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Processing and secretion by *Escherichia coli* of a recombinant form of the immunogenic protein MPB70 of *Mycobacterium bovis*

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The gene encoding an immunodominant secreted antigen, MPB70, of *Mycobacterium bovis* was cloned into the plasmid vector pBluescript II KS$^+$ along with its native ribosome-binding site. In this construct translation of the protein in *Escherichia coli* was from the native AUG initiation codon and was directed by the mycobacterial ribosome-binding site. Two different molecular mass forms (26 kDa and 22 kDa) of MPB70 were observed in whole-cell pellets of recombinant *E. coli*. The difference in size indicates cleavage of the signal peptide of MPB70 by an endopeptidase of *E. coli*. MPB70 was secreted into the periplasm of recombinant *E. coli*, where the 22 kDa form of the protein was predominant. The culture filtrate contained only the 22 kDa form of the protein, which was soluble. The passage of MPB70 from the periplasm into the growth medium was found to be due, at least in part, to non-specific leakage of periplasmic proteins across the outer membrane associated with the expression of recombinant MPB70.

Introduction

Improvement of immunodiagnostic screening for *Mycobacterium bovis* infection relies on the identification and purification of antigens exhibiting a high degree of specificity (Daniel & Janicki, 1978; Auer, 1987; Fifis *et al.*, 1992). In order to identify such antigens for diagnosis in cattle, Fifis *et al.* (1992) purified a number of proteins from *M. bovis* and tested these antigens with panels of sera from *M. bovis*-infected cattle, *M. bovis* culture-negative cattle, and cattle infected with other mycobacteria. Their study identified a number of promising candidate antigens, of which the protein MPB70 gave greatest specificity for *M. bovis* infection.

MPB70 is an immunodominant antigen of *M. bovis* (Fifis *et al.*, 1989) containing *M. bovis* species-specific epitopes (Wood *et al.*, 1988). The protein is an active component of bovine PPD (Harboe *et al.*, 1990) and is able to elicit a delayed-type hypersensitivity (DTH) response (Nagai *et al.*, 1981; Miura *et al.*, 1983; Harboe *et al.*, 1986; Hasløv *et al.*, 1987), and to stimulate T lymphocyte proliferation (Fifis *et al.*, 1989; Griffin *et al.*, 1991) and antibody production (Fifis *et al.*, 1992; Wood *et al.*, 1992) in *M. bovis*-infected animals. The protein is a major component of *M. bovis* culture filtrate (Nagai *et al.*, 1981; Harboe *et al.*, 1986; Abou-Zeid *et al.*, 1987; Fifis *et al.*, 1991) and is secreted from the cell following cleavage of a 30-amino-acid signal peptide which directs translocation of the hydrophobic molecule across the cytoplasmic membrane (Terasaka *et al.*, 1989). MPB70 has been cloned and sequenced (Terasaka *et al.*, 1989; Radford *et al.*, 1990).

The use of isolated *M. bovis* antigens, such as MPB70, in immunodiagnostic assays is hindered by difficulties in growing the organism and purifying the antigens. Recombinant proteins may provide a realistic alternative. Previous reports have indicated that bulk purification of mycobacterial recombinant proteins in *Escherichia coli* may be achieved by fusion of the protein of interest to a second protein for which column purification procedures are available. However, this often results in the accumulation of the fusion protein as an insoluble protein mass within the bacterial cell (Vordermeier *et al.*, 1991; Ashbridge *et al.*, 1992), making purification difficult.

This report describes the production of a plasmid construct which generates a form of MPB70 with retained antigenicity, that is secreted across the cytoplasmic membrane of *E. coli*. The results indicate that the leader peptide of the recombinant mycobacterial protein is cleaved by an endopeptidase of *E. coli*, resulting in the accumulation of a soluble, mature form of the protein in the periplasm and growth medium, thus facilitating purification.

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Methods

Bacterial strains and vectors. Escherichia coli strain DH5α (F^- *ϕ80lacZ*ΔM15 Δ(*lacZYA–argF*)U169 *recA1 endA1 hsdR17* (r_k^- m_k^+) *supE44* λ^- *thi-1 gyrA relA1*) was used as host for plasmid vectors pBluescript II KS^+ (Stratagene), pUC18 and pUC19 (Gibco-BRL). The *M. bovis* DNA used in this study was extracted from strain AN5 (Central Veterinary Laboratory, Weybridge, UK).

Immunological reagents. M. bovis sonicated extract was prepared by the method of Morris *et al.* (1985). Rabbits were immunized subcutaneously at multiple sites with *M. bovis* sonicated extract (100 μg) in Freund's incomplete adjuvant. The rabbits were boosted twice at 14 d intervals. Murine anti-MPB70 monoclonal antibody, SB10 (Wood *et al.*, 1988), was purchased from Agen Biomedical (Queensland, Australia). Rabbit antibody directed against β-lactamase of *E. coli* was purchased from CP Laboratories (Hertfordshire, UK).

Polymerase chain reaction (PCR). DNA was extracted from *M. bovis* strain AN5 by the method of Whipple *et al.* (1987). PCR was performed in 50 μl reaction mixtures containing 50 mM-KCl, 10 mM-Tris/HCl (pH 8·8), 0·01% (w/v) gelatin, 3 mM-$MgCl_2$, 200 μM of each dinucleotide triphosphate (dNTP), 2·5 U of thermostable *Taq* DNA polymerase (Perkin-Elmer Cetus) and 20 pmol of each oligonucleotide primer. The DNA sequence of the primers was as follows: 5′-AAAGAATTCGGACGGCTCCGAAGAAATC-3′ and 5′-CCCGGATCCTTACGCCGGAGGCATTAGCAC-3′. The reaction mixture was overlaid with 50 μl mineral oil and DNA samples (10 ng) were pipetted through the oil into the mixture. The parameters for amplification were: denaturation at 94 °C for 3 min for one cycle, followed by 30 cycles at 94 °C for 1·5 min, 56 °C for 1·5 min and 72 °C for 2 min. A final extension at 72 °C for 10 min was also included. A reagent blank (negative control) was as described above but without the addition of DNA sample.

Sequence analysis. DNA sequencing of double-stranded template was performed using the primer-extension dideoxy chain-termination method (Sanger *et al.*, 1977) as recommended by the manufacturer (Sequenase, United States Biochemicals).

Other genetic procedures. Restriction endonucleases and other enzymes were used according to the manufacturers' instructions. Other genetic manipulations were as described by Sambrook *et al.* (1989).

Osmotic shock procedure. The method used to obtain periplasmic and cytoplasmic cell fractions was a modification of that described by Koshland & Botstein (1980). A 10 ml overnight culture of bacterial cells was grown in Luria–Bertani broth, with 0·1% (w/v) glucose added (LBG; Sambrook *et al.*, 1989), in the presence of 100 μg ampicillin ml^{-1}. The culture was harvested by centrifugation at 5000 *g*, 15 min, 20 °C. The supernatant was discarded and the cells washed twice in 40 ml LBG broth. The final pellet was resuspended in 10 ml LBG broth and 1 ml was inoculated into 100 ml LBG broth with 50 μg ampicillin ml^{-1} in a 250 ml conical flask. The cells were incubated at 37 °C, 200 r.p.m. for 2–4 h until an OD_{550} of 0·5–0·7 was obtained (i.e. exponential-phase growth).

The OD_{550} of the cells was standardized to 0·5 by dilution in LBG broth. The cell suspension (50 ml) was harvested by centrifugation; the supernatant was removed and filtered twice through 0·2 μm filters. The filtrate was concentrated to 0·09 volumes (i.e. 11-fold concentration) by ultrafiltration (Centricon, Amicon) and saved as the culture filtrate fraction. The cell pellet was resuspended in 4·5 ml ice-cold 20% (w/v) sucrose, 10 mM-Tris/HCl (pH 7·5). EDTA solution (0·5 M, pH 8, 150 μl) was added and incubation was continued on ice for 10 min. All subsequent steps were carried out at 4 °C. A sample (1·5 ml) was removed for use as the untreated control. The remaining cell suspension was dispensed in 1·5 ml volumes into Eppendorf tubes and centrifuged for 5 min at 13000 *g*, 4 °C. The supernatant fluid was removed quickly and the pellet rapidly resuspended by vigorous agitation in 1 ml ice-cold distilled water. The mixture was incubated for 10 min on ice and then centrifuged for 10 min at 13000 *g*, 4 °C. The supernatant fluid was recovered (periplasmic fraction). Residual fluid was removed carefully from the cells and the remaining cell pellet was resuspended in 1 ml distilled water (the cytoplasmic fraction). All cellular fractions were stored at −20 °C prior to use.

Determination of β-lactamase activities. The hydrolysis of the chromogenic cephalosporin nitrocefin (O'Callaghan *et al.*, 1972) by β-lactamase at 37 °C was monitored at 550 nm (Angus *et al.*, 1982). The molar absorption coefficient for nitrocefin was 10·7 l $mmol^{-1}$ cm^{-1}. The reaction mixture (final volume 1 ml) contained 5 mM-$MgCl_2$/25 mM-MOPS (pH 7·4) and 13–126 μM-nitrocefin. The reaction was started by the addition of 10 μl of osmotic shock fraction. Initial rates of nitrocefin hydrolysis by the β-lactamase present in each osmotic shock fraction were determined by the chord-drawing method of Waley (1981). Values for K_m and V_{max} of the β-lactamase in each osmotic shock preparation were determined using linear regression analysis of Lineweaver–Burk plots. From these, a mean value of the K_m for nitrocefin hydrolysis by the β-lactamase encoded by pBluescript II KS^+ was obtained. To enable direct comparison of V_{max} for all experiments, the mean of the K_m values was used to calculate V_{max} from initial rates of nitrocefin hydrolysis using the Michaelis–Menten equation.

SDS-PAGE. Protein samples (50 μl) were solubilized by heating at 100 °C for 3 min in 50 μl sample loading buffer [75 mM-Tris/HCl, pH 6·8, 5% (v/v) 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol, 0·002% (w/v) bromophenol blue]. Fractions were then separated on a 12·5% (w/v) SDS-polyacrylamide gel using a discontinuous Tris/HCl buffer system as described by Laemmli (1970). Rainbow protein markers (Amersham) were run as molecular mass standards.

Immunoblotting. Proteins were transferred to nitrocellulose by electroblotting as described by Matsudaira (1987). Membranes were blocked with 3% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min, washed in PBS with 0·05% (w/v) Tween 20 (PBST) and incubated for 1 h with rabbit antiserum directed against sonicated *M. bovis* (diluted 1 in 500 in PBST), or with rabbit antiserum directed against β-lactamase (diluted 1 in 500 in PBST). Membranes were washed and incubated for 30 min at 20 °C with alkaline-phosphatase-conjugated anti-rabbit immunoglobulin (Sigma; diluted 1 in 8000 in PBST). After washing, bound alkaline phosphatase was detected using Nitro Blue Tetrazolium as described by Blake *et al.* (1984).

Results

Cloning of the gene encoding MPB70 and its expression in E. coli

PCR was used to amplify the gene encoding MPB70 from chromosomal DNA extracted from *M. bovis* AN5. The oligonucleotide primers used in this reaction were designed to give a product of 678 bp encompassing 660 bp of mycobacterial DNA encoding the MPB70 structural gene with an additional 78 bp upstream of the AUG initiation codon and restriction endonuclease sites at each end to allow cloning into the plasmid vector. The upstream region of DNA included a putative ribosome-binding site at positions −13 to −9 from the initiation codon (Terasaka *et al.*, 1989; Radford *et al.*, 1990). Thus

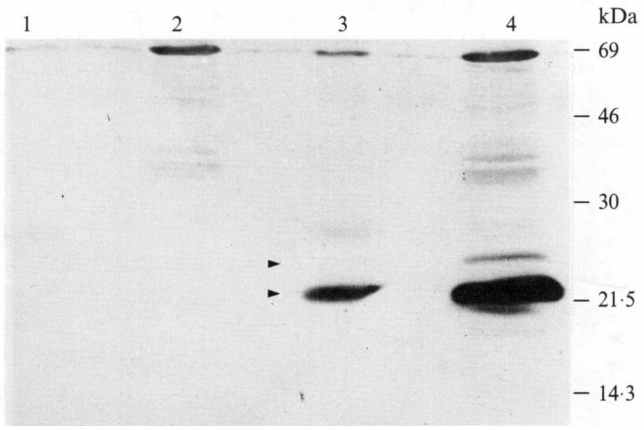

Fig. 1. Western blots of recombinant *E. coli* clones incubated with polyclonal antisera directed against sonicated *M. bovis*. pBluescript II KS$^+$ is the original plasmid vector. Plasmid pVW500 was constructed by cloning the gene encoding MPB70 into pBluescript II KS$^+$ as described in Results. Lanes: 1, *E. coli* DH5α(pBluescript II KS$^+$) culture filtrate; 2, *E. coli* DH5α(pBluescript II KS$^+$) cell pellet; 3, *E. coli* DH5α(pVW500) culture filtrate; 4, *E. coli* DH5α(pVW500) cell pellet. Arrowheads indicate the positions of the two forms of MPB70.

it was envisaged that by cloning the PCR product into the vector pBluescript II KS$^+$ transcription of the gene encoding MPB70 would occur from the *lacZ* promoter of the vector and that translation of MPB70 would occur from ribosomes binding to the cloned ribosome-binding site. A 678 bp product was obtained from the PCR and was ligated into pBluescript II KS$^+$ using the *Eco*RI and *Bam*HI restriction endonuclease sites encoded by the oligonucleotide primers. The recombinant plasmid was transformed into *E. coli* DH5α. The identity of the mycobacterial insert in *E. coli* transformed with the recombinant plasmid was confirmed by DNA sequence analysis. The recombinant plasmid was designated pVW500.

Expression of MPB70 was detected by Western blotting using polyclonal rabbit antisera directed against sonicated *M. bovis*. Two forms of MPB70 were observed in the cell pellet (Fig. 1, lane 4). These polypeptides were not detected in the cell pellet of control recombinant *E. coli* containing plasmid without the mycobacterial DNA insert (Fig. 1, lane 2). One form of MPB70 gave an apparent molecular mass of 26 kDa, the other, predominant form, gave an apparent molecular mass of 22 kDa as estimated by denaturing PAGE. The expression of MPB70 was confirmed by Western blot analysis of the recombinant proteins using SB10, a monoclonal antibody specific for MPB70 (Wood *et al.*, 1988). SB10 bound both forms of recombinant MPB70, although its affinity for the polypeptides was not as high as that of the polyclonal antisera (data not shown).

In order to determine whether transcription of MPB70 was from a promoter present in the 78 bp of *M. bovis* DNA upstream of the MPB70 initiation codon or from the *lacZ* promoter of the plasmid vector, the entire DNA fragment was excised from pVW500 using *Eco*RI and *Bam*HI endonuclease digestion and ligated into the plasmid vectors pUC18 and pUC19. This procedure introduced the DNA fragment into pUC in both orientations relative to the *lacZ* promoter. Western blot analysis using the rabbit antisera raised against sonicated *M. bovis* showed that MPB70 was only expressed by recombinant *E. coli* containing the pUC18 construct, i.e. in the same orientation as pVW500, suggesting that expression was under control of the *lacZ* promoter.

Secretion of MPB70 by E. coli

Western blotting of concentrated culture filtrate using rabbit antisera raised against sonicated *M. bovis* detected the presence of only the 22 kDa form of recombinant MPB70 in the culture medium (Fig. 1, lane 3). This suggested that MPB70 was expressed and secreted by *E. coli* via a mechanism involving cleavage of the secretory signal peptide. In order to confirm this, osmotic shock was used to obtain cytoplasmic and periplasmic fractions of the recombinant cells. These fractions, and the culture filtrates from recombinant *E. coli*, with and without the MPB70 DNA insert, were Western blotted with antisera directed against sonicated *M. bovis* (Fig. 2*a*). The majority of the 26 kDa form of MPB70 was restricted to the cytoplasmic fraction of recombinant *E. coli* (lane 3), whereas the 22 kDa form was detected in the culture filtrate, the cytoplasm and the periplasm (lanes 1–3).

The cellular fractions were also analysed for the presence of β-lactamase (a periplasmic protein of 29 kDa that is synthesized as a preprotein with a typical signal peptide of 23 amino acids: Sutcliffe, 1978; Koshland & Botstein, 1980), which was encoded by the plasmid vector. β-Lactamase was detected by Western blotting using specific rabbit antisera (Fig. 2*b*). A similar pattern of β-lactamase secretion to that of MPB70 was observed. Two forms of β-lactamase (of approximately 31·5 kDa and 29 kDa) were detected in the cytoplasm and periplasm (lanes 2, 3, 5 and 6), although the lower molecular mass form was predominant in the periplasm (lanes 2 and 5). The lower molecular mass form was also observed in the culture filtrate (lanes 1 and 4), suggesting some leakage across the outer membrane.

Measurement of β-lactamase leakage across the outer membrane of recombinant E. coli

In order to establish whether the presence of MPB70 in the culture filtrate was due to active secretion via a specific pathway, or to a general increase in the leakage of proteins through the outer membrane, the β-lactamase

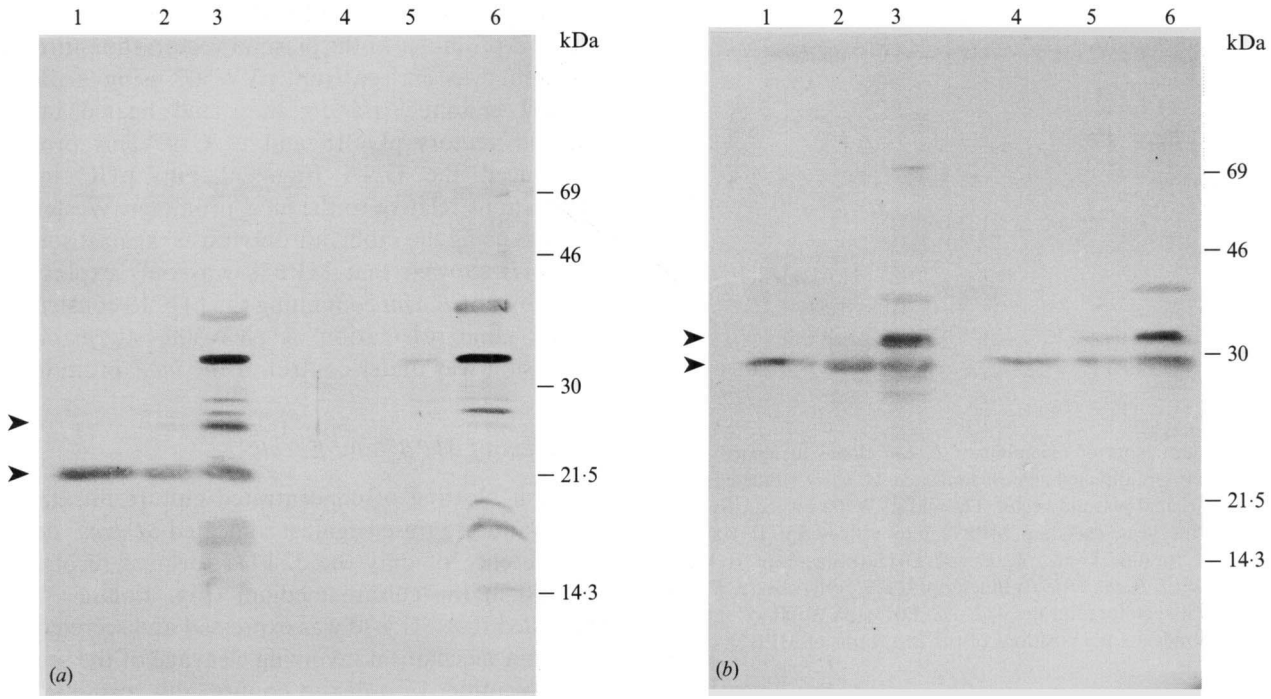

Fig. 2. (*a*) Western blots of cellular fractions of recombinant *E. coli* clones incubated with polyclonal sera raised against sonicated *M. bovis*. Plasmid pBluescript II KS$^+$ is the original plasmid vector. Plasmid pVW500 was constructed by cloning the gene encoding MPB70 into pBluescript II KS$^+$ as described in Results. Lanes: 1, *E. coli* DH5α(pVW500) culture filtrate; 2, *E. coli* DH5α(pVW500) periplasmic fraction; 3, *E. coli* DH5α(pVW500) cytoplasmic fraction; 4, *E. coli* DH5α(pBluescript II KS$^+$) culture filtrate; 5, *E. coli* DH5α(pBluescript II KS$^+$) periplasmic fraction; 6, *E. coli* DH5α(pBluescript II KS$^+$) cytoplasmic fraction. Arrowheads indicate the positions of the two forms of MPB70. (*b*) Western blots of cellular fractions of recombinant *E. coli* clones incubated with polyclonal sera raised against β-lactamase. Lanes 1 to 6 as for (*a*). Arrowheads indicate the positions of the two forms of β-lactamase.

Table 1. *β-Lactamase activities of cellular fractions of recombinant E. coli*

Bacteria were grown to early exponential phase growth in LBG broth. The cells were harvested and the cellular fractions obtained by osmotic shock treatment. Hydrolysis of nitrocefin was monitored spectrophotometrically (550 nm) at 37 °C in 5 mM-$MgCl_2$/25 mM-MOPS (pH 7·4). Initial rates of hydrolysis were determined by the chord-drawing method of Waley (1981) and were used to calculate K_m and V_{max} using Lineweaver–Burk plots. A mean K_m value of 35·7 ± 8·4 μM ($n = 10$) was obtained. This value was used to calculate V_{max} [nmol min^{-1} (mg dry mass)$^{-1}$] from each initial rate of hydrolysis using the Michaelis–Menten equation. The SEM was calculated from the values obtained using either 27 (*) or 12 (†) initial rates of nitrocefin hydrolysis in the concentration range 10–120 μM-nitrocefin from three separate experiments.

Plasmid in *E. coli* DH5α	V_{max} culture filtrate	V_{max} periplasmic	V_{max} cytoplasmic	V_{max} culture filtrate/ V_{max} periplasmic
pBluescript II KS$^+$	1017 (±424*)	3111 (±184*)	178 (±47†)	0·33
pVW500	2785 (±808*)	1656 (±274*)	164 (±127†)	1·68

activity was measured in each of the cellular fractions. The results of three experiments comparing the β-lactamase activities in the cellular fractions of recombinant *E. coli* expressing MPB70 and *E. coli* containing plasmid without the MPB70 insert are shown in Table 1. The β-lactamase activities in the culture filtrate were consistently higher for *E. coli* expressing MPB70 [$V_{max} = 2785 \pm 808$ nmol min^{-1} (mg dry mass)$^{-1}$; mean ± SEM, $n = 27$] than the activities observed for recombinant *E. coli* that did not express MPB70 [$V_{max} = 1017 \pm 424$ nmol min^{-1} (mg dry mass)$^{-1}$; $n = 27$]. The ratio V_{max} culture filtrate/V_{max} periplasm was 1·68 for *E. coli* expressing MPB70 whereas for cells not expressing MPB70 it was only 0·33.

Discussion

The intention of this study was to produce recombinant antigens for the development of sensitive and specific blood-based immunodiagnostic tests of *M. bovis*

infections in domestic and wild animals. Antigen production in *E. coli* rather than in *M. bovis* reduces the problems associated with the slow generation time of *M. bovis*, the infectivity of the pathogen and the more complex biochemical composition of mycobacteria. The immunogenic protein MPB70 is a suitable candidate antigen for diagnosis since it has been shown to be the most specific antigen for serodiagnosis of *M. bovis* infection in cattle (Fifis *et al.*, 1992). Fortuitously, the recombinant MPB70 produced in this study was found to be secreted across the cytoplasmic membrane of *E. coli* and to accumulate in the periplasm and culture medium of the organism.

Since the plasmid construct encoding MPB70 (pVW500) also contained 81 bp of mycobacterial DNA upstream from the initiation codon, it was possible that transcription of MPB70 was initiated from a mycobacterial promoter present in this DNA fragment. However, as expression was only observed in one orientation of the mycobacterial DNA insert relative to the *lacZ* promoter, we conclude that it is most likely that transcription is directed from the *lacZ* promoter of the plasmid vector. This result is consistent with the sequencing data of Terasaka *et al.* (1989), which identified putative promoter sequences at positions −148 to −143 and −124 to −119 upstream of the MPB70 initiation codon (i.e. outside the DNA region contained in pVW500). Moreover, insertion of the PCR product into the *lacZ* gene of pBluescript II KS$^+$ and pUC18 introduced a stop codon in the *lacZ* gene at positions −8 to −6 from the initiation codon of the MPB70 structural gene, thus creating a two-cistron expression system (Schoner *et al.*, 1984). It therefore seems likely that expression of MPB70 was achieved by translational coupling (Schoner *et al.*, 1984). In this system it is envisaged that transcription is initiated from the *lacZ* promoter, the *E. coli* ribosomes bind the putative ribosome-binding site (Terasaka *et al.*, 1989; Radford *et al.*, 1990) encoded by the *M. bovis* DNA insert and MPB70 is translated from its native AUG initiation codon. In some cases this type of two-cistron construction has been shown to improve the efficiency of translation of heterologous genes in *E. coli* (Schoner *et al.*, 1984).

Two forms (26 kDa and 22 kDa) of the recombinant MPB70 were produced by *E. coli* containing the plasmid construct pVW500. Analysis of the protein sequence of MPB70 reveals the presence of a 30-amino-acid secretory signal sequence at the amino-terminus of the protein (Terasaka *et al.*, 1989; Radford *et al.*, 1990). This is cleaved from the mature protein during secretion by *M. bovis* (Radford *et al.*, 1990). The signal peptide of MPB70 conforms to the physico-chemical properties observed for other bacterial signal peptides (Von Heijne, 1988) in that the peptide has a positively charged amino-terminal region, a central hydrophobic region and a more polar carboxy-terminal with an Ala-X-Ala endopeptidase recognition site. The difference in molecular mass of the two forms of MPB70 can therefore be explained by cleavage of the 30-amino-acid secretory signal peptide (Terasaka *et al.*, 1989; Radford *et al.*, 1990) from the 26 kDa form of MPB70 by an endopeptidase of *E. coli*. The molecular mass of mature MPB70 predicted from the amino acid sequence is 16·3 kDa (Terasaka *et al.*, 1989; Radford *et al.*, 1990) rather than the observed 22 kDa. However, mature native MPB70 from *M. bovis* also displays aberrant mobility in denaturing PAGE, giving an apparent molecular mass of 18–23 kDa (Miura *et al.*, 1983; Nagai *et al.*, 1986; Abou-Zeid *et al.*, 1987; Hasløv *et al.*, 1987; Fifis *et al.*, 1989). Therefore, when the signal peptide is removed, both native and recombinant MPB70 display similar aberrant mobilities. Preliminary unpublished data suggest that the pI of the recombinant form of MPB70 (4·8) is similar to the value of 4·5–5·0 reported for native MPB70 (Nagai *et al.*, 1986; Fifis *et al.*, 1989).

Secretion of recombinant MPB70 into the periplasm of *E. coli* was observed. To our knowledge this is the first report of the secretion of a mycobacterial protein by a Gram-negative bacterium. Moreover, the 22 kDa form of MPB70 was detected in both the periplasm and culture filtrate of recombinant *E. coli* in the exponential phase of growth. Since the signal peptide of MPB70 was removed following translocation across the cytoplasmic membrane, it seemed likely that the appearance of MPB70 in the culture medium was either due to non-specific leakage across the outer membrane or to a specific, signal-peptide-independent process. Recent studies indicate that several pathways exist for the secretion of proteins across the outer membrane (Pugsley, 1989). However, our results show that the expression of MPB70 in *E. coli* gives rise to an increase in leakage of the normally periplasmic β-lactamase across the outer membrane into the culture medium. Therefore the accumulation of recombinant MPB70 in the culture medium of *E. coli* is due, at least in part, to non-specific leakage of periplasmic proteins across the outer membrane. A likely explanation for this leakage is that hydrophobic interactions between the outer membrane and MPB70, which is a hydrophobic molecule (Terasaka *et al.*, 1989), cause some disruption of the outer membrane. However, expression of MPB70 did not affect the growth rate of the cells in culture (unpublished data).

This processing and secretion of the mycobacterial antigen MPB70 by *E. coli* may be exploited for the purpose of antigen production. Production of MPB70 in this way has several advantages. Secretion precludes

proteolysis of the protein by housekeeping cytoplasmic peptidases, which would normally remove abnormal proteins from the cytoplasm, and separates it from contaminating cytoplasmic proteins. Moreover, processing of the protein by *E. coli* results in the production of a mature, soluble form of MPB70 which resembles the native molecule, whereas recombinant proteins are often produced as chimeric fusion proteins which might alter the conformation of the protein.

The ability of *E. coli* to secrete MPB70 raises the possibility that *E. coli* and attenuated oral vaccine strains of salmonella might be able to secrete a range of mycobacterial antigens. This possibility is currently under investigation.

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