Identification and characterization of a putative serine protease expressed *in vivo* by *Mycobacterium avium* subsp. *paratuberculosis*

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A putative serine protease expressed *in vivo* by *Mycobacterium avium* subsp. *paratuberculosis* was isolated from a lambda gt11 genomic expression library by screening with serum from a naturally infected sheep. The gene was contained in two overlapping clones, which were shown by antibody elution to encode a protein of 34 kDa in *M. a. paratuberculosis*. The clones were sequenced and database searches detected a motif identical to the active serine site in trypsin, and 30% homology to the putative serine proteases (HtrA proteins) of *Escherichia coli*, *Salmonella typhimurium*, *Brucella abortus* and *Rochalimaea henselae*.

Keywords: *Mycobacterium avium*, paratuberculosis, serine protease, 34 kDa antigen, HtrA

INTRODUCTION

Paratuberculosis (syn. Johne's disease) is a chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* and, occasionally, by *M. avium* subsp. *silvaticum* (Chiodini *et al.*, 1984; Matthews & McDiarmid, 1979). The outcome of infection of the host is variable, ranging from complete recovery in the majority, to clinical disease in a small proportion. The interaction between host and bacterium is poorly understood due, in part, to inadequate knowledge of the properties of these mycobacteria. Consequently, we have focussed our interest on *M. a. paratuberculosis* genes and gene products that are involved in the bacterium–host interaction, particularly those proteins which are immunogenic in the natural ruminant host, and which may be involved in pathogenesis.

Individual proteins have been identified in several mycobacteria by producing monoclonal antibodies (mAbs) (Engers *et al.*, 1985; Kolk *et al.*, 1989), and using these to screen genomic expression libraries (Young *et al.*, 1985; Morris *et al.*, 1988, 1990; Rouse *et al.*, 1991). However, this approach has identified principally heat shock proteins (Husson & Young, 1987; Lu *et al.*, 1987; Young & Mehler, 1989), as the use of mycobacterial lysates in immunization procedures does not reflect accurately the immune response of the natural host to infection. To

circumvent this problem, we chose to construct an *M. a. paratuberculosis* genomic expression library and screen with serum from a naturally infected animal. By utilizing the natural immune response of the infected host to *M. a. paratuberculosis* we aimed to detect antigens expressed *in vivo* that may be absent from, or very poorly expressed in, bacteria grown *in vitro*, suggesting a possible involvement in pathogenesis (Donachie & Gilmour, 1988; Jonson *et al.*, 1989). A similar approach in which *M. leprae* lambda gt11 genomic libraries were screened with pooled sera from leprosy patients, resulted in the identification of several new proteins which were not detected by mouse monoclonal antibodies or rabbit hyper-immune serum (Clark-Curtiss *et al.*, 1990; Sathish *et al.*, 1990; Wiele *et al.*, 1994). The present report describes the characterization of two overlapping clones isolated from an *M. a. paratuberculosis* lambda gt11 library, which were found to encode a putative serine protease.

METHODS

Bacterial strains, bacteriophages, plasmids and growth conditions. Strain JD88/107 of *M. a. paratuberculosis*, used throughout the experiments, was isolated from a commercially farmed red deer (*Cervus elaphus*) and propagated on Stuart's medium (Stuart, 1965) supplemented with mycobactin J. *Escherichia coli* strains NM522, JM101, Y1088 and Y1089 were grown in Luria–Bertani (LB) medium or LB solidified with 1.5% (w/v) agar (Sambrook *et al.*, 1989). Lambda recombinant phages were propagated as described by Sambrook *et al.* (1989). Expression plasmid pMS1S (Scherf *et al.*, 1990) was kindly supplied by M. Schreiber and J. Scaife. Plasmids pUC8 and

Abbreviation: mAb, monoclonal antibody.

The EMBL accession number for the sequence reported in this paper is Z23092.

pUC18 (Yanisch-Perron *et al.*, 1985) were purchased from Life Technologies.

Library construction and screening. Two hundred milligrams (wet wt) of *M. a. paratuberculosis* cells were lysed in 4 M guanidine isothiocyanate using an Eaton press (Eaton, 1962). DNA was purified using standard laboratory procedures (Sambrook *et al.*, 1989). The lambda gt11 library was constructed as described by Young *et al.* (1985) using *Eco*RI-cleaved lambda gt11 DNA and Gigapack II Gold packaging extracts from Stratagene. Amplification was performed using *E. coli* Y1088.

The library was screened with antiserum from a sheep with Johne's disease (JD87/40) using the procedure described by Young *et al.* (1985) with a few modifications. Nitrocellulose filters were incubated with the recombinant phage overnight at 37 °C and were blocked in 50 mM Tris pH 7, 150 mM NaCl, 0.05% (v/v) Tween 20, 50% (v/v) horse serum for 2 h. Antibody was absorbed with 1 ml of a concentrated lysate of induced *E. coli* Y1090 cells. Bound antibody was detected using horseradish peroxidase-conjugated donkey-anti sheep IgG.

DNA manipulations and sequencing. Recombinant phage DNA was prepared from plate lysates and subsequent subcloning into the plasmid vectors pUC8, pUC18 and pMS1S was carried out using standard procedures described by Sambrook *et al.* (1989). Subcloning from lambda gt11 to pMS1S conserved the open reading frame and allowed expression of the recombinant protein. Plasmid DNA was prepared using the alkaline lysis procedure and purified by equilibrium centrifugation in caesium chloride-ethidium bromide gradients (Sambrook *et al.*, 1989). Both strands of DNA from the S4 and S8 plasmid clones were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase version 2.0 (United States Biochemicals), the M13 universal primer, a pMS1S specific primer (ACGTACGGTACCAAGCT), and a series of internal 17-mer oligonucleotides. The sequencing procedure used was that recommended by USB. dCTP labelling mix and [³⁵S]-dCTP α S were used to achieve better incorporation into the G-C rich mycobacterial DNA. The Sequenase enzyme was diluted 1:4. Reactions were run on 40% (v/v) formamide, 7 M urea, 8% (w/v) acrylamide gels in 'glycerol-tolerant' sequencing buffer (1.78 M Tris, 0.57 M taurine, 0.01 M EDTA) to eliminate compressions, as recommended by USB.

Purification of recombinant fusion protein. A culture of *E. coli* NM522 containing the pMS1S/S4 or pMS1S/S8 plasmid was grown to an OD₆₀₀ of 0.5, and induced with 10 mM IPTG. After a further 4 h growth, cells were pelleted, resuspended in 10 ml 2 mM PMSF and lysed in a mini French pressure cell at 1500 p.s.i. (10.35 MPa). The lysate was clarified by centrifugation at 100 000 g and adjusted to 300 mM NaCl, 50 mM Tris/HCl pH 8. Recombinant fusion protein was purified using a 2 ml column of aminobenzyl thiogalactopyranoside (ABTG) agarose (Sigma). The column was washed with 10 ml 300 mM NaCl, 50 mM Tris/HCl pH 8, 5 ml 600 mM NaCl, 50 mM Tris/HCl pH 8 and 10 ml 50 mM Tris/HCl pH 8 and the fusion protein eluted with 10 ml 0.1 M borate pH 10. The eluate was collected on ice into 1 ml of 2 M Tris/HCl pH 7, dialysed overnight against 10 mM Tris/HCl pH 8, and concentrated to 2–3 ml with PEG 20000 (Sigma). Total protein concentration was estimated with the Pierce BCA micro protein assay as recommended by the manufacturer.

SDS-PAGE. Protein samples were boiled for 90 s in an equal volume of 2×Laemmli sample buffer [250 mM Tris/HCl pH 6.8, 2% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 20% (w/v) sucrose, 0.002% (w/v) bromophenol blue] and separated on a discontinuous SDS-polyacrylamide gel [3% (w/v) stacking gel, 10% resolving gel] using the buffering system of Laemmli

(1970). Molecular mass standards (BDH Electran; 12–78 kDa) were run on each gel.

Western blotting. Proteins were transferred from SDS-polyacrylamide gels onto nitrocellulose by electroblotting in Tris/glycine blot buffer [20 mM Tris, 153 mM glycine, 20% (v/v) methanol, pH 8.3] at 100 V for 1 h. After blotting, the membranes were stained with Ponceau S (Sigma) for 5 min to confirm transfer, and washed for 30 min in PBS containing 0.5% (v/v) Tween 20, 1 mM EDTA, 350 mM NaCl, pH 7.2 (PBS-Tween). Membranes were probed for 1 h at room temperature with either clinical sheep serum (absorbed with lysed *E. coli* NM522) diluted 1:40 in PBS-Tween, specifically eluted clinical sheep antibodies, or mAb 5.8 (neat tissue culture supernate) raised against S4 fusion protein. Membranes were washed and incubated for 1 h at room temperature in horseradish peroxidase-conjugated swine anti-sheep IgG, or rabbit anti-mouse IgG (Dako) diluted 1:2000 in PBS-Tween. Bands were developed with 0.1 M Tris/HCl pH 7.5, 0.15% (v/v) H₂O₂, 0.2% (w/v) diaminobenzidine.

Antibody elution studies were performed as described by Beall & Mitchell (1986).

Monoclonal antibody production. Purified pMS1S/S4 fusion protein solubilized in 10 mM Tris/HCl pH 8 was inoculated intraperitoneally into 8–10 week old Balb/c mice three times over two week intervals. The mice were finally boosted 3 d before fusion with the myeloma cell line P3 X63 Ag.8.6.5.3 using standard procedures (Goding, 1980).

Hybridoma culture supernates were screened in ELISAs in microtitre plates coated with either S4 fusion protein or β -galactosidase. Clones reacting with S4 fusion protein but not β -galactosidase were selected.

Two Balb/c mice were inoculated intraperitoneally with 5×10^6 hybridoma cells, and the resulting ascitic fluid was collected after 14 d. IgG1 was purified from the ascites on a protein A-Sepharose column (Pharmacia), and coupled to cyanogen bromide-activated Sepharose (Pharmacia) as recommended by the manufacturer. The column was then equilibrated in PBS supplemented with 0.5 M NaCl.

Purification of 34 kDa protein from *M. a. paratuberculosis*.

One gram (wet wt) of *M. a. paratuberculosis* cells was resuspended in 5 ml of PBS supplemented with 0.5 M NaCl, and lysed in an Eaton Press. After sonication to shear DNA, the lysate was mixed with the monoclonal affinity column matrix overnight at 4 °C. The column was then washed with 20 ml PBS supplemented with 0.5 M NaCl and the protein eluted with 10 ml of 0.1 M glycine pH 2.3. The eluate was then dialysed against 10 mM Tris pH 8, concentrated to 2–3 ml with PEG 20000 (Sigma) and further to 500–1000 μ l with a Millipore Ultrafree microconcentrator (10 kDa cutoff).

N-terminal sequencing. Approximately 2 μ g of purified *M. a. paratuberculosis* 34 kDa protein was run on SDS-PAGE, and Western blotted onto PVDF (polyvinylidene difluoride) membrane using borate blot buffer [50 mM sodium borate pH 8, 20% (v/v) methanol, 0.02% (v/v) mercaptoethanol]. The blot was stained with Coomassie blue, and the bands excised. The first 20 amino acids were sequenced from this membrane by Edman degradation at the microchemical facility, IAPGR, Babraham, Cambridge, UK.

RESULTS

Library screening

The genomic library of *M. a. paratuberculosis* (strain JD88/107) constructed in bacteriophage lambda gt11 was shown to contain 8×10^5 individual phage. Subsequent

Fig. 1. Western blot demonstrating relationship between S4 fusion protein and a 34 kDa protein in *M. a. paratuberculosis*. Lane 1, pMS15/S4 fusion protein probed with sheep serum JD87/39; lane 2, *M. a. paratuberculosis* lysate probed with JD87/39 antibody eluted from pMS15/S4 fusion protein; lane 3, *M. a. paratuberculosis* lysate probed with sheep serum JD87/39; lane 4, *M. a. paratuberculosis* lysate probed with mAb 5.8 raised against pMS15/S4 fusion protein. M, Molecular mass markers stained with Ponceau S (Sigma).

amplification in *E. coli* Y1088 yielded a plate stock of 10^9 p.f.u. ml $^{-1}$. The amplified library contained 65% recombinants with an average insert size of 4 kb. Statistical analysis showed this library to be representative of the *M. a. paratuberculosis* genome (Old & Primrose, 1989).

Approximately 125000 plaques were screened with anti-serum JD87/40 obtained from a sheep with naturally acquired Johne's disease and eight recombinant clones, designated S1 to S8, were isolated. The DNA inserts from two of these recombinant phage (S4 and S8) were subcloned into the plasmid vector pMS1S and the encoded antigens expressed in *E. coli* as β -galactosidase fusion proteins.

Due to the random generation of DNA fragments during library construction, recombinant clones often contain only part of an open reading frame (ORF) and, consequently, express only a part of the bacterial antigen. Therefore, the size of the *M. a. paratuberculosis* protein encoded by the clones was determined by an antibody elution procedure. Western blots of S4 and S8 fusion proteins were incubated with serum from a sheep (JD87/39) with clinical paratuberculosis that had been shown previously to recognize both clones. Specific antibody bound by the fusion proteins was eluted and used to probe a Western blot of *M. a. paratuberculosis* lysate (Fig. 1). Antibodies eluted from both S4 and S8 fusion proteins detected a 34 kDa protein in *M. a. paratuberculosis*, and also recognized the reciprocal fusion protein after absorbing with β -galactosidase.

Sequencing of DNA inserts

DNA sequencing revealed that both the S4 and S8 clones contained the same C-terminal portion of a gene encoding a predicted protein of 210 amino acids. The S8 clone also contained a further 480 bp upstream of this region, and was thought to represent the complete gene. This contained a possible Shine-Dalgarno sequence (AGGA-

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10 SD 30 50
CCGCGCAGCGAGGAGGGTGACGGGATGAGCAAAATCGCACCAACCGCTCGGTCTGGTGG
M S K S H H H R S V W W
70 90 110
TCATGGTTGGTGGTGTGCTGACCGTGGTGGGCTGGGCTGGGGTGGGGTCCGGCGTG
S W L V G V L T V V G L G L G L G S G V
130 150 170
GGGCTGGCGCGGGTCCGGGCGACCGTGGGCGTGGCGTGGACCGGTTCCGGGATCGG
G L A P A S A A P S G L A L D R F A D R
190 210 230
CCGCTGGCGCGGATCGACCGCTCGGCCATGGTGGTGGGCGCGCAAGTGGTCAAC
P L A P I D P S A M V G Q V G P Q V V N
250 270 290
ATCGACACCAAGTTCGGGTACAACAACGGGTGGGCGCGGTACCGGCATCGTATCGAC
I D T K F G Y N N A V G A G T G I V I D
310 330 350
CCGAACGGCGTGGTGGTACCAACAACACCGTCATCTCGGGCGGCGCAAAATCAGCGCG
P N G V V L T N N H V I S G A D R F A D R
370 390 410
TTCGACGTGGGCAACGGGCGAGACCTACCGCGTCCAGCTGGTGGTGGTGGTGGTGGT
F D V G N G Q T Y A V D V V G Y D R T Q
430 450 470
GACATCGCGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
D I A V L Q L R G A A G L P T A T I G G
490 510 530
GAGGCCACGGTGGGCGAGCCCATCGTGGCGCTGGCAACCGTGGGCGGCGAGGGCGGCGC
E A T V G E P I V A L G N V G G Q G G T
550 570 590
CCCAACCGGTGGCGGCAAGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGG
P N A V A G K V V A L N Q S V S A T D T
610 630 650
CTGACCGGCGCGGAGAGAACCTCGGCGGCGGTGATCCAGGCGGCGGCGGCGGCGGCGG
L T G A Q E N L G G L I Q A D A P I K P
670 690 710
GGCGACTCGGTGGGCGGATGGTGAACAGCGCGGCGGCGGCGGCGGCGGCGGCGGCGG
G D S G G P M V N S A G Q V I G V D T A
730 750 770
GCCACCGACAGTACAAGATGTCCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG
A T D S Y K M S G G Q G F A I P I G R A
790 810 830
ATGGCGGTGGCAACAGATCGGCTCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG
M A V A N Q I R S G A G S N T V H I G P
850 870 890
ACCGCATTCGGGCTGGGCGTGACGAGCAACAACCGCAACCGGCGGCGGCGGCGGCGGCGG
T A F L G L G V T D N N G N G A R V Q R
910 930 950
GTGGTCAACACCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG
V V N T G P A A A A G I A P G D V I T G
970 990 1010
GTGACACCGTCCCGATCAACGGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG
V D T V P I N G A T S M T E V L V P H H
1030 1050 1070
CCCGGTGACACCATCGCGGTGCACCTTCGGTCCGTCGACGGGCGGCGGCGGCGGCGGCGG
P G D T I A V H F R S V D G G E R T A N
1090 1110
ATCACCTGGCGGAGGGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG
I T L A E G P P A *

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Fig. 2. Nucleotide sequence of the DNA inserts of clones S4 and S8, encoding the 34 kDa protein of *M. a. paratuberculosis*. The deduced amino acid sequence is shown in single-letter code. The putative Shine-Dalgarno sequence (SD) is underlined. The N-terminus of the native protein is marked by $\uparrow$ and underlined. Possible signal cleavage sites after amino acids 37 and 39 marked by $\uparrow$. The histidine, aspartate and serine residues homologous to those in the HtrA proteins are boxed. This sequence has been assigned the EMBL accession number Z23092.

GGG) and start codon (ATG) preceding a single ORF of 1083 bp encoding a predicted protein of 361 amino acids (Fig. 2). The first 40 amino acids of this sequence were highly hydrophobic, suggesting the protein may contain a leader or signal sequence which is removed after translation.

Database searches using the complete deduced amino acid sequence were run on SEQNET at the SERC Daresbury

computing facility. A SWEEP search (Akrigg *et al.*, 1988) of the OWL protein database (Bleasby & Wootton, 1990) identified four bacterial proteins, the HtrA proteins of *E. coli* (Lipinska *et al.*, 1988), *Salmonella typhimurium* (Johnson *et al.*, 1991), *Brucella abortus* (Roop *et al.*, accession no. L09274) and *Rochalimaea henselae* (Anderson *et al.*, accession no. L20127), which displayed 30% homology to the probe sequence. A MOTIFS search using the PROSITE dictionary (Bairoch, 1991) detected a motif 'GDSGG' at amino acid position 213–217, which showed 100% homology to the residues surrounding the active serine in a trypsin-like serine protease.

Purification of native protein

The native 34 kDa protein identified by antibody elution was purified from lysates of *M. a. paratuberculosis* on an affinity column that was prepared using a monoclonal antibody (mAb 5.8) raised against S4 fusion protein. This monoclonal antibody also recognized the 34 kDa protein on Western blots of *M. a. paratuberculosis* (Fig. 1). Both eluted antibody and mAb 5.8 detected a second band of 90–100 kDa on Western blots of *M. a. paratuberculosis* lysate (Fig. 1). This 90–100 kDa protein is unlikely to be a dimer/trimer, due to the reducing conditions of the gel; and other studies (unpublished results) suggest that the reaction is due to a cross-reactive epitope. Approximately 40 µg of pure 34 kDa protein was recovered from 1 g (wet wt) of *M. a. paratuberculosis*. The N-terminal sequence of this protein was derived by Edman degradation, and was found to be identical to amino acids 55–74 of the S8 deduced protein sequence (Fig. 2).

DISCUSSION

This paper describes the identification and characterization of a putative serine protease expressed *in vivo* by *M. a. paratuberculosis*. The protein was encoded by two overlapping clones, which were extracted from a lambda gt11 genomic expression library of *M. a. paratuberculosis* by screening with serum from a naturally infected sheep. The isolated clones therefore were known to encode a mycobacterial protein expressed *in vivo* which was also involved in the ovine immune response to infection with *M. a. paratuberculosis*.

Genes encoding 34 kDa proteins have been described in both *M. a. paratuberculosis* (De Kesel *et al.*, 1993) and *M. tuberculosis* (Barnini *et al.*, EMBL accession no. X69463). Comparison of the previously described *M. a. paratuberculosis* 34 kDa protein with S4 and S8 fusion proteins by reciprocal reactions with polyclonal antisera and monoclonal antibodies failed to demonstrate any relationship between them (unpublished results). This lack of homology has been confirmed further by the absence of any homology between the recently published genomic sequence (Gilot *et al.*, 1993) and that obtained from S8. Similarly, no homology was detected between the S8 clone and the nucleotide sequence of the *M. tuberculosis* 34 kDa protein. The gene contained in the S8 clone therefore has not been described previously in any of the *Mycobacteriaceae*.

The deduced amino acid sequence of the S8 clone contains a highly hydrophobic N-terminus. This is characteristic of a signal peptide, and two possible signal cleavage sites of Ala-X-Ala are situated at positions 35–37 and 37–39 in the protein. The first of these, Ala³⁵-Pro³⁶-Ala³⁷, can be dismissed, as proline is a 'forbidden' residue and cannot be part of a signal cleavage site (von Heijne, 1984). Cleavage after Ala³⁷-Ser³⁸-Ala³⁹ is more likely, but leaves a sequence of 15 amino acids between the cleavage site and the N-terminus of the protein as determined by Edman degradation. It is doubtful that the entire peptide of 54 amino acids would be cleaved after Arg⁵²-Pro⁵³-Leu⁵⁴, as these residues will not be recognized by a signal peptidase (von Heijne, 1984). The 15 amino acid sequence may have been removed during purification of the native protein, as elution was performed in 0.1 M glycine pH 2.3, which may have resulted in hydrolysis of the protein before dialysis against Tris pH 8. However, it is also possible that the protein contains a pro-peptide of 15 amino acids which, although shorter, is similar to that observed with the alpha-lytic serine protease of *Lysobacter enzymogenes* (Epstein & Wensink, 1988). Both the signal peptide of 39 amino acids and the pro-peptide of 15 amino acids would then be removed during post-translational modification and possible secretion of the protein.

Database searches have shown the S8 protein to resemble most closely a trypsin-like serine protease. This class of protease has been well characterized in eukaryotes, and X-ray crystallography has revealed the active site to be comprised not only of serine, but also of histidine and aspartate, due to the three dimensional structure of the molecule. The classical trypsin active site triad is His⁵⁷ Asp¹⁰² Ser¹⁹⁵, and any protein matching this pattern may be regarded as a potential serine protease (Neurath, 1989; Dunn, 1989). Although the S8 protein contains a motif that completely matches the trypsin serine active site (amino acid position 215), no corresponding histidine and aspartate residues occur at positions 77 and 122. The absence of any significant homology with trypsin, excluding the serine active site, suggests the S8 protein is not a form of trypsin. However, other serine proteases such as subtilisin maintain the His-Asp-Ser components of the active site, despite differing from trypsin in both amino acid composition and tertiary structure (Neurath, 1989). Thus the active serine in the conserved motif in the S8 protein may combine with a histidine and aspartate residue elsewhere in the sequence to form the electrostatically important active site.

Further evidence for the S8 protein being a serine protease was provided by homology with four possible bacterial serine proteases: the HtrA proteins of *E. coli* (Lipinska *et al.*, 1988), *S. typhimurium* (Johnson *et al.*, 1991), *Br. abortus* (Roop *et al.*, OWL accession no. L09274), and *R. henselae* (Anderson *et al.*, OWL accession no. L20127). As these proteins have been described only recently, there are as yet no X-ray crystallographic data, and their active sites are not well defined. However, an endopeptidase activity of the *E. coli* HtrA has been reported recently (Lipinska *et al.*, 1990). Although only 30% homology exists between the entire S8 protein and these four bacterial proteases, a

E.coli	126	V	V	T	N	N	H	V	V	D	N	A	136
S.typhimurium	127	V	V	T	N	N	H	V	V	D	N	A	137
B.abortus	147	V	V	T	N	N	H	V	V	S	D	G	157
R.henselae	138	I	V	T	N	N	H	V	I	S	D	G	148
M.paratuberculosis	97	V	L	T	N	N	H	V	I	S	G	A	107

E.coli	157	D	P	R	S	D	I	A	L	I	Q	I	167
S.typhimurium	158	D	P	R	S	D	I	A	L	I	Q	I	168
B.abortus	178	D	P	R	T	D	I	A	V	L	K	I	188
R.henselae	169	D	P	R	T	D	I	A	V	L	K	V	179
M.paratuberculosis	129	D	R	T	Q	D	I	A	V	L	Q	L	139

E.coli	231	I	N	R	G	N	S	G	G	A	L	V	241
S.typhimurium	232	I	N	R	G	N	S	G	G	A	L	V	242
B.abortus	152	V	N	K	R	G	N	S	G	P	A	F	162
R.henselae	242	V	N	R	G	N	S	G	P	T	F		252
M.paratuberculosis	210	T	K	P	G	D	S	G	P	M	V		220

Fig. 3. Partial homology of the deduced amino acid sequences of the S8 protein and the HtrA proteins of *Escherichia coli*, *Salmonella typhimurium*, *Brucella abortus* and *Rochalimaea henselae*. Black boxing indicates complete identity; shaded boxing represents conserved amino acids. Active histidine, aspartate and serine are marked *.

higher degree of homology (50–80%) is observed at three sites, containing histidine, aspartate and serine respectively (Fig. 3). The high conservation of amino acids at these three sites suggests that they probably represent the three important active residues which are brought together to form the active site of the protease.

The native 34 kDa protein is highly immunogenic in infected sheep, goats and deer. Western blots have shown the S4 recombinant fusion protein to be recognized strongly by serum from 8/14 (57%) sheep, 2/2 goats, and 3/3 deer with clinical Johne's disease. The other six sheep reacted weakly (unpublished results). Although this protein appears to be important in the immune response to *M. a. paratuberculosis* infection, only 40–50 µg is recovered from 1 g of *M. a. paratuberculosis* cells grown *in vitro*. The HtrA proteins of *E. coli* and *S. typhimurium* have been shown to be stress proteins (Lipinska *et al.*, 1989; Johnson *et al.*, 1991), induced by heat shock and oxidative stress respectively. *In vivo*, mycobacteria undoubtedly are exposed to several types of stress during intracellular growth, and it is possible that, as with other HtrA proteins, levels of expression of the 34 kDa protein may increase under these conditions. During less stressful growth *in vitro* the protein may be rapidly degraded intracellularly, or its expression accordingly down-regulated. This would account for the poor yield observed during the purification of the native protein from organisms grown *in vitro*.

Serine proteases have not been described previously in the *Mycobacteriaceae*, and possible functions of the 34 kDa protein are speculative. However, recent reports suggest that serine proteases are important components of the pathogenic mechanisms of other intracellular microorganisms. *Plasmodium falciparum* is thought to use a serine protease to gain entry into red blood cells during the blood-borne stage of malaria (Braun Breton & Pereira da Silva, 1993). The *P. falciparum* gp76 and *Plasmodium chabaudi* gp68 appear to be serine proteases which specifically degrade the erythrocyte band 3 protein, thereby altering the erythrocyte cytoskeleton, and allowing entry of the parasite.

The HtrA serine protease of *S. typhimurium* also has been linked with virulence of the intracellular pathogen. Mutants with the transposon *TnpA* inserted in the

protease gene show a decreased ability to survive oxidative stress, similar to the killing mechanisms employed by macrophages (Johnson *et al.*, 1991). This protein therefore may be important in allowing the organism to survive and replicate in this usually hostile environment.

M. a. paratuberculosis also is an intracellular pathogen, which persists and replicates in macrophages, and the mechanisms by which it evades killing within these cells are currently unknown. If, however, the native 34 kDa protein uses the same physiological substrate as the *S. typhimurium* serine protease, the two proteins may perform a similar function that contributes to the intracellular survival of these pathogens.

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