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Characterization of a Novel *Mycobacterium bovis* Secreted Antigen Containing PGLTS Repeats

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Serum from naturally infected cattle was used to identify a novel *Mycobacterium bovis* antigen from an expression library. The first recombinant product identified was a fusion protein with *lacZ* (55 kDa). A clone containing the whole gene was also obtained. This clone expressed a 38-kDa protein. A rabbit serum against the recombinant antigen reacts in *M. bovis* supernatants with two proteins of 36 and 34 kDa. The new protein was called P36/P34. The gene cloned has a deduced amino acid sequence with a predicted molecular mass of 28 kDa, showing a characteristic signal sequence for exportation. The protein bears partial homology to a 28-kDa protein from *M. leprae*. An interesting feature of the P36/P34 sequence is that it contains several PGLTS repeats, which are not present in the *M. leprae* protein. Antigenic determinants seem also to be conserved between the two proteins because sera from leprosy patients recognized the recombinant *M. bovis* protein. The discrepancy among the molecular mass deduced from the sequence (28 kDa), that of the recombinant protein in *Escherichia coli* (38 kDa), and that of the native protein in *M. bovis* (36 and 34 kDa) could be attributed to posttranslational modifications or to the high proline content that may alter the migration properties of the protein. This antigen seems to be immunodominant during bovine tuberculosis, because 8 of 9 serum specimens from diseased cattle are reactive. The homology among the *M. leprae* 28-kDa protein, the protein described in this article, and a recently described *M. tuberculosis* protein suggests the existence of a new protein family in mycobacteria.

Mycobacterium bovis is the causative agent of zoonotic tuberculosis (28). *M. bovis* and *M. tuberculosis* are highly related bacteria. Together with *M. africanum* and *M. microti* they form a taxonomic group called the *M. tuberculosis* complex (24).

The extremely long doubling time and the pathogenicity of these bacteria have delayed for years the identification of antigens and virulence factors of mycobacteria. Application of both molecular biology and immunological tools, such as cloning and monoclonal antibodies, have recently led to important progress in knowledge of *M. tuberculosis* and *M. leprae* antigens and about cellular and humoral immune responses against them (13, 33). 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 (10, 31).

Purification and characterization of individual antigenic proteins are essential for understanding the pathogenic mechanisms of mycobacteria and the immune response against them. This may also contribute to an understanding of the pathogenesis of other intracellular bacteria with which cellular immunity is also involved.

In contrast to the situation with *M. tuberculosis*, little work has been performed on the characterization of *M. bovis* antigens (32). Most antigens were found by searching *M. bovis* BCG genomic expression libraries with monoclonal antibodies. Secreted protein antigens such as MPB64, MPB70, and antigen 85 were characterized (9, 11, 12, 22), and it was shown that they are recognized by sera from infected cattle (7). While most antigens are also present in *M. tuberculosis*, MPB70 is an *M. bovis*-specific antigen (22). Many of these findings have been made by studying *M. bovis* BCG, but little is known about antigens produced by pathogenic *M. bovis*. In recent articles, the main *M. bovis* antigens recognized by cattle are described (4, 8).

Despite the extensive use of monoclonal antibodies in the screening of genomic libraries to identify antigens, there are few examples of the use of sera from naturally infected hosts (5).

This work deals with the characterization of a novel *M. bovis*-secreted antigen, which was identified by using sera from cattle suffering from tuberculosis.

MATERIALS AND METHODS

Bacterial strains, media, and cloning vectors. Two *M. bovis* isolates from animals, obtained from local bacteriology services, were used to obtain DNA to prepare the genomic library. *M. bovis* AN5 was used to prepare whole-cell extracts and culture supernatants. Mycobacteria were cultivated in Stonebrink or Dubos medium. *Escherichia coli* XL blue1 (Stratagene, La Jolla, Calif.) was used as the main recipient for recombinant plasmids and was grown in LB medium (25). Lambda ZAP phage and pBluescript KSII (Stratagene) were used as cloning vectors.

Mycobacterial DNA preparation. Preparation of mycobacterial DNA was done according to the method of van Soolingen et al. (30).

Construction of an *M. bovis* expression library. *M. bovis* DNA from a wild-type local isolate (6 μg) was partially digested with *Eae*I (1 h, 37°C, 1 U), an enzyme with frequent recognition sites in mycobacterial DNA. Fragments ranging from 1.5 to 8 kb were prepared from the agarose gel by electrodialysis, resuspended in ligase buffer, and ligated to dephosphorylated *Not*I-digested lambda ZAP arms (*Not*I and *Eae*I leave compatible ends). Pilot ligations with 1 μg of vector were done to determine the optimal vector/insert ratio. We chose a 6/1 (by mass) vector/insert ratio. The ligation mixture was scaled up to 1 μg of vector and packaged in vitro. *E. coli* XL blue 1 was then infected and plated on LB agar. The

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primary library contains $3 \cdot 10^5$ PFU/ml. The library was amplified to 10^8 PFU/ml.

Library screening. The bovine pooled sera were first absorbed from *E. coli* antibodies as described by Young et al. (33). Phages ($5 \cdot 10^4$) were plated on a bioassay dish (24 by 24 cm; Nunc) and incubated for 5 h at 37°C. Plates were overlaid with a nitrocellulose filter soaked in 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated 2 h more at 37°C. Filters were stripped and blocked with milk-TBS (100 mM Tris HCl [pH 9.5], 100 mM NaCl, and 5 mM $MgCl_2$) for 30 min at 37°C. Bovine sera (first antibody, 1/100 dilution) were added, and the membranes were incubated for 2 h at 37°C. After being rinsed with TBS, filters were incubated for 2 h at 37°C with alkaline phosphatase-conjugated anti-bovine immunoglobulin G (secondary antibody). Color was allowed to develop for 30 min by the addition of 5-bromo-4-chloro-3-indolylphosphate and toluidinum nitroblue tetrazolium as substrates.

Extract and supernatant preparation. *E. coli* cultures were induced for 2 h by addition of IPTG up to 5 mM. Cells were harvested by centrifugation and resuspended in loading buffer (2% sodium dodecyl sulfate [SDS], 0.125 M Tris HCl [pH 6.8], 1% 2-mercaptoethanol, 0.02% bromophenol blue, 10% glycerol). The extract was obtained by boiling for 3 min. *M. bovis* culture supernatant and sonic extracts were obtained as previously described (4).

Western blots (immunoblots). Proteins (50 μg) were developed by electrophoresis in 12% polyacrylamide–SDS gels by the technique of Laemmli (15) and electrotransferred onto a nitrocellulose sheet by the semidry method (14). Transfer yield was visualized by transient staining with Ponceau Rouge. The membranes were incubated with the first antibody (when directed against *E. coli* antigens, the sera were absorbed from *E. coli* antibodies) overnight at 4°C and with an alkaline phosphatase-conjugated secondary antibody for 2 h at 37°C. The color reaction was then performed as described for library screening.

Sera. Cattle sera were obtained from cattle with macroscopical lesions from which *M. bovis* had been isolated as already described (3); a pool of five of these serum specimens was used for library immunoscreening. This pool was used at a 1/100 dilution.

Rabbit immunization with *M. bovis* antigens was done as follows. *E. coli* recombinant extracts (100 μg) were loaded in a large 10-cm-diameter well and developed on an SDS-polyacrylamide gel electrophoresis (PAGE) gel. The gel region where the recombinant band migrates (as determined by Western blot) was excised. The gel strip was ground, mixed with Freund incomplete adjuvant, and injected in three doses into one rabbit. The doses were given at 2-week intervals. Sera from leprosy patients were obtained from A. Segal-Eiras of the School of Medicine, La Plata University, La Plata, Argentina.

Sequencing. Plasmid DNA prepared by the Wizard Minipreps kit (Promega Biotec) was used as a template. Sequencing was performed by using T3, T7, and sequence-deduced primers that initiated dideoxy-nucleotide chain termination reactions (26). The TaqTrack sequencing kit (Promega Biotec) with deaza analogs and polyacrylamide gels with 40% formamide were used. Sequencing was also performed with clones obtained by ExoIII digestions (2).

Molecular genetics procedures. Standard procedures (2, 25) were used for the preparation of plasmid DNA, restriction enzyme digestions, ligations, and ExoIII digestions.

Sequence analysis. Sequence analyses were performed by using DNA Strider 1.2 (17) software. Nucleotide sequence searches were performed by using MacVector software with the Entrez (National Center for Biotechnology Information, Bethesda, Md.) database. Homology determinations were performed with DNASIS software (Hitachi Corp.).

Nucleotide sequence accession number. The nucleotide sequence of the gene encoding P36/P34 has been submitted to the EMBL sequence database under accession no. Z48749.

RESULTS

Selection and characterization of recombinant phages. To identify *M. bovis* antigens recognized by sera from infected cattle, an *M. bovis* expression library was constructed. A pool of five serum specimens from infected cattle was used for library immunoscreening. When 20% of the library was screened, we found a clone giving an intense signal. This clone was termed λ4-1. The recombinant plasmid contained in the λ4-1 clone was obtained by the lambda ZAP automatic plasmid excision method (27). This plasmid, which was termed pMBA41, has an insert size of 0.8 kb (Fig. 1). *E. coli* XL blue1(pMBA41) yielded a blue color on IPTG–5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (XGal) plates, suggesting an in-frame fusion with the α fragment of the vector β-galactosidase. The expression of pMBA41 recombinant product is induced by IPTG (data not shown). The promoter seems to be inactive in *E. coli*, because when the insert was oriented in the opposite direction, no expression could be observed (data not shown).

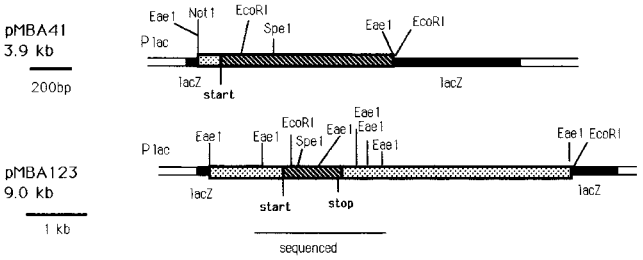

FIG. 1. Restriction maps of inserts of pMBA41 and pMBA123. The *M. bovis* inserts are depicted as thick boxes. The putative P36/P34 gene is depicted by hatched boxes, and the rest of the *M. bovis* DNA is depicted by dotted boxes. The vector is depicted by thinner boxes; *lacZ* sequences are depicted by black boxes, and the rest of the vector is depicted as open white boxes. The regions of the putative start GTG and stop codons are shown. The sequenced segment is indicated by a line.

Characterization of the recombinant protein. The size of the recombinant protein encoded by clone λ4-1 was determined by Western blotting with the bovine pooled sera. These sera recognized not a single band but several bands ranging from 55 to 20 kDa (Fig. 2, lane 1). To observe if these multiple bands were due to proteolysis, pMBA41 was transformed into *E. coli* CAG1139, a *lon* strain that lacks a major *E. coli* protease (21). Degradation decreased, but the expression level was reduced as well (Fig. 2, lane 4). The molecular mass (55 kDa) of the recombinant protein also suggests that a fusion was formed between the *M. bovis* protein and the α fragment of the β-galactosidase.

Identification of protein in *M. bovis*. To determine the molecular weight and the localization of the native protein in *M. bovis*, a specific serum was obtained in rabbits. The gel region where the recombinant antigen migrates was excised and injected into rabbits. This antiserum (anti-P55) reacted with a protein doublet of 36 and 34 kDa in culture supernatants (Fig. 2, lane 6). The protein was thus termed P36/P34. No protein was detected in cell extracts (Fig. 2, lane 7). P36/P34 seems not to be a major protein because bands of 36 and 34 kDa were not visible in Coomassie blue-stained gels. When directed against *E. coli* XL blue1(pMBA41) extracts, the anti-P55 serum yields a band pattern identical to that obtained with the bovine pooled sera (Fig. 2, lane 3), indicating that the rabbit and bovine antibodies recognized the same protein.

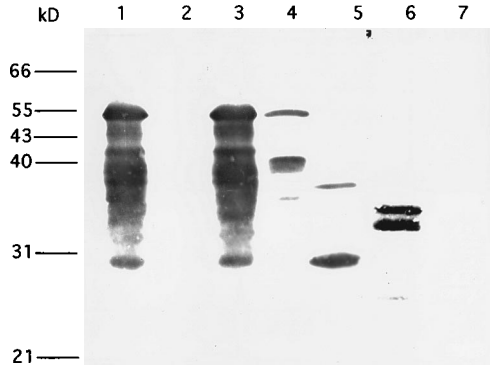

FIG. 2. Western blot. Lane 1, *E. coli* XL blue1(pMBA41); lane 2, *E. coli* XL blue1(pBluescript SKII) (negative control); lane 3, *E. coli* XL blue1(pMBA41); lane 4, *E. coli* CAG1139(pMBA41); lane 5, *E. coli* XL blue1(pMBA123); lane 6, *M. bovis* culture supernatant; lane 7, *M. bovis* whole-cell extract. Rabbit anti-P55 serum was used as first antibody in lanes 2 to 7. A pool of sera from infected cattle was used as first antibody in lane 1. The numbers on the left are molecular sizes in kilodaltons.

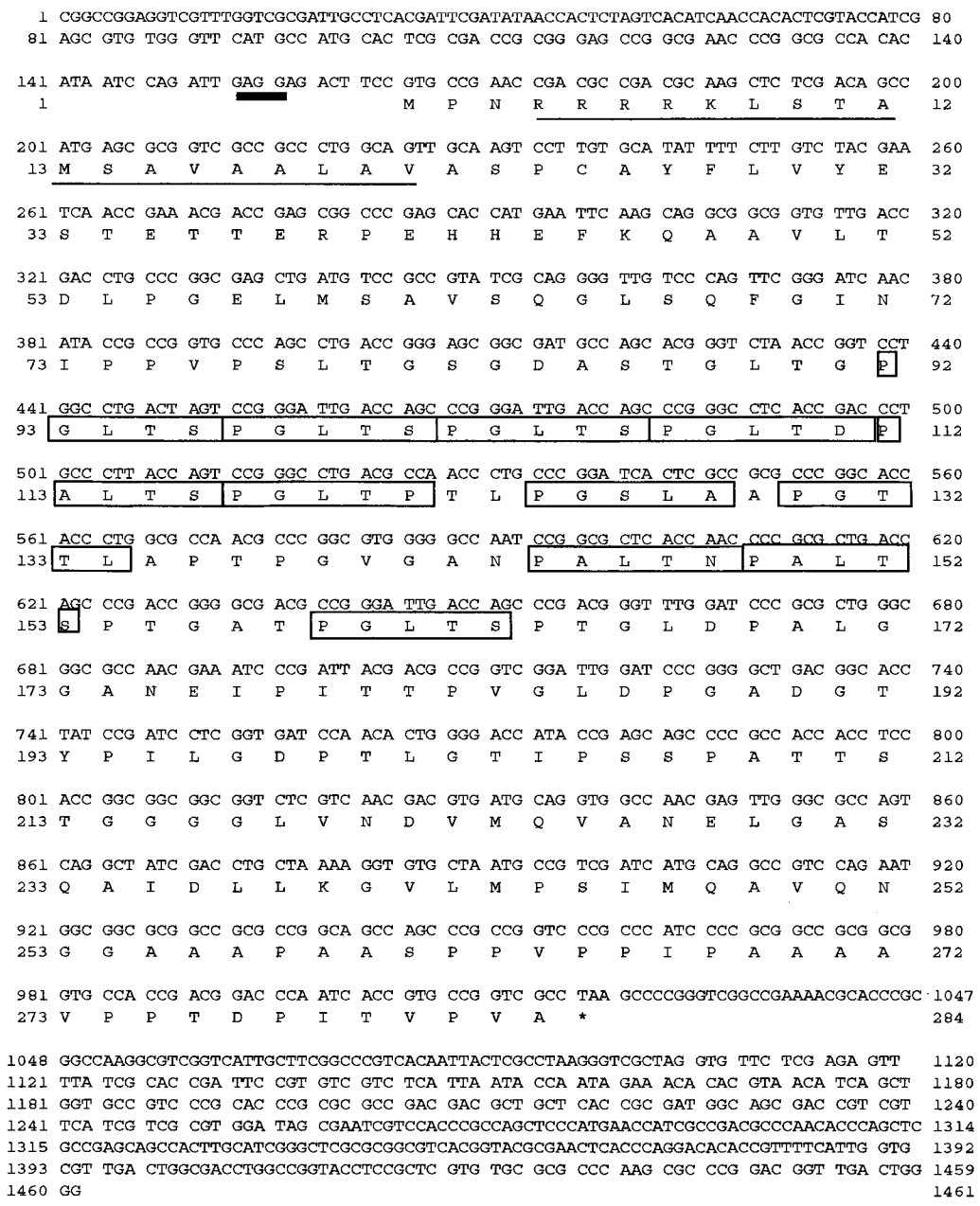

FIG. 3. Nucleic acid sequence of the P36/P34 coding region and deduced amino acid sequence. The sequences which resemble the *E. coli* ribosomal binding site (thick line) and signal sequences (thin lines) are indicated. Numbers indicate base pairs and amino acid positions. The asterisk indicates the termination codon. PGLTS and related amino acid sequences are shown boxed.

A clone with the entire coding region. With further library screening, another positive clone (λ12-3) with a larger insert (6 kb) was obtained. The recombinant plasmid of λ12-3 was then obtained and termed pMBA123. The overlapping restriction maps of pMBA123 and pMBA41 (Fig. 1), and the reactivity of the protein encoded by pMBA123 with the anti-P55 antiserum, indicate that pMBA123 contains an insert extending to both ends of pMBA41. pMBA123 must contain the entire P36/P34 coding region. This clone yields white color on IPTG–X-Gal plates. A protein of 38 kDa is recognized by the anti-P55 antiserum in *E. coli* XL blue1(pMBA123) extracts (Fig. 2, lane 5). This result reasonably agrees with the molecular mass of the protein detected in *M. bovis* (36 and 34 kDa). Some degradation of this protein was also observed.

Determination and analysis of the nucleotide sequence. The nucleotide sequence of the P36/P34 coding region was determined by using pMBA123 and pMBA41 as templates (Fig. 3). An open reading frame comprising 852 bp and extending from a putative GTG start (position 165) and ending at a TAA stop codon (position 1016) was selected as the putative gene. The overall G+C content is 68%. The molecular mass of the deduced protein is 27,669 Da, and it comprises 284 amino acids (aa). The selected direction of transcription and the reading phase of the P36/P34 putative coding gene are the same as

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  1 MPNRRRRKLSTAMSAVAALAVASPCAYFLVYESTETTERPEH-H-EFKQA      50
    |||||| ||||| | || || |||||||||||| |     |   | |||||
  1 MPNRRRCKLSTAISTVATLAIASPCAYFLVYEPTASAK-PAAKHYEFKQA      50

 51 AVLTDLPGE-LMSAVSQGLSQFGINIPPVPSLTGSGDASTGLTGPGLTSP     100
    |   ||||| |  | ||||||||||| ||||||||  |       ||
 51 ASIADLPGEVLD-AISQGLSQFGINLPPVPSLTGTDD-------PGN---     100

101 GL-TSPGLTSPGLTDPALTSPGLTPTLPGSLAAPGTTLAPTPGVGANPAL     150
    || | |||||| ||   |   | || |    | |||       |       |
101 GLRT-PGLTSPDLTNQEL---G-TPVLT---A-PGT------G------L     150

151 TNPALTSP-TGATPGLTSPTGLDPALGGAN--EIPITTPVGLDPGADGTY     200
    | |    | ||  |  | |  |   |||    | |||||  |||| ||||
151 T-P----PVTGS-PICTAPD-LN--LGGTCPSEVPITTPISLDPGTDGTY     200

201 PILGDP-TLG-TIPSSPATTSTGGGGLVNDVMQVANELGASQAIDLLKGV     250
    |||||| ||| | | |  | | |   ||||   ||| |||||  || |||
201 PILGDPSTLGGTSPIS--TSS-GE--LVNDLLKVANQLGASQVMDLIKGV     250

251 LMPSIMQAVQNGGAAAPAASPPVPPIPAAAAVPPTDPITVPVA.......     300
     ||  || ||||  |    |  | |  ||       |  ||
251 VMPAVMQGVQNGNVAGDL-SGSVTP--AAISL-----I--PVT.......     300
```

FIG. 4. Comparison of primary structure of *M. bovis* P36/P34 (top sequence) with the primary structure of the 28-kDa protein from *M. leprae* (bottom sequence). Identical residues are indicated by vertical lines. The region containing the PGLTS repeats is underlined. The numbers on the left and right indicate amino acid positions in the respective proteins.

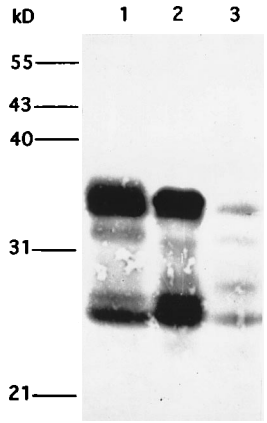

FIG. 5. Western blot of culture supernatants from different species of the *M. tuberculosis* complex. Lanes 1, *M. bovis* AN5; lane 2, *M. bovis* BCG; lane 3, *M. tuberculosis* H_{37}Rv. Rabbit anti-P55 serum was used as first antibody. Molecular masses of markers are indicated on the left.

those of the *lacZ* reading frame in pMBA41. In the other phases there are no other long open reading frames. The proposed start codon is homologous to that of the *M. leprae* 28-kDa protein gene (5) (see below). Two other putative start codons are present in the same phase in which the proposed start codon is located, GTG at position 84 and ATG at position 99, but in both cases the translated products are devoid of signal sequences. The sequence of the insert of pMBA41 confirms the fusion with *lacZ*. There is a characteristic signal sequence (5, 18) comprising the first 4 charged aa followed by 14 nonpolar aa and then a probable cleavage site for the signal peptidase. The signal sequence extends from R (aa 4) to A (aa 22). A typical ribosomal binding site motif (5) (GAGG) occurs 12 bp upstream from the GTG start codon.

A search for homologous sequences in the Entrez database was performed. Homology to the gene of a 28-kDa protein from *M. leprae* was found (5). The overall homology is 52%. The N-terminal and C-terminal portions show greater homology, 72 and 64%, respectively. While the sequence is essentially conserved at both the C-terminal and N-terminal regions (Fig. 4), in the central segment, 11 repeated PGLTS peptides are present in the *M. bovis* P36/P34 protein but not in the *M. leprae* 28-kDa protein. Four pentapeptides match exactly, while the others are degenerated. A search in PROSITE (3) did not reveal any homology to PGLTS (data not shown). A search for other homologous proteins showed that besides the *M. leprae* 28-kDa protein, proteins showing similarities were chicken elastin and human salivary mucin.

P36/P34 localization in *M. tuberculosis* and *M. bovis* BCG. The presence of the P36/P34 protein in other members of the *M. tuberculosis* complex was studied by Western blot. We used culture supernatants from 3-week cultures. The anti-P55 serum reacted with two protein bands of 36 and 34 kDa in *M. bovis* BCG, *M. tuberculosis* H_{37}Rv, (Fig. 5), and an *M. bovis* bovine isolate (data not shown). *M. tuberculosis* bands were less intense, although an equal amount of protein was loaded in the *M. tuberculosis* lane, as shown by Coomassie blue staining (data not shown). Some P36/P34 proteolysis was also observed in the three supernatants, although to a lesser extent than in *E. coli* XL blue1(pMBA41).

Recognition by sera from *M. bovis*-infected cattle. A panel of nine serum specimens from infected cattle and five serum specimens from healthy cattle was absorbed from anti-*E. coli* antibodies and directed against *E. coli* CAG1139(pMBA41) cell extracts in Western blots to determine the frequency of recognition by sera from infected cattle. Eight of nine serum specimens from infected cattle recognized the recombinant antigen, as shown by the reaction with the 55-kDa recombinant band (Fig. 6). Some proteolytic products are also detected, especially two major bands of 40 and 38 kDa. These major proteolytic products are produced even in *E. coli* CAG1139 (Fig. 2, lane 4). Three of four serum specimens from healthy cattle did not show recognition. The other one (Fig. 6, lane 3) reacted against the 55-kDa recombinant band (not readily visible in the figure). A band of 58 kDa was detected in several lanes; this band is from *E. coli*, because it was detected in CAG1139(pBluescript) cell extracts (data not shown). No additional bands were detected in CAG1139(pBluescript) cell extracts.

Recognition by sera from leprosy patients. In order to determine if antigenic determinants are conserved between *M. bovis* P36/P34 and the *M. leprae* 28-kDa protein, the reactivities of five serum specimens from patients suffering from lepromatous leprosy were also studied. Three serum specimens reacted with bands corresponding to the 55-kDa protein and also against the degradation products (Fig. 7). The other visible

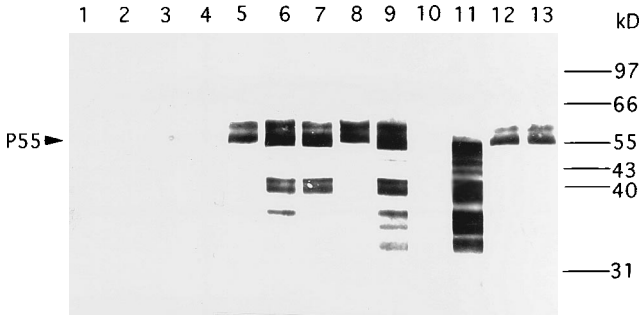

FIG. 6. Western blot of recombinant P36/P34 with individual sera from *M. bovis*-infected and healthy cattle. *E. coli* CAG1139(pMBA41) extract was loaded onto an SDS-PAGE gel and transferred to nitrocellulose. Lanes 1 to 4, five different serum specimens from healthy cattle; lanes 6 to 13, different serum specimens from infected cattle. The arrowhead denotes the position of P55. Molecular masses of markers are indicated on the right.

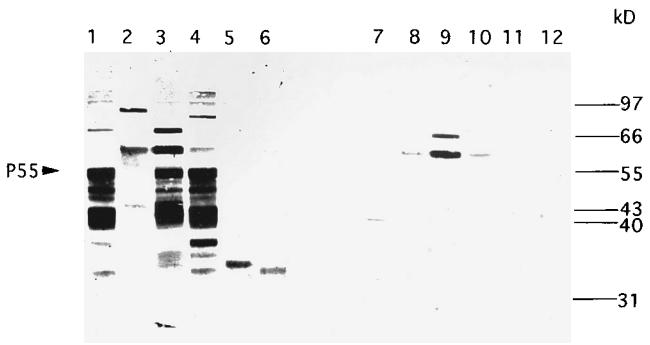

FIG. 7. Western blot of recombinant P36/P34 with individual sera from human leprosy patients. *E. coli* CAG1139(pMBA41) (lanes 1 to 6) and *E. coli* CAG1139(pBluescript SKII) (lanes 7 to 12) extracts were loaded onto an SDS-PAGE gel and transferred to nitrocellulose. Lanes 1 to 5 and 7 to 11, different sera from leprosy patients; lanes 6 and 12, serum from a healthy individual. The arrowhead denotes the position of P55. Molecular masses of markers are indicated on the right.

bands were also present in *E. coli*(pBluescript) extract lanes, indicating an incomplete absorption of the sera.

DISCUSSION

We used sera from infected cows to clone the gene of a novel *M. bovis* antigen. From a lambda ZAP library, two clones were characterized; one of them, λ4-1, encodes a form of the antigen that lacks the C-terminal end and that is fused to the α fragment of the β-galactosidase. The fused protein of λ4-1 has a molecular mass of 55 kDa and is strongly recognized by the sera of the infected cattle. The promoter seems to be inactive in *E. coli*, because when the insert of the coding plasmid (pMBA41) was oriented in a direction opposite to the *lacZ* promoter, no expression was observed. The 55-kDa protein is highly unstable because of proteolysis. The transfer of pMBA41 to a *lon E. coli* strain reduced but did not abolish degradation. The other positive clone, λ12-3, is not fused and covers the complete coding region. The protein encoded by λ12-3 has a molecular mass of 38 kDa.

The identified gene has regions of extensive homology to that of the *M. leprae* 28-kDa protein described by Cherayil and Young (5). It is interesting that both proteins were identified by screening genomic libraries with sera from naturally infected hosts. The conserved regions occur in both ends of the protein. The middle portion of the *M. bovis* antigen contains 11 repeated PGLTS peptides, 4 of which are perfectly conserved while the others are degenerated. These pentapeptides are absent in the *M. leprae* 28-kDa protein. What is the functional and structural significance of this pentapeptide? The search for homologous sequences in the Entrez database demonstrated that, following the protein with the highest homology score, which is the *M. leprae* 28-kDa protein, the other proteins showing similarities were mainly mucins and elastins. However, with our present knowledge, it is difficult to speculate about the functional significance of this homology. The homology between the two proteins is further emphasized by the fact that sera from lepromatous leprosy patients react with the recombinant antigen.

The protein described here is a secreted one or a protein shed from the cell surface. On the other hand, the subcellular localization the *M. leprae* 28-kDa protein was not clearly established because of the difficulties in working with *M. leprae*. On the basis of its sequence, the authors postulated that the *M. leprae* 28-kDa protein is an integral membrane protein (5).

Lim et al. have recently cloned an *M. tuberculosis* gene homologous to that encoding the *M. leprae* 28-kDa protein by a new method for the identification of exported proteins (16). The sequence differs from the *M. bovis* protein by some amino acids at the signal sequence (16). The *M. tuberculosis* protein also has PGLTS repeats (21a). Taken together, these results suggest the existence of a new family of mycobacterial proteins.

A specific rabbit serum against the *E. coli* recombinant 55-kDa protein (anti-P55) reacts with a pair of proteins of 34 and 36 kDa in *M. bovis* supernatants. The extracellular localization is consistent with the signal peptide deduced from the nucleotide sequence. We don't know why two protein bands are present in *M. bovis* supernatants. Either the smaller protein could be a degraded product of the larger one or the two proteins could represent different stages of a posttranslational modification, as with MPB70 (7, 9, 12). The two proteins could also be different but share antigenic determinants, as with the antigen 85 complex. The determination of the N-terminal sequence of both proteins will distinguish between these possibilities. Tentatively, we designated this protein P36/P34.

P36/P34 is also produced in supernatants from *M. tuberculosis* $H_{37}Rv$, *M. bovis* BCG, and an *M. bovis* bovine isolate. In *M. tuberculosis*, less intense signals were observed. This could be attributed to a lower level of P36/P34 gene expression in *M. tuberculosis*, at least with the culture time and media used.

The immunodominance of P36/P34 during bovine tuberculosis infection is suggested by the fact that eight of the nine serum specimens from infected cattle tested reacted with the recombinant band. However, more sera should be tested to confirm these observations. The immunodominance is also suggested by the fact that clones expressing this protein or truncated forms of it were frequently found during the library screening (data not shown). This last characteristic was also observed with the *M. leprae* 28-kDa protein (5).

A protein of 28 kDa is deduced from the P36/P34 gene sequence. In *E. coli* a protein of 38 kDa is expressed. Finally, the anti-P55 serum reacted with a pair of proteins of 34 and 36 kDa in *M. bovis* supernatants. The reasons for these discrepancies are unknown, but even considering that SDS-PAGE is not an accurate method to estimate molecular mass, the discrepancies could be attributed to posttranslational modifications that take place in *M. bovis* (6) or to the high proline content that might provoke alterations in the migration properties of the protein (5, 23, 29). Similar deviations were observed with regard to the *M. leprae* 28-kDa protein. The DNA sequence-derived molecular mass of the *M. leprae* 28-kDa protein is 24 kDa (Cherayil and Young [5] stated 28 kDa, but our calculations with the Strider and DNASIS software packages gave a result of 24 kDa), and the proteins detected by specific antibodies are 28 kDa in *M. leprae* and 31 kDa *E. coli.*

Several lines of evidence indicate that secreted proteins from mycobacteria are essential in the protective immune response. First is the observation that living bacteria are more protective than dead bacteria (19), second is the fact that secreted proteins are the main target of the protective immune response (20), and third is the observation that secreted proteins may elicit by themselves a protective immune response (1). Secreted proteins in *M. bovis* are beginning to be studied, and we hope that further studies of the protein described here will contribute to the comprehension of the *M. bovis-M. tuberculosis*–host interaction.

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