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Heterologous Expression of a Gene Encoding a 35 kDa Protein of *Mycobacterium avium paratuberculosis* in *Escherichia coli*

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ABSTRACT

The full-length open reading frame coding for a potentially immunogenic 35 kDa protein of *Mycobacterium avium paratuberculosis* was generated using polymerase chain reaction technology. The gene was inserted in-frame into *Escherichia coli* expression plasmid pQE32. The resulting recombinant plasmid pPMP35 was transformed into *E. coli* M15. Analysis of the *E. coli* induced with isopropyl-β-D-thiogalactopyranoside revealed that the protein accumulated into the cytoplasm as insoluble inclusion bodies. The level of expression of the recombinant 35 kDa protein (P35) was more than 30% of the total protein of *E. coli* cells. Expression of the recombinant protein was confirmed by immunoblotting. The P35 reacted with a rabbit antiserum raised against a sonicate of *M. a. paratuberculosis*. The protein was also recognized by serum from a goat with clinical paratuberculosis. Further, a polyclonal antiserum against P35 recognized a 35 kDa band in a membrane fraction of *M. a. paratuberculosis*. Also, the protein provoked a significant skin reaction in outbred guinea pigs sensitized with *M. a. paratuberculosis*, as well as in those sensitized with *Mycobacterium avium*. The results indicate that the 35 kDa protein of *M. a. paratuberculosis* is a membrane protein, having a role in the cellular immune response.

Keywords: Mycobacterium avium paratuberculosis, 35 kDa protein, heterologous expression

Abbreviations: CAPS, 3-[cyclohexylamino]-1-propanesulphonic acid; DIG, digoxigenin; DTH, delayed-type hypersensitivity reaction; DTT, dithiothreitol; EMBL, European Molecular Biology Laboratory; His, histidine; IFA, incomplete Freund's adjuvant; IPTG, isopropyl β-D-thiogalactopyranoside; *lac*, lactose; LB, Luria–Bertani; ORF, open reading frame; P35, recombinant 35 kDa protein of *M. a. paratuberculosis*; PBS, phosphate-buffered saline, pH 7.4; PCR, polymerase chain reaction; PMSF, phenylmethylsulphonyl fluoride; PPD, purified protein derivative; pQE, plasmid QIA expression; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulphate; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; SCC, saline-sodium citrate (pH 7)

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* is an intracellular pathogen causing chronic enteric disease in cattle and other ruminant animals, known as Johne's disease (Cocito *et al*., 1994). The organism has also been associated with cases of Crohn's disease in humans (Loftus *et al*., 1998; Naser *et al*., 2000). Paratuberculosis, with its

worldwide occurrence, causes significant ecnomic losses to the livestock industry (Whipple *et al*., 1992; Whitlock, 1992). *M. a. paratuberculosis* infection generally remains subclinical and thus may remain unnoticed for years (Kreeger, 1991). About 10% of infected animals become clinically ill after a prolonged incubation period. At present, there is no easy and reliable means of diagnosis, treatment or prevention, so the disease continues to constitute a threat to livestock farming in all parts of the world.

The present basis for a definitive diagnosis of Johne's disease is the cultural isolation of the causative organism from tissues or faeces (Collins *et al*., 1990). Cultural procedures, however, require incubation periods of about 3–6 months (Gwozdz *et al*., 1997). In addition, strains infecting sheep may be extremely difficult to grow (Collins *et al*., 1993). Lack of suitable diagnostic tests has hindered the attempts to control the disease. This situation led to renewed interest in the development of improved diagnostic tests for paratuberculosis, particularly in an ELISA based on partially purified proteins or recombinant proteins of *M. a. paratuberculosis* (Sugden *et al*., 1989; De Kesel *et al*., 1993; Mutharia *et al*., 1997; El-Zaatari *et al*., 1997). However, as seroconversion occurs relatively late in the course of infection (Cocito *et al*., 1994), the use of serological tests is only helpful in confirmation of suspected clinical cases. During the early subclinical stages of infection, *M. a. paratuberculosis* elicits a cell-mediated immune response by the host that can be characterized by delayed-type (type IV) hypersensitivity (DTH) reactions, lymphocyte proliferation and production of cytokines by stimulated T lymphocytes (Stabel, 2000). Also, for the development of effective vaccines, it is important to know the T-cell epitopes relevant to protective immunity. Hence, as the identification of specific antigens recognized by T cells is required for quick and early diagnosis, and for vaccine development, efforts are under way to evaluate the immunoreactivity of various antigens of *M. a. paratuberculosis.*

Vaccination with a 35 kDa antigen of *Mycobacterium avium*, which stimulates T cells, was reported to protect against virulent infection (Martin *et al*., 2000). Recently, Banasure and colleagues (2001) reported the identification of a gene specific to *M. a. paratuberculosis*. In the present study, we describe the heterologous expression, purification and preliminary characterization of this 35 kDa protein of *M. a. paratuberculosis.*

MATERIALS AND METHODS

Bacterial strains and plasmid

M. a. paratuberculosis strain 316F and *M. bovis* AN5 were obtained from Centraal Diengenees Kundig Tieh Instituut, Lelystad, The Netherlands, and maintained at the Biological Products Division, IVRI, Izatnagar, India. *Mycobacterium avium* (human isolate) was obtained from the Mycobacterial Repository Centre, Jalma Institute for Leprosy, Agar (India). The mycobacteria were grown and maintained at 37°C on Middlebrook 7H10 agar (Difco Laboratories, Detroit, MI, USA) enriched with 0.1%

glycerol (v/v) and 10% oleic acid dextrose catalase (Difco). For the growth of *M. a. paratuberculosis*, an additional supplement of 2 mg/L mycobactin J (Allied Monitor, Fayette, MO, USA) was included.

Escherichia coli (strain M15, pREP4), supplied by Qiagen (Valencia, CA, USA), was grown at 37°C in Luria–Bertani broth containing kanamycin (25 μg/ml), as the strain carries a kanamycin-resistant plasmid.

The expression vector plasmid pQE32 was purchased from Qiagen. The plasmid contains a T5 promoter and a 6 × His-tag coding sequence at 5′ to the multiple cloning region. The plasmid also contains an ampicillin resistance marker.

PCR amplification and cloning

The gene encoding a 35 kDa protein of *M. a. paratuberculosis* was amplified by a polymerase chain reaction. A set of specific oligonucleotide primers, (forward) 5′-CCGAGCTCTGACGTCGGCTCAGAATG-3′ and (reverse) 5′-CCAAGCTTT-CACTTGTACTCATGGAAC-3′, based on the nucleotide sequence information for the 35 kDa protein of *M. a. paratuberculosis* (EMBL accession no. AJ250887) was used for specific amplification. The primers had, respectively, linkers at the 5′ end for *Sac*I and *Hin*dIII restriction endonuclease sites. Appropriate nucleotides were also included in the primers to obtain the specific insert in-frame with the 6 × His coding sequence of the pQE32 expression vector. The amplification reaction was performed in a 50 μl reaction volume containing 100 ng of *M. a. paratuberculosis* DNA; 5 μl of *Taq* DNA polymerase buffer (10 mmol/L Tris HCl pH 9.0, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$ and 0.01% (w/v) gelatin), 200 μmol of each dNTP; 0.5 μmol each of forward and reverse primer, and 1.5 units of *Taq* DNA polymerase. The final volume was made up with sterile distilled water and overlaid with two drops of mineral oil.

The reaction was carried out in a PTC-100 reactor (MJ Research Inc., Waltham, MA, USA) for 30 cycles; each cycle consisted of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. The amplified product was analysed by submarine gel electrophoresis (Genei, Bangalore, India) (Sambrook *et al*., 1989) on 1% agarose gel.

The amplified gene was purified from agarose gel using a QIAEXII gel extraction kit (Qiagen). For the purpose of cloning, about 2 μg of the PCR product was double digested with *Sac*I and *Hin*dIII, purified and ligated into the plasmid pQE32 expression vector. The recombinant clone, designated as pPMP35, was transformed into competent *E. coli* M15 cells. The *E. coli* harbouring the recombinant plasmid were screened by plating on LB agar (Sambrook *et al*., 1989) containing ampicillin (75 μg/ml) and kanamycin (25 μg/ml). Ampicillin- and kanamycin-resistant transformants were further screened by restriction enzyme analysis of the plasmids with *Hin*dIII and *Sac*I (Sambrook *et al*., 1989). Plasmid pUC18, digested with *Sau*3AI and *Taq*I, was used as the standard molecular weight marker for agarose gel electrophoresis (Genei).

DNA hybridization

Positive recombinant plasmids were digested with *Sac*I and *Hin*dIII enzymes and electrophoresed on 1% agarose gel. The gel was then denatured and blotted onto Hybond N^+ membrane (Amersham Biosciences, Amersham, Bucks, UK). Hybridization was carried out at 68°C, using the 35 kDa gene fragment, amplified by PCR and labelled with DIG (Boehringer Mannheim, Mannheim, Germany), as a probe. High-stringency washing was done at 68°C in 0.015 mol/L SSC and detection of the hybridization signals was carried out following the manufacturer's instructions.

Expression and localization of the recombinant 35 kDa protein

E. coli cells containing the recombinant pPMP35 plasmid were induced with 1.0 mmol/L IPTG for 4–6 h. The secretory, periplasmic and cytosolic fractions of the *E. coli* with the recombinant plasmid were separated following the procedures described by Simmons and Yansura (1996) and Connaris and colleagues (1999), with certain modifications. Briefly, the culture supernatant (secretory fraction) and the cells were separated by centrifugation at 4000*g* at 4°C for 15 min. The culture supernatant was then filtered through a 0.22 μm filter (Millipore, Bedford, MA, USA). The filtrate was precipitated with 30% (v/v) trichloroacetic acid and resuspended in PBS containing 10 mmol/L phosphate buffer, pH 7.4, and 150 mmol/L sodium chloride. The cellular pellet was resuspended in Tris-sucrose-EDTA (20 mmol/L Tris-HCl pH 8.0, 20% sucrose, 1 mmol/L EDTA) buffer and incubated on ice for 10 min. It was again centrifuged at 10 000*g* for 10 min and the material in the pellet was osmotically shocked by resuspending it in 1 mmol/L $MgCl_2$ in 20 mmol/L Tris-HCl (pH 8.0). The cellular material was then centrifuged for a further 10 min at 10 000*g*, resulting in a clear supernatant that constituted the periplasmic fraction, while the pellet represented the cytosolic fraction.

Similarly, the soluble and insoluble fractions were obtained following the procedure of Wingfield (1995). *E. coli* cells harbouring the recombinant plasmid pPMP35 were induced with IPTG and pelleted. The pellet was resuspended in PBS and sonicated. The crude sonicates of *E. coli* with the plasmid pPMP35 were centrifuged at 4000*g* for 15 min at 4°C, and the pellet was resuspended in isotonic lysis buffer (Tris-HCl 10 mmol/L, NaCl 140 mmol/L, $MgCl_2$ 0.5 mmol/L, $CaCl_2$ 1 mmol/L, DTT 1 mmol/L, EDTA 1 mmol/L, Triton X-100 0.5%, PMSF 1 mmol/L, pH 9.0) and incubated on ice for 15 min. The suspension was centrifuged again at 10 000*g* for 15 min to obtain the soluble (supernatant) and insoluble (pellet) fractions.

The fractions were subjected to 12.5% SDS-PAGE to indicate the localization and solubility of the expressed recombinant 35 kDa protein. The molecular size of the recombinant 35 kDa protein was determined by comparing it with the standard protein molecular weight marker (Genei).

Purification of recombinant 35 kDa protein

The purification of recombinant protein under denaturing conditions was carried out by single-step affinity chromatography using Ni-NTA (nickel–nitrilotriacetate) agarose (Qiagen), following the manufacturer's recommendations. The recombinant protein contains a polyhistidine tag at the N-terminus that allowed single-step purification by nickel affinity chromatography. Briefly, the induced cell suspension was disrupted by sonication in an ice bath, dissolved in lysis buffer (8 mol/L urea, 0.1 mol/L NaH_2PO_4, 0.01 mol/L Tris-HCl, pH 8.0) and centrifuged at 10 000*g* for 10 min. The supernatant was then equilibrated with Ni-NTA resin. The resin was washed 10 times with wash buffer (8 mol/L urea, 0.1 mol/L NaH_2PO_4, 0.01 mol/L Tris-HCl, pH 6.3) and the recombinant protein P35 was eluted with a similar buffer at pH 4.5. The fractions containing recombinant 35 kDa protein were extensively dialysed at 4°C against PBS, to renature the protein. The protein was analysed on an SDS-PAGE gel and the final concentration was determined spectrophotometrically (Lowry *et al.*, 1951). The protein solution was sterilized by filtration and aliquots were stored at –70°C, until used.

Antibody production

Four New Zealand White rabbits were obtained from the Laboratory Animals Resource Section, IVRI, Izatnagar, and allowed to acclimatize for a week before their use in the study. Standard guidelines for the care and use of laboratory animals were followed.

The 8-week-old rabbits were used to raise antibody against the recombinant P35 and also against a sonicate of native *M. a. paratuberculosis*. For each of the proteins, two rabbits were immunized subcutaneously with 150 μg of immunogen in incomplete Freund's adjuvant (IFA) (Genei) and boosters of 100 μg of the immunogens with IFA were given intramuscularly after 3 weeks and again 2 weeks later. The animals were bled 10 days after the second booster and the sera were separated. All the sera used in this study were preabsorbed with *E. coli* antigens following the procedure of Harlow and Lane (1988).

In brief, to isolate monospecific antibodies, P35 antigen was initially electrophoresed and transferred to nitrocellulose membrane. The specific protein band was excised from the blot and incubated with polyclonal rabbit serum diluted 1:3 in buffer I (0.1 mol/L NaH_2PO_4 and 0.01 mol/L Tris-HCl, pH 7.5) at 37°C, overnight. Following washing of the membrane in buffer I, the monospecific antibodies were eluted in buffer II (0.1 mol/L NaH_2PO_4 and 0.01 mol/L Tris-HCl, pH 6.3). These were further utilized in Western blotting experiments.

Goat serum was obtained from a goat with clinical paratuberculosis, confirmed by Ziehl–Nielsen staining of faecal smears and also by PCR amplification of the *M. a. paratuberculosis*-specific IS900 sequence (Collins *et al.*, 1989) from the faecal sample.

Subcellular fractionation of M. a. paratuberculosis

Cytoplasmic, cell wall and membrane fractions of *M. a. paratuberculosis* were obtained following the procedure of Hunter and colleagues (1990). The culture supernatant and cells were separated by centrifugation at 4000*g* for 15 min. The proteins in the culture supernatant were precipitated with 10% (v/v) trichloroacetic acid. The precipitated material was resuspended in 1/20 of the original volume of PBS containing 0.05% Tween-20, forming the secretory fraction. The cellular fraction was resuspended in 5 ml PBS and sonicated in an ice bath 10 times with 30 s pulses with intervening pauses of 10 s. RNase and DNase were added to the sonicate at concentrations of 100 μg/ml, kept overnight at 4°C and centrifuged at 27 000*g* for 30 min. The pellet so obtained was treated with 2% SDS at 56°C for 2 h to obtain the cell wall fraction, while the supernatant was again centrifuged at 100 000*g* for 2 h. The resultant pellet was resuspended in 1/20 of the original volume of PBS containing 0.05% Tween-20 and centrifuged again. The washed pellet was rich in membrane vesicles and represented the cytoplasmic membrane fraction of *M. a. paratuberculosis*. The supernatant from the 100 000*g* centrifugation contained the soluble cytoplasmic contents.

SDS-PAGE and western blotting

SDS-PAGE was carried out on lysates from *E. coli*, subcellular fractions of *M. a paratuberculosis*, sonicated antigens of *M. a. paratuberculosis*, and purified recombinant P35 in a vertical slab mini apparatus (Genei), using polyacrylamide gels run under denaturing conditions, as described by Laemmli (1970). The 12.5% separating and 4% stacking polyacrylamide gels contained 0.1% SDS. The protein content of each sample was estimated (Lowry *et al.*, 1951) and about 20 μg was boiled in an equal volume of 2× sample loading buffer prior to loading. Electrophoresis was carried out with Tris-glycine electrode buffer (Tris 25 mmol/L, glycine 250 mmol/L, 0.1% SDS pH 8.3) at 90 V for 2 h. The gels were stained overnight with Coomassie brilliant blue G 250 (Sigma, St Louis, MO, USA).

For western blotting, the proteins from the gels were transferred to nitrocellulose membranes (0.45 μm), using semidry electroblotting (Atto, Tokyo, Japan) at 0.8 mA/cm^2, following the method of Bjerrum and Schafter-Nielsen (1986). The blots were blocked with 2% skimmed milk powder in PBS-T buffer (PBS containing 0.1% Tween-20) for 2 h at room temperature. After then washing three times with PBS-T buffer, the membranes were incubated for 2 h at 37°C with rabbit anti-P35 protein antiserum (1:2000 in PBS) or 1:200 hyperimmune serum raised against sonicated antigens of *M. a. paratuberculosis*. Following further washing, the blots were incubated with a 1:500 dilution of HRP-labelled goat anti-rabbit IgG (Genei) for 1 h. The reactivity of P35 with the clinically positive goat serum (1:50 dilution) was recognized using a 1:500 dilution of a rabbit anti-goat HRP conjugate (Genei). Antigens were visualized on the blots by incubation with 0.02% diaminobenzidine suspended in PBS containing 0.03% hydrogen peroxide.

Deduced amino acid analysis

The deduced amino acid sequence of the gene encoding the 35 kDa protein from *M. a. paratuberculosis* (accession no. AJ250887) was analysed for hydrophobic domains according to the Kyte and Doolittle algorithm (Kyte and Doolittle, 1982) using Lasergene software (DNASTAR, Madison, WI, USA), and the transmembrane domain was predicted according to Rost and colleagues (1996) utilizing the facility available at URL (http://cubic.bioc.columbia.edu). These two programs analyse for the distribution of polar and apolar amino acid residues along the protein sequence.

N-terminal sequencing of the purified protein was performed by the Edman degradation technique using transfer of the protein for 2 h at 300 mA on PVDF membrane in CAPS buffer (10 mmol/L CAPS, pH 11.0; 10% methanol). The protein band was excised, stained with Coomassie blue and sequenced on a protein/peptide sequencer (Applied Biosystems, Branchburg, NJ, USA).

Measurement of delayed-type hypersensitivity

Twenty-four female guinea-pigs (8–10 weeks old) were divided into four groups each consisting of 6 animals. The animals in each group were subcutaneously immunized once with different heat-killed mycobacteria (2 mg/animal in PBS) mixed with sterile incomplete Freund's adjuvant (IFA) (1:1). Group 1 was immunized with heat-killed *M. a. paratuberculosis* (316F); group 2 with *M. avium*; group 3 with *Mycobacterium bovis* (AN5); and group 4 (control) with PBS-IFA alone. After 4 weeks, each animal was shaved on the back and given an intradermal injection of 10 μg of purified recombinant P35 solution in 0.1 ml PBS. In groups 1, 2 and 3, respectively, 10 μg of Johnin PPD, *M. avium* sonicate or tuberculin PPD was used to monitor for positive reactions. PBS was injected intradermally into all the animals as a negative control. The results of the local skin reactions (DTH) were read after 24 h by measuring two transverse diameters of the erythema, the mean ($\pm$SEM) of which is quoted in the results. Differences between the treatment means were assessed for significance by Student's *t*-test, at a significance level of $p < 0.05$.

RESULTS

PCR amplification and construction of recombinant pPMP35

A PCR product of 938 bp was obtained on amplification. Restriction digestion of the recombinant plasmid pPMP35 with *Hin*dIII and *Sac*I released an identical size of fragment on a 1% agarose gel (Figure 1). Upon Southern blot hybridization using a PCR-amplified 35 kDa gene probe labeled with DIG, a positive signal corresponding to the gene was detected.

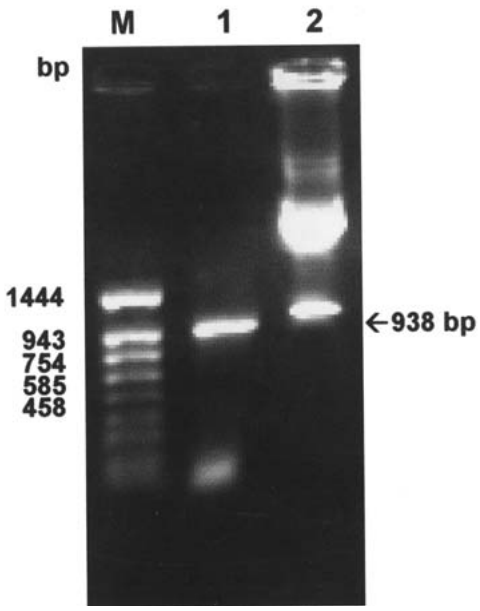

Figure 1. Agarose gel electrophoresis of cloned 35 kDa gene of *M. a. paratuberculosis*. Lane M, plasmid pUC 18 digested with *Taq*I and *Sau*3AI as DNA molecular size markers; lane 1, PCR-amplified 35 kDa gene; lane 2, released 35 kDa gene insert of 938 bp and linear pQE vector of 3.46 kb, upon digestion of pPMP 35 with *Sac*I and *Hin*dIII

Expression of recombinant 35 kDa protein in E. coli

When *E. coli* M15 cells harbouring the recombinant plasmid pPMP35 were induced with 1 mmol/L IPTG and analysed by 12.5% SDS-PAGE and Coomassie staining, it was observed that expression of the recombinant P35 protein could be detected as early as 30 min after induction. The expression increased steadily, with the maximum level of expression at 3–6 h after induction. After that, the level of expression remained almost constant. A predominant band of 35 kDa, corresponding to that predicted for the P35 fusion protein, was detected in the total cell extract of the *E. coli* (Figure 2, lane 3). The recombinant protein formed a major (more than 30%) portion of the total *E. coli* extract. No such protein band was observed with *E. coli* M15 cells or in uninduced *E. coli* M15 cells harbouring a recombinant plasmid pPMO35 (Figure 2, lanes 1 and 2).

Purification of the recombinant 35 kDa protein

The purified protein, when analysed by SDS-PAGE, showed a monomeric band of about 35 kDa size (Figure 2, lane 6). Purification of the recombinant protein was nearly 90%, as visualized on SDS-PAGE. The yield of the pure recombinant protein on

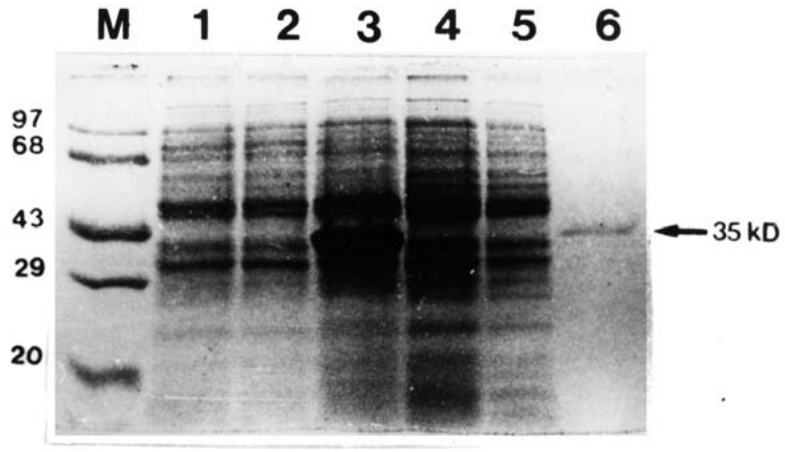

Figure 2. SDS-PAGE of Ni-NTA affinity purification of heterologously expressed *M. a. paratuberculosis* 35 kDa protein under denaturing conditions. Lane M, protein molecular weight marker; lane 1, whole-cell extract of non-recombinant *E. coli* M15; lane 2, whole-cell extract of uninduced recombinant *E. coli* M15; lane 3, IPTG-induced recombinant whole-cell extract of *E. coli* M15; lane 4, column flow-through; lane 5, column wash; lane 6, protein eluate

a laboratory scale was about 15–20 mg/L of culture. The N-terminal sequence of the recombinant protein was identical to the expected sequence.

Localization and solubility of the expressed recombinant 35 kDa protein.

Fractionation of the induced culture of *E. coli* M15 cells harbouring the recombinant plasmid pPMP 35 and analysis by 12.5% SDS-PAGE and Coomassie staining revealed that the expressed recombinant 35 kDa protein was present in neither the culture supernatant (secretory fraction) nor the periplasmic space but was accumulated in the cytosol (Figure 3). The analysis of the soluble and insoluble fractions of *E. coli* harbouring recombinant plasmid pPMP 35 showed that the recombinant protein was found in the insoluble fraction (Figure 4).

Immunoreactivity of the recombinant protein

The pooled polyclonal antiserum raised against P35 in rabbits could bind to recombinant protein as well as to the native protein from *M. a paratuberculosis* (Figure 5A). Furthermore, the P35 protein was recognized by the polyclonal serum against *M. a. paratuberculosis* sonicate and also by the serum from a goat naturally infected with *M. a. paratuberculosis* (Figure 5B and C).

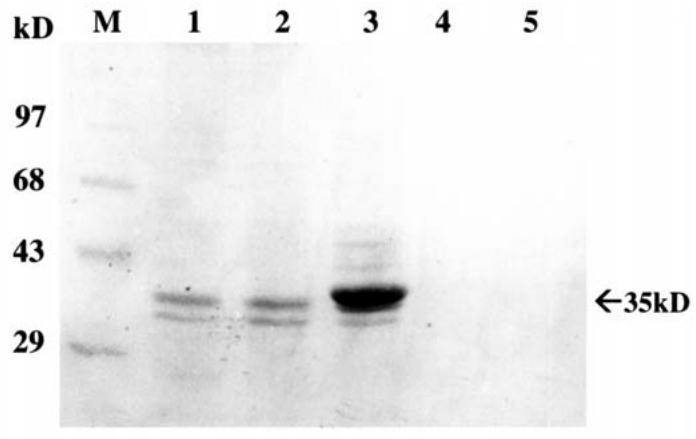

Figure 3. SDS-PAGE showing the localization of recombinant 35 kDa protein in *E. coli*. Lane M, protein molecular weight marker; lane 1, whole-cell extract of non-recombinant *E. coli* M15; lane 2, whole-cell extract of uninduced recombinant *E. coli* M15; lane 3, cytosolic fraction; lane 4, periplasmic fraction; lane 5, secretory fraction

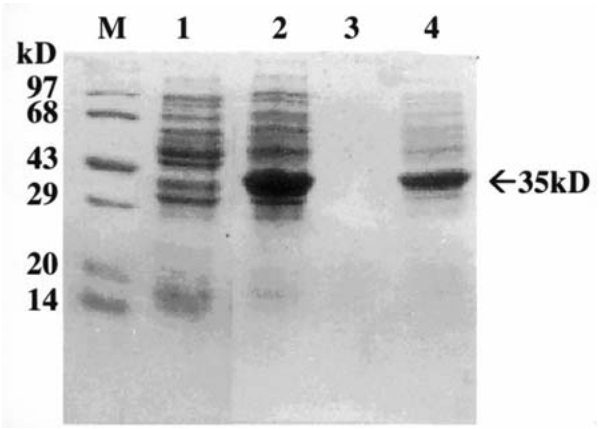

Figure 4. SDS-PAGE showing the insolubility of recombinant 35 kDa protein in *E. coli*. Lane M, protein molecular weight marker; lane 1, whole-cell extract of non-recombinant *E. coli* M15; lane 2, IPTG-induced recombinant whole cell extract of *E. coli* M15; lane 3, soluble fraction; lane 4, insoluble fraction

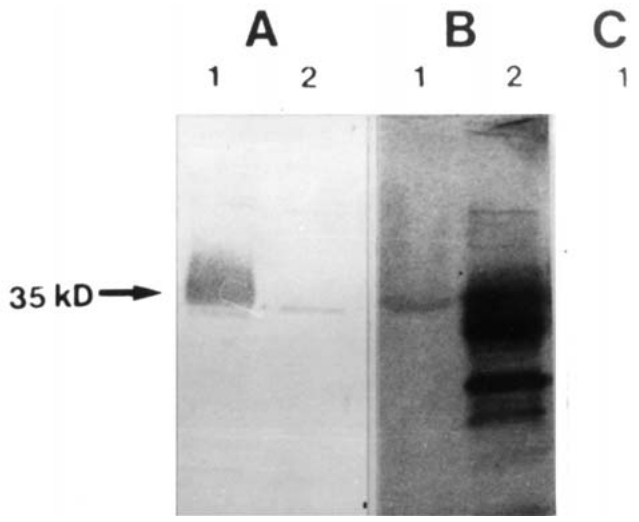

Figure 5. Immunoreactivity of recombinant 35 kDa protein. Lanes 1 and 2 indicate recombinant P35 and *M. a. paratuberculosis* (316F) sonicate, respectively. (A) Immunoblot using rabbit anti-P35 specific serum; (B) immunoblot using rabbit anti-*M. a. paratuberculosis* sonicate; (C) immunoblot using clinically positive goat serum

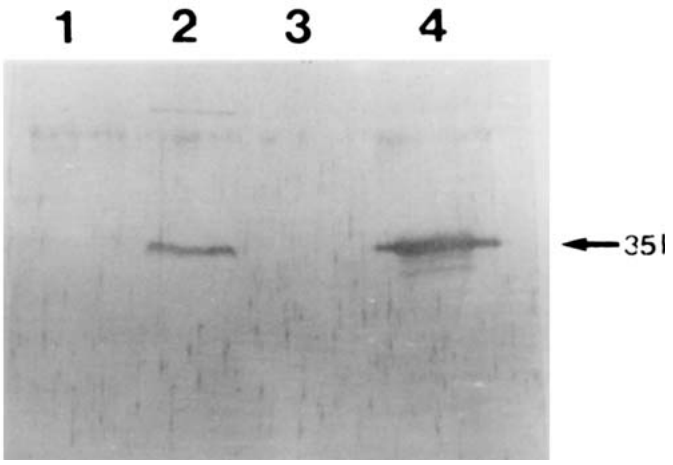

Figure 6. Immunoblot analysis of fractions of *M. a. paratuberculosis* (316F) cell extract by anti-P35 specific serum. Lane 1, cell wall fraction; lane 2, membrane fraction; lane 3, cytoplasmic fraction; lane 4, purified recombinant protein P35

Subcellular localization of 35 kDa protein in M. a. paratuberculosis

When the culture filtrate and sonicate fractions of *M. a paratuberculosis* were dotted on nitrocellulose paper and probed with anti-P35 polyclonal serum, only the *M. a paratuberculosis* sonicate reacted with the serum. Furthermore, upon immunoblotting of different subcellular fractions from *M. a paratuberculosis*, the anti-P35 polyclonal serum recognized a 35 kDa band in the membrane fraction (Figure 6).

Deduced amino acid analysis

Analysis of the deduced amino acid sequence (307 amino acids) of the *M. a paratuberculosis* 35 kDa protein, indicated that the protein contained five major hydrophobic regions (amino acids 33–50, 179–190, 199–210, 212–220 and 269–299). Furthermore, it was predicted to contain a transmembrane helix (amino acids 269–286), with an N-terminal outside region and a C-terminal inside region.

Induction of DTH response

Guinea-pigs sensitized with recombinant P35, *M. a paratuberculosis* (316F) or *M. avium* reacted with P35, giving skin reactions. However, the responses to Johnin PPD or to an avian sonicate, in the respective groups, were greater than those to the recombinant P35, and unsensitized guinea-pigs and those sensitized with *M. bovis* did not show significant skin reactions to P35 (Table I).

DISCUSSION

Understanding the immune response to *M. a paratuberculosis* requires the identification of the antigen(s) or epitope(s) involved in protection and/or in the pathogenesis of paratuberculosis. A few antigens of *M. a paratuberculosis* have been reported to elicit humoral immune responses (De Kesel *et al.*, 1993; El-Zaatari *et al.*, 1997; Naser *et al.*, 2000). However, the antigens of *M. a paratuberculosis* that induce a cellular immune response remained to be characterized. In our laboratory, a gene coding for a 35 kDa protein of *M. a paratuberculosis* was identified, homologous to the T-cell-specific 35 kDa protein of *Mycobacterium leprae* and *M. avium* (Banasure *et al.*, 2001). In the present study, we used an efficient expression system, based on the pQE32 vector, to produce the 35 kDa protein of *M. a paratuberculosis* in *E. coli*, so as to facilitate further characterization of the protein.

Based on the sequence information on the ORF of the gene encoding the 35 kDa protein of *M. a paratuberculosis* (EMBL accession no. AJ250887) and also the information on the multiple cloning site of the plasmid vector pQE32, restriction sites for *Sac*I and *Hin*dIII were incorporated into the specific primers to facilitate directional cloning. In the design of the forward primer, consideration was given to inserting the amplified gene of 35 kDa protein in-frame into the pQE32 vector under

TABLE I
Delayed-type hypersensitivity elicited by P35 protein in *Mycobacterium*-sensitized guinea-pigs: mean diameter of erythema (±SEM) upon recall with 10 μg of the indicated antigen

	Antigen				
Sensitizing organism	P35	Johnin PPD	Avium sonicate	Tuberculin PPD	PBS
M. a. paratuberculosis (316F)	10.5±0.5	12.3±0.4	9.0±0.3	2.3±0.5	1.6±0.2
M. avium	9.8±0.7	7.8±0.5	10.7±0.5	2.4±0.3	1.8±0.3
M. bovis (AN5)	2.7±0.3	2.3±0.5	2.0±0.2	9.7±0.9	1.7±0.3
Unsensitized	2.0±0.2	2.0±0.2	1.8±0.1	1.7±0.2	1.8±0.1

the T5 promoter. The resulting plasmid pPMP35 contained an open reading frame encoding successively 6×His polypeptide and the 35 kDa protein. The recombinant pPMP35 clones were confirmed by release of the insert by double digestion with *Sac*I and *Hin*dIII enzymes. and Southern hybridization. A similar strategy of cloning in pQE series expression vector system was applied to generate high levels of *Clostridium perfringens* epsilon toxin (Goswami *et al.*, 1996) and of an outer membrane protein of *Pasteurella multocida* (Luo *et al.*, 1997). Expression of recombinant proteins is induced by IPTG, which binds to the *lac* repressor protein, inactivating it and leading to transcription of sequences downstream of the promoter.

The insolubility of the recombinant protein may be due to its high concentration in *E. coli*, leading to aggregation. The formation of inclusion bodies is advantageous in that the protein is protected from proteolysis (Mukhija *et al.*, 1995; Goswami *et al.*, 1996). No degradation of the recombinant protein was detected in our study.

Expression of the gene facilitated the production of large amounts of the recombinant protein for immunological studies. The 6×His tag is of 840 Da size. It rarely interferes with protein immunogenicity, with protein functional structure or with secretion (Sisk *et al.*, 1994). Hence, the tag was not removed by protease cleavage, although this could be done if necessary.

When the culture filtrate and sonicate fractions of *M. a. paratuberculosis* were probed with the rabbit polyclonal serum raised against the purified 35 kDa recombinant protein, it was found that only the sonicate fraction and not the culture filtrate reacted with the serum. This not only confirmed the heterologous expression of the 35 kDa protein, but also indicated that it is not a protein secreted by *M. a. paratuberculosis*. The immunoblot assays clearly identified the expression of the recombinant protein. These results also suggested that the recombinant fusion protein had retained its antigenicity. Furthermore, the reactivity of P35 with the clinical serum indicates that the protein could be a useful antigen during infection in animals.

The 35 kDa protein was found to be localized in the membrane fraction of *M. a. paratuberculosis*. Also, analysis of the deduced amino acid sequence, according to the Kyte and Doolittle algorithm, showed the presence of five highly hydrophobic regions, which could be the membrane-spanning segments of the protein. Furthermore, analysis according to Rost and colleagues (1996) predicted the occurrence of a transmembrane domain. These observations suggest that the 35 kDa protein was associated with the membrane in *M. a. paratuberculosis*.

The recombinant P35 had retained the ability to elicit a DTH response. DTH reactions were observed in all the animals sensitized with either *M. a. paratuberculosis* or *M. avium*. The cross-reactivity of the P35 with *M. avium* indicates the conserved nature of the 35 kDa protein among related mycobacteria. This finding is in agreement with the earlier report by Triccas and colleagues (1998), who reported the presence of the 35 kDa protein in *M. avium*. The greater responses to the Johnin PPD or an avium sonicate in the respective groups may be due to polyclonal activation of T cells by multiple antigenic components in these preparations compared to the single antigenic component of the P35 protein. The role of P35 in eliciting DTH response in naturally infected animals needs to be investigated.

This high-level expression of the recombinant 35 kDa protein of *M. a. paratuberculosis* provides an opportunity for its further characterization. Apart from possessing seroreactive epitopes, the protein stimulated cell-mediated immune responses. However, its cross-reactivity with *M. avium* necessitates further study. Specific B-cell- and T-cell-stimulatory epitopes from the 35 kDa protein in *M. a. paratuberculosis* need to be investigated, using overlapping synthetic peptides, as these could be of value for diagnostic and/or immunoprophylactic uses.

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