* cells containing the recombinant pQE30::*mtc28* plasmid was purified by sequential chromatography with metal chelate affinity, size exclusion, and anion-exchange columns. This figure shows a Western blot analysis of culture filtrate proteins of *M. tuberculosis* $H_{37}Rv$ (lane 1) and of purified recombinant MTC28 protein (lane 2) with anti-MTC28 rabbit antiserum. Purified recombinant MTC28 is shown in lane 3 (silver staining of gradient SDS–4 to 20% PAGE). Molecular mass (in kilodaltons) is indicated on the left. We do not know why the mature MTC28 protein migrates in SDS-PAGE more slowly (~49 kDa) than the product of the *lacZ* fusion expressed by the original clone (~42 kDa [Fig. 1B]). It may be that a proline-rich stretch has a stronger effect on protein mobility when it is located at the protein's end (as in MTC28) than when it is placed internally (as in the longer fusion protein).

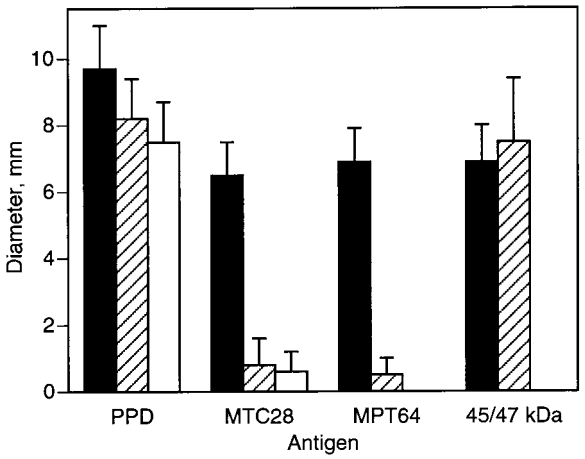

FIG. 5. DTH responses to selected *M. tuberculosis* antigens in guinea pigs immunized with live *M. bovis* BCG (solid bars), live *M. avium* (striped bars), and killed *M. tuberculosis* (open bars). Three groups of six guinea pigs were immunized as described in Materials and Methods and skin tested 5 weeks after immunization by intradermal injection of 10 TU of PPD and 2 μg of purified recombinant antigens. Results are expressed as the means (plus standard deviations) of the diameters (in millimeters) of erythema measured 24 h after antigen injection in each group of animals.

In contrast to MTC28, the proline-rich 45/47-kDa antigen complex elicited DTH reactions both in BCG- and in *M. avium*-immunized animals (Fig. 5), indicating that this antigen complex contains T-cell epitopes cross-reactive with *M. avium* proteins.

Antibody responses to MTC28 were also measured in the same three groups of guinea pigs immunized with live *M. bovis* BCG, with live *M. avium*, and with killed *M. tuberculosis* $H_{37}Rv$. As observed with DTH responses, antibodies were produced against MTC28 in the BCG-immunized group (Fig. 6, solid bars) but not in the animals immunized with *M. avium* (Fig. 6, striped bars). Titers of anti-MTC28 antibodies exceeded those measured against the 38-kDa antigen of *M. tuberculosis* (Fig. 6, solid bars), a reference antigen that induces strong, *M. tuberculosis* complex-specific antibody responses (16).

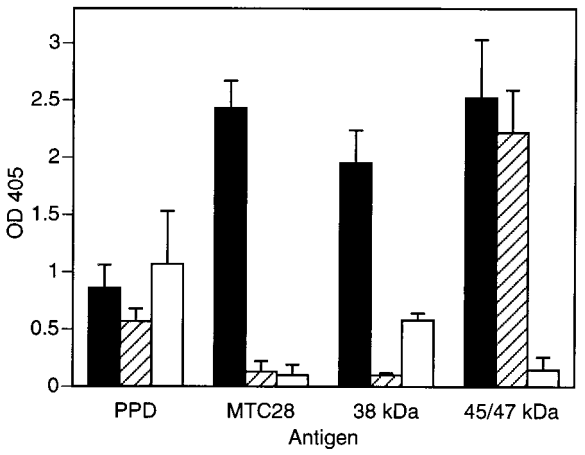

FIG. 6. Antibody responses to selected *M. tuberculosis* antigens in guinea pigs immunized with live *M. bovis* BCG, live *M. avium*, and killed *M. tuberculosis*. Sera from guinea pigs subjected to skin testing were collected 8 weeks after immunization and tested in ELISA with purified recombinant antigens of *M. tuberculosis* as described in Materials and Methods. Data are expressed as OD_{405} readings (means plus standard deviations) obtained for each group of animals. Bars are as for Fig. 5.

In contrast to MTC28, the 45/47-kDa antigen elicited similar levels of antibody responses in both BCG- and *M. avium*-immunized guinea pigs, indicating extensive serological cross-reactivity of this antigen. The presence of cross-reactive B-cell epitopes in the 45/47-kDa antigen complex has also been recently demonstrated by using murine monoclonal antibodies (20).

Neither DTH nor antibody responses to MTC28 were detected in guinea pigs immunized with killed *M. tuberculosis* (Fig. 5 and 6, open bars). This is typical of proteins that are rapidly secreted by *M. tuberculosis* into the culture medium and, therefore, scarcely if at all present in preparations of killed and washed mycobacterial cells (18, 24, 29). In contrast, the 38-kDa antigen, which remains associated with the bacterial cell wall prior to release into the external environment (1), evokes antibody responses, albeit at a low level, also in guinea pigs sensitized with killed *M. tuberculosis* (Fig. 6).

Taken together, results presented in this section show that the secreted MTC28 antigen of *M. tuberculosis* contains no epitopes that cross-react with a common environmental mycobacterial species, such as *M. avium*.

DISCUSSION

We report the molecular cloning of the gene encoding MTC28, a 28-kDa extracellular protein of *M. tuberculosis*. Nucleotide sequence analysis of the gene revealed an ORF encoding a protein of 310 aa residues. Comparison of the nucleotide and deduced amino acid sequences with sequences in the database revealed no homology with known genes and proteins, indicating that the gene designated *mtc28* isolated in the present studies is a novel gene.

Three sets of findings presented in this paper provide strong evidence that MTC28 is actively secreted by *M. tuberculosis*. First, analysis of the deduced amino acid sequence of the MTC28 protein strongly suggests that the protein is composed of a putative NH_2-terminal 32-aa secretion signal peptide and a mature, extracytoplasmic form of 278 aa (M_r, 28,660). This conclusion will be confirmed by NH_2-terminal sequencing of the extracellular form of the MTC28 protein obtained from *M. tuberculosis* cultures. Second, MTC28 is found in the early culture filtrate of *M. tuberculosis*. Third, MTC28 elicits immune responses only in guinea pigs immunized with live, not killed, mycobacteria, like many of the extracellular proteins of *M. tuberculosis* (18, 24, 29).

An interesting feature of the MTC28 polypeptide sequence is the high proline content in the NH_2-terminal (34%) and COOH-terminal (30%) regions of the protein. Proline richness, which in MTC28 is associated with the presence of the short repetitive motif $(AP)_n$, is a feature shared by several mycobacterial antigens. One is the secreted 45/47-kDa antigen complex of *M. tuberculosis* (24). In this antigen, as in MTC28, proline residues primarily localize at the protein's ends (40% [29 of 71] proline residues in the NH_2-terminal region and 39% [16 of 41] at the COOH terminus), where they are also associated with $(AP)_n$ motifs. It can be speculated that the terminal proline-rich stretches in the MTC28 and 45/47-kDa antigens constitute stiff arms that can mediate nonspecific binding to other proteins and/or cells, as reported for other proteins carrying $(AP)_n$ repeats (39). Another example is the 36-kDa proline-rich antigen of *Mycobacterium leprae* (35), which is characterized by a high number of proline residues in its NH_2-terminal portion that are present primarily as polyproline stretches (P_{2-6}) rather than $(AP)_n$ motifs. A high proline con-

tent is also found at the COOH termini of two homologous, presumably cell-wall-associated proteins, ERP of *M. tuberculosis* (7) and IRG of *M. leprae* (25).

A property common to the proline-rich MTC28 and 45/47-kDa antigens of *M. tuberculosis* and the 36-kDa antigen of *M. leprae* is their abnormally slow migration in SDS-PAGE. The calculated molecular weight of MTC28 is 28,660, while the protein migrates in SDS-PAGE with an apparent molecular mass of 49 kDa. Likewise, the calculated molecular weights of the 45/47-kDa antigen of *M. tuberculosis* and the 36-kDa antigen of *M. leprae* (named on the basis of their apparent molecular masses in SDS-PAGE) are only 28,779 and 26,290, respectively. The discrepancies between calculated and apparent molecular weights of these antigens are attributable to the rigidity and reduced mobility characteristic of proline-rich stretches (39). Low electrophoretic mobility is observed when the MTC28 protein is obtained both from *M. tuberculosis* and from *E. coli* cells, thus ruling out an involvement of posttranslational modifications, such as glycosylation (15), in this phenomenon.

Our studies indicate that MTC28 is one of the immunologically active components of the culture filtrate of *M. tuberculosis*. MTC28 elicited strong immune responses in BCG-immunized guinea pigs comparable with those elicited by immunodominant secreted antigens (MPT64 for DTH reactions [30] and the 38-kDa antigen for humoral responses [16]). We also determined that sera from 2 of 10 guinea pigs aerosol infected with virulent *M. tuberculosis* tested strongly positive for antibodies against MTC28 (data not shown). Further, about 25% of patients with active pulmonary TB mounted strong antibody responses to MTC28 (25a).

The strong immunological activity of MTC28 is associated with specificity for mycobacterial species in the *M. tuberculosis* complex. Not only is the *mtc28* gene absent in several nontubercular mycobacteria tested by Southern blot analysis, but more importantly, MTC28 shares no T- and B-cell epitopes with *M. avium* proteins. The immunological specificity of MTC28 for the *M. tuberculosis* complex strongly encourages further evaluation of this antigen as a reagent for TB-specific immunodiagnostic assays.

ACKNOWLEDGMENTS

We are indebted to several colleagues for providing reagents used in this work: Harald Wiker for a rabbit antiserum raised against an early culture filtrate of *M. tuberculosis* $H_{37}Rv$, John Belisle for culture filtrates and gamma-irradiated cells of *M. tuberculosis* $H_{37}Rv$, David McMurray for sera of guinea pigs aerosol infected with *M. tuberculosis* $H_{37}Rv$, and Jeannie Dubnau for the *M. tuberculosis* $H_{37}Rv$ cosmid library. We also thank Barry Kreiswirth and the PHRI TB Center staff for providing mycobacterial DNAs and for helping with genomic Southern blot analysis, Jerrold Weiss for performing LPS assays on purified recombinant proteins used in this study, and Karl Drlica and Carol Lusty for comments on the manuscript.

This work was supported by NIH grant AI-36896 (M.L.G.). C.M. was a Fellow in the Graduate Program of Microbial Technologies, Department of Agricultural Sciences, University of Sassari, Sassari, Italy. R.C. was the recipient of an AIDS training fellowship from the Istituto Superiore di Sanità, Rome, Italy.

REFERENCES

1. **Andersen, A. B., and P. Brennan.** 1994. Proteins and antigens of *Mycobacterium tuberculosis*, p. 307–327. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C.
2. **Andersen, B. A., and E. B. Hansen.** 1989. Structure and mapping of antigenic domains of protein antigen b, a 38,000-molecular-weight protein of *Mycobacterium tuberculosis*. Infect. Immun. **57:**2481–2488.

2a. **Andersen, P.** Personal communication.

3. **Andersen, P., and I. Heron.** 1993. Specificity of a protective memory immune response against *Mycobacterium tuberculosis*. Infect. Immun. **61:**844–851.
4. **Ashbridge, K. R. A., R. J. Booth, J. D. Watson, and R. B. Lathigra.** 1989. Nucleotide sequence of the 19kDa antigen gene from *Mycobacterium tuberculosis*. Nucleic Acids Res. **17:**1249.
5. **Bassey, E. O. E., D. Catty, D. S. Kumararatne, and C. Raykundalia.** 1996. Candidate antigens for improved serodiagnosis of tuberculosis. Tubercle Lung Dis. **77:**136–145.
6. **Bassey, E. O. E., P. F. Life, D. Catty, J. S. H. Gaston, and D. S. Kumararatne.** 1996. T-cell response to mycobacterial proteins: a comparative study of tuberculous and control immunoblots of *M. tuberculosis* and *M. bovis* BCG. Tubercle Lung Dis. **77:**146–153.
7. **Berthet, F.-X., J. Rauzier, E. M. Lim, W. Phillip, B. Gicquel, and D. Portnoi.** 1995. Characterization of the *Mycobacterium tuberculosis erp* gene encoding a potential cell surface protein with repetitive structures. Microbiology **141:** 2123–2130.
8. **Bifani, P. J., B. B. Plikaytis, V. Kapur, K. Stockbauer, X. Pan, M. L. Lutfey, S. L. Moghazeh, W. Eisner, T. M. Daniel, M. H. Kaplan, J. T. Crawford, J. M. Musser, and B. N. Kreiswirth.** 1996. Origin and interstate spread of a New York City multidrug-resistant *Mycobacterium tuberculosis* clone family. JAMA **275:**452–457.
9. **Bigi, F., A. Alito, J. C. Fisanotti, M. I. Romano, and A. Cataldi.** 1995. Characterization of a novel *Mycobacterium bovis* secreted antigen containing PGLTS repeats. Infect. Immun. **63:**2581–2586.
10. **Bloom, B. R., and C. J. L. Murray.** 1992. Tuberculosis: commentary on a reemergent killer. Science **257:**1055–1064.
11. **Boesen, H., B. N. Jensen, T. Wilcke, and P. Andersen.** 1995. Human T-cell responses to secreted antigen fractions of *Mycobacterium tuberculosis*. Infect. Immun. **63:**1491–1497.
12. **Colangeli, R., A. Heijbel, A. Williams, C. Manca, J. Chan, K. Lyashchenko, and M. L. Gennaro.** Three-step purification of lipopolysaccharide-free, polyhistidine-tagged recombinant antigens of *Mycobacterium tuberculosis*. Submitted for publication.
13. **Content, J., A. de la Cuvellerie, L. de Wit, V. Vincent-Levy-Frebault, J. Ooms, and J. D. Bruyn.** 1991. The genes coding for the antigen 85 complexes of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG are members of a gene family: cloning, sequence determination, and genomic organization of the gene coding for antigen 85-C of *M. tuberculosis*. Infect. Immun. **59:**3205–3212.
14. **Cooper, A. M., and J. L. Flynn.** 1995. The protective immune response to *Mycobacterium tuberculosis*. Curr. Opin. Immunol. **7:**512–516.
15. **Dobos, K. M., K. H. Khoo, K. M. Swiderek, P. J. Brennan, and J. T. Belisle.** 1996. Definition of the full extent of glycosylation of the 45-kilodalton glycoprotein of *Mycobacterium tuberculosis*. J. Bacteriol. **178:**2498–2506.
16. **Harboe, M., and H. G. Wiker.** 1992. The 38-kDa protein of *Mycobacterium tuberculosis*: a review. J. Infect. Dis. **166:**874–884.
17. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Hasløv, K., Å. Andersen, S. Nagai, A. Gottschau, T. Sørensen, and P. Andersen.** 1995. Guinea pig cellular immune responses to proteins secreted by *Mycobacterium tuberculosis*. Infect. Immun. **63:**804–810.
19. **Heym, B., Y. Zhang, S. Pulet, D. Young, and S. T. Cole.** 1993. Characterization of the *katG* gene encoding a catalase-peroxidase required for the isoniazid susceptibility of *Mycobacterium tuberculosis*. J. Bacteriol. **175:**4255–4259.
20. **Horn, C., P. Pescher, F. Romain, and G. Marchal.** 1996. Characterization of murine monoclonal antibodies specific for the 45/47 kDa antigen (APA) of *Mycobacterium tuberculosis*, *M. bovis* and BCG. J. Immunol. Methods **197:** 151–159.
21. **Horwitz, M. A., B.-W. E. Lee, B. J. Dillon, and G. Harth.** 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **92:** 1530–1534.
22. **Hubbard, R. D., C. M. Flory, and F. M. Collins.** 1992. Immunization of mice with mycobacterial culture filtrate proteins. Clin. Exp. Immunol. **87:**94–98.
23. **Katz, S. S., K. Chen, S. Chen, M. E. Doerfler, P. Elsbach, and J. Weiss.** 1996. Potent CD14-mediated signalling of human leukocytes by *Escherichia coli* can be mediated by interaction of whole bacteria and host cells without extensive prior release of endotoxin. Infect. Immun. **64:**3592–3600.
24. **Laqueyrerie, A., P. Militzer, F. Romain, K. Eiglmeier, S. Cole, and G. Marchal.** 1995. Cloning, sequencing, and expression of the *apa* gene coding for the *Mycobacterium tuberculosis* 45/47-kilodalton secreted antigen complex. Infect. Immun. **63:**4003–4010.
25. **Lim, E. M., J. Rauzier, J. Timm, G. Torrea, A. Murray, B. Gicquel, and D. Portnoi.** 1995. Identification of *Mycobacterium tuberculosis* DNA sequences encoding exported proteins by using *phoA* gene fusions. J. Bacteriol. **177:** 59–65.

25a. **Lyashchenko, K., R. Colangeli, and M. L. Gennaro.** Unpublished data.

26. **Mahairas, G. G., P. J. Sabo, M. J. Hickey, D. C. Singh, and C. K. Stover.** 1996. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. J. Bacteriol. **178:**1274–1282.
27. **Manca, C., K. Lyashchenko, H. G. Wiker, D. Usai, R. Colangeli, and M. L. Gennaro.** 1997. Molecular cloning, purification, and serological character-

ization of MPT63, a novel secreted antigen of *Mycobacterium tuberculosis*. Infect. Immun. **65:**16–23.
28. **Matsumoto, S., T. Matsuo, N. Ohara, H. Hotokezaka, M. Naito, J. Minami, and T. Yamada.** 1995. Cloning and sequencing of a unique antigen MPT70 from *Mycobacterium tuberculosis* H_{37}Rv and expression in BCG using *E. coli*-Mycobacteria shuttle vector. Scand. J. Immunol. **41:**281–287.
29. **Miura, K., S. Nagai, M. Kinomoto, S. Haga, and T. Tokunaga.** 1983. Comparative studies with various substrains of *Mycobacterium bovis* BCG on the production of an antigenic protein, MPB70. Infect. Immun. **39:**540–545.
30. **Oettinger, T., A. Holm, I. M. Mtoni, Å. B. Andersen, and K. Hasløv.** 1995. Mapping of the delayed-type hypersensitivity-inducing epitope of secreted protein MPT64 from *Mycobacterium tuberculosis*. Infect. Immun. **63:**4613–4618.
31. **Ohara, N., H. Kitaura, H. Hotokezaka, T. Nishiyama, N. Wada, S. Matsumoto, T. Matsuo, M. Naito, and T. Yamada.** 1995. Characterization of the gene encoding the MPB51, one of the major secreted protein antigens of *Mycobacterium bovis* BCG, and identification of the secreted protein closely related to the fibronectin binding 85 complex. Scand. J. Immunol. **41:**433–442.
32. **Roberts, A. D., M. G. Sonnenberg, D. J. Ordway, S. K. Furney, P. J. Brennan, J. T. Belisle, and I. M. Orme.** 1995. Characteristics of protective immunity engendered by vaccination of mice with purified culture filtrate protein antigens of *Mycobacterium tuberculosis*. Immunology **85:**502–508.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Sørensen, A. L., S. Nagai, G. Houen, P. Andersen, and Å. B. Andersen.** 1995. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. Infect. Immun. **63:**1710–1717.
35. **Thole, J. E. R., L. F. E. M. Stabel, M. E. G. Suykerbuyk, M. Y. L. De Wit, P. R. Klatser, A. H. J. Kolk, and R. A. Hartskeerl.** 1990. A major immunogenic 36,000-molecular-weight antigen from *Mycobacterium leprae* contains an immunoreactive region of proline-rich repeats. Infect. Immun. **58:**80–87.
36. **Watson, M. E. E.** 1984. Compilation of published signal sequences. Nucleic Acids Res. **12:**5145–5164.
37. **Williamson, M. P.** 1994. The structure and function of proline-rich regions in proteins. Biochem. J. **297:**249–260.
38. **World Health Organization.** 1996. WHO report on the tuberculosis epidemic. World Health Organization, Geneva, Switzerland.
39. **Yamaguchi, R., K. Matsuo, A. Yamazaki, C. Abe, S. Nagai, K. Terasaka, and T. Yamada.** 1989. Cloning and characterization of the gene for immunogenic protein MPB64 of *Mycobacterium bovis* BCG. Infect. Immun. **57:**283–288.
40. **Young, D. B., S. H. E. Kaufmann, P. W. M. Hermans, and J. E. R. Thole.** 1992. Mycobacterial protein antigens: a compilation. Mol. Microbiol. **6:**133–145.

Editor: S. H. E. Kaufmann