Antigenicity of Recombinant Maltose Binding Protein-\textit{Mycobacterium avium} subsp. \textit{paratuberculosis} Fusion Proteins with and without Factor Xa Cleaving

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\textit{Mycobacterium avium} subsp. \textit{paratuberculosis} causes Johne’s disease (JD) in ruminants. Proteomic studies have shown that \textit{M. avium} subsp. \textit{paratuberculosis} expresses certain proteins when exposed to \textit{in vitro} physiological stress conditions similar to the conditions experienced within a host during natural infection. Such proteins are hypothesized to be expressed \textit{in vivo}, are recognized by the host immune system, and may be of potential use in the diagnosis of JD. In this study, 50 recombinant maltose binding protein (MBP)-\textit{M. avium} subsp. \textit{paratuberculosis} fusion proteins were evaluated using serum samples from sheep infected with \textit{M. avium} subsp. \textit{paratuberculosis}, and 29 (58%) were found to be antigenic. Among 50 fusion proteins, 10 were evaluated in MBP fusion and factor Xa-cleaved forms. A total of 31 proteins (62%) were found to be antigenic in either MBP fusion or factor Xa-cleaved forms. Antigenicity after cleavage and removal of the MBP tag was marginally enhanced.

Johne’s disease (JD) in ruminants is a chronic infection of the intestines caused by \textit{Mycobacterium avium} subsp. \textit{paratuberculosis}. Economic losses arise due to culling, reduced production of milk and wool, and mortalities (1, 2). The disease is characterized by a long incubation period, and subclinical infection creates a potential source of infection for uninfected animals. The most common method of detection of JD is by measuring immune responses in the infected host. The two widely used assays to measure cell-mediated and antibody-mediated immune responses are the gamma interferon (IFN-γ) assay and the enzyme-linked immunosorbent assay (ELISA), respectively. These depend on \textit{M. avium} subsp. \textit{paratuberculosis}-specific antigens for stimulating IFN-γ from memory T cells or detecting specific antibodies in blood samples. Currently used antigens in these assays are French-pressed proteins or purified protein derivatives (PPDs) derived from whole cells of \textit{M. avium} subsp. \textit{paratuberculosis}, containing a large number of antigens. The diagnostic specificity of commercial antibody ELISAs is generally high, but sensitivity is poor (3, 4). The accuracy of an ELISA also can be adversely affected by potential cross-reactions due to exposure of the host to environmental mycobacteria. The specificity of an assay can be enhanced by absorbing the serum against \textit{Mycobacterium phlei} proteins (5, 6). However, it is difficult to enhance ELISA sensitivity due to the poor Th2 responses during the lengthy latent period of the disease and the large population of infected animals with latent subclinical infections in an exposed flock or herd. Therefore, to enhance assay performance, new \textit{M. avium} subsp. \textit{paratuberculosis}-specific antigens that are expressed during latency need to be identified.

Evidence of dormancy in \textit{M. avium} subsp. \textit{paratuberculosis} when the organism was present in the soil/pasture environment was reported (7). \textit{In vitro} studies simulating the stress conditions of natural infection reported dormancy-associated proteins in \textit{M. avium} subsp. \textit{paratuberculosis} (8–10). These findings led to a hypothesis that \textit{M. avium} subsp. \textit{paratuberculosis} expresses stress/dormancy-related proteins during infection of the host. The use of \textit{M. avium} subsp. \textit{paratuberculosis} proteins that are expressed \textit{in vivo} following pathogen entry into the host as diagnostic antigens may be of value in the detection of an early stage of \textit{M. avium} subsp. \textit{paratuberculosis} infection. Indeed, some of the \textit{M. avium} subsp. \textit{paratuberculosis} proteins known to be differentially regulated under stress conditions were found to be antigenic in serum collected from sheep infected with \textit{M. avium} subsp. \textit{paratuberculosis}, and these findings support the hypothesis that stress proteins expressed \textit{in vitro} are also expressed \textit{in vivo} (11–13).

A large number of recombinant \textit{M. avium} subsp. \textit{paratuberculosis} antigens have been investigated for their diagnostic potential in cell- and antibody-mediated assays (11, 13–22). Some of these \textit{M. avium} subsp. \textit{paratuberculosis} proteins were from groups of proteins that were differentially regulated under physiological stress conditions. Although many proteins were found to be antigenic, no obvious candidate has yet been identified as having suitable diagnostic sensitivity and specificity.

A major limitation for characterization of recombinant \textit{M. avium} subsp. \textit{paratuberculosis} proteins is their expression as inclusion bodies or insoluble proteins, especially when prepared using histidine (His) as an affinity purification tag (13). Production of antigens from insoluble proteins involves processes that may be detrimental to biological activity. Expression of maltose binding protein (MBP) fusion proteins facilitates maintenance of the solubility, structure, and functions of recombinant proteins through downstream processing (23, 24). Several recombinant MBP-\textit{M. avium} subsp. \textit{paratuberculosis} fusion proteins (MBP fusion proteins) were found to be antigenic in sheep, cattle, and mice infected with \textit{M. avium} subsp. \textit{paratuberculosis} (15, 16, 25). How-

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ever, MBP alone, with a molecular mass of about 42.5 kDa, is known to have a small amount of seroreactivity; because of this, it must be used as a control in ELISAs (23). Furthermore, it is not known if the MBP protein masks the immune recognition of a protein of interest. Therefore, cleavage of the MBP tag from the recombinant M. avium subsp. paratuberculosis proteins may be beneficial for their use.

Factor Xa is a protease that specifically cleaves after the arginine residue in its preferred site Leu-(Glu or Asp)-Gly-Arg sequence (26) and can be used to separate the MBP affinity purification tag from the protein of interest following expression and purification (27, 28). This protease was used in the current study to molecular masses of MBP and the relevant MBP fusion proteins.

**TABLE 1** Evaluation and comparison of recombinant M. avium subsp. paratuberculosis proteins (MBP fusion and His-tagged proteins)

| Protein type and function | Protein(s) |
|---------------------------|------------|
| MBP fusion proteins       |            |
| Phosphate metabolism      | MAP2487c, MAP3567 |
| Cold shock protein        | MAP0217c, MAP1272c, MAP1339 |
| Putative virulence factor | MAP2398c, MAP2658c |
| Universal stress proteins | MAP339 |
| Cell wall synthesis       | MAP3968c |
| Signal recognition        | MAP2964c, MAP3200 |
| ATP and purine biosynthesis | MAP4200c, MAP3393c |
| Cell division             | MAP0068, MAP1889c |
| Heat shock protein chaperone | MAP3268, MAP3701c |
| Response regulators       | MAP0834c, MAP3200 |
| Protein synthesis         | MAP1027c, MAP4125 |
| Proteolysis               | MAP1834c, MAP2280c, MAP2281c |
| Amino acid metabolism     | MAP1293, MAP1297, MAP1846c, MAP2864c |
| Antioxidant enzymes       | MAP1588c, MAP1589c, MAP1653, MAP3635c, MAP4340 |
| Hypothetical protein      | MAP0593c, MAP0184c, MAP1586, MAP3555, MAP3864 |
| Fatty acid metabolism     | MAP3538c, MAP3567 |
| Cellular processes        |            |
| MAP2487c, MAP3567         | |
| His-tagged proteins       |           |
| Putative virulence factor | MAP1272c |
| Fatty acid metabolism     | MAP2698c |
| Cellular processes        | MAP2487c, MAP3567 |

**MATERIALS AND METHODS**

Antigens. The M. avium subsp. paratuberculosis proteins in this study (Table 1) were selected based on their expression in response to in vitro physiological stress conditions (8–10). Fifty M. avium subsp. paratuberculosis recombinant proteins used in this study were produced as MBP fusion proteins at the Bacterial Diseases of Livestock Research Unit, USDA-ARS Agricultural Research Service National Animal Disease Center (NADC) (Ames, IA), and one (MAP1272c) was produced as a His-tagged recombinant M. avium subsp. paratuberculosis protein (29). The MBP fusion proteins were produced as described previously (30). Briefly, the full-length coding sequence of the M. avium subsp. paratuberculosis protein was amplified using gene-specific primers and was cloned into the pMAL-c2 translational fusion expression vector. The vector and amplified products were digested with XbaI and HindIII, and the ligated products were transformed into Escherichia coli DH5α cells. The overexpressed proteins were extracted and purified by affinity chromatography with amylose resin columns (New England BioLabs). Three His-tagged recombinant M. avium subsp. paratuberculosis proteins (MAP2698c, MAP2487c, and MAP3567) were produced at the Faculty of Veterinary Science, University of Sydney (Sydney, Australia), as previously described (12). Briefly, gene-specific primers were designed to include attB1 and attB2 sites at the 5′ end of each sequence. The complete open reading frames of each gene were amplified by PCR using Gateway technology (Invitrogen, Australia). Amplified and purified PCR products were cloned into the donor vector pDONR221 (Invitrogen, Australia) to produce an entry clone. Purified entry clones were subcloned into the destination vector pET160-DEST with an N-terminal 6×His and Lumio tag (Champion pET160 Gateway expression kit with Lumio technology; Invitrogen, Australia) and transformed into One Shot TOP10 chemically competent E. coli cells (Invitrogen, Australia) to produce an expression clone. One Shot BL21 Star (DE3) cells (Invitrogen, Australia) were transformed with the purified expression clone. The transformed culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside to express the recombinant proteins. Recombinant proteins were extracted and purified by affinity liquid chromatography (AKTApurifier system; GE Healthcare). The four His-tagged recombinant M. avium subsp. paratuberculosis proteins (1 from the NADC and 3 from the University of Sydney) were compared with the corresponding MBP fusion proteins.

**Proteolytic cleavage of MBP fusion proteins.** A pilot study was performed with MBP fusion proteins (MAP0435c, MAP1846c, and MAP1017c) to determine the optimal time for enzymatic cleavage of the M. avium subsp. paratuberculosis protein from the MBP tag. Factor Xa (Amersham Biosciences) was reconstituted to a final concentration of 1 μl/ml in nuclease-free water at 4°C. MBP fusion proteins were diluted to 1 mg/ml in phosphate-buffered saline (PBS). Factor Xa (1 μl/ml) of MBP fusion proteins was added to the MBP fusion protein (1 mg) in a 1.5-ml screw-cap tube and mixed briefly with a vortex mixer. Reaction buffer (100 μl) was added to the mixture, which was vortex mixed briefly and incubated for 16 to 40 h at room temperature (RT) (22 to 24°C). Fifteen-microliter aliquots were collected after 16, 18, 20, 25, 30, 35, and 40 h of incubation for SDS-PAGE analysis. The optimal cleavage time was determined for each protein based on the appearance of two bands that corresponded to the expected molecular masses of MBP and the relevant M. avium subsp. paratuberculosis protein.

Due to the limited volume (500 μl) of MBP fusion proteins, only 22 proteins with volumes and concentrations adequate for cleavage and antigenicity evaluation were available. A reaction mixture containing 1 ml of MBP fusion protein (1 mg), 10 μl (10 units) of factor Xa, and 1 ml of reaction buffer in a 5-ml screw-cap tube was prepared, vortex mixed briefly, and incubated for up to 40 h at RT. Aliquots of 15 μl were collected at various incubation times (16, 18, 20, 25, 30, and 40 h) and examined by SDS-PAGE analysis. The SDS-PAGE analysis. SDS-PAGE analyses of proteins were performed using 12% precast polyacrylamide gels (Mini-PROTEAN TGX precast gel, product no. 456–1043; Bio-Rad). Briefly, a 15-μl aliquot of each protein sample (MBP-LaCZ, MBP-M. avium subsp. paratuberculosis fusion protein, factor Xa-cleaved M. avium subsp. paratuberculosis protein, pu-
rifed MBP, or purified *M. avium* subsp. *paratuberculosis* protein) was placed in a 1.5-ml screw-cap tube. The protein samples were mixed with 3 μl of reducing sample buffer and heated for 5 min in a boiling water bath. Protein samples (18 μl) were loaded onto a precast gel, and electrophoresis was performed at a constant voltage of 180 V (SmartPower 4000 power pack) for 65 min or until the visible line of bromphenol blue reached the bottom of the gel, using a Mini-PROTEAN 3 cell system (Bio-Rad). Protein bands were stained with Coomassie brilliant blue (0.1% Coomassie brilliant blue G-250, 3% ortho-phosphoric acid, 10% ammonium sulfate, and 20% methanol). The protein bands were visualized with a Geldoc system (Bio-Rad).

**Affinity chromatography purification of cleaved proteins.** Purification of cleaved MBP fusion proteins was performed with an AKTApurifier fast performance liquid chromatography (FPLC) system (GE Healthcare) using a MBPTrap HP dextran-Sepharose high performance column (5 ml; GE Healthcare), following the manufacturer’s manual. Briefly, the column was equilibrated with 5 column volumes (CVs) of binding buffer at a flow rate of 2 ml/min. A cleaved protein sample solution (1 ml) was applied using a 1-ml sample loop, followed by washing with 5 CVs of binding buffer to remove unbound protein (*M. avium* subsp. *paratuberculosis* protein), at a flow rate of 5 ml/min. The flowthrough protein peaks were collected in 2-ml fractions. The bound MBP was eluted with 5 CVs of elution buffer at a flow rate of 5 ml/min, and protein peaks were collected in 2-ml fractions. The column was reequilibrated with 5 CVs of binding buffer at a flow rate of 5 ml/min. The cleaved and purified *M. avium* subsp. *paratuberculosis* proteins were dialyzed against PBS overnight at 4°C using a 12-kDa-cutoff membrane tube. Proteins were concentrated by centrifuging the dialyzed protein solution at 2,000 × g for 10 min at 4°C (AllegraX-12R; Beckman Coulter), using Amicon Ultra-15 centrifugal filter units (nominal molecular mass limit of 10 kDa). The protein yield in the retentate was estimated using a spectrophotometer (NanoDrop 1000; Thermo Scientific) set at 280 nm.

**Serum samples.** A total of 46 sheep serum samples were obtained from the serum archive maintained at the Faculty of Veterinary Science, University of Sydney. Twenty-three serum samples were from unexposed/uninfected sheep from Western Australia (31) that were certified to be free of JD based on the negative test results for their flocks. Another 23 serum samples were obtained from exposed/infected sheep that either tested positive by tissue/fecal culture or had histopathological lesions consistent with ovine JD. Serum samples obtained from infected animals also tested positive with the Institut Pourquier ELISA (32), with a sample-to-positive ratio of >70%, and were categorized as strong reactors (n = 8), medium

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**FIG 1** SDS-PAGE analysis of pilot scale factor Xa cleavage of MBP fusion proteins. (A) MBP-MAP0435c; (B) MBP-MAP1017c; (C) MBP-MAP1846. Lanes 1, molecular mass markers (PageRuler Plus prestained protein ladder); lanes 2, MBP-LacZ (54 kDa); lanes 3, MBP-*M. avium* subsp. *paratuberculosis* fusion protein before cleavage; lanes 4 to 10, MBP-*M. avium* subsp. *paratuberculosis* fusion proteins cleaved at different time points, i.e., 16 h (lanes 4), 18 h (lanes 5), 20 h (lanes 6), 25 h (lanes 7), 30 h (lanes 8), 35 h (lanes 9), and 40 h (lanes 10).
reactors (n = 8), or low reactors (n = 7). The infected sheep were fecal culture positive (shedderv; n = 15) or negative (nonshedder; n = 8). Histopathological lesions were categorized according to a previously described method (33), as follows: no or low-grade lesion (grade 0 to 2; n = 4), paucibacillary lesion (grade 3a plus grade 3c; n = 2 + 8), or multibacillary lesion (grade 3b; n = 9).

Positive- and negative-control sheep sera were obtained from sheep from the University of Sydney that were in an independent experimental infection trial (34). The negative-control serum was obtained from a sheep not exposed to M. avium subsp. paratuberculosis that had tested negative by fecal culture, biopsied tissue culture, histopathological analysis, and ELISA. Positive-control serum was obtained from a Gudair vaccine-immunized sheep, and the serum was determined to be positive by the Institut Pourriol ELISA.

ELISA methodology. Antibody ELISA was performed to evaluate the seroreactivity of MBP fusion proteins, cleaved M. avium subsp. paratuberculosis proteins, and His-tagged recombinant M. avium subsp. paratuberculosis proteins, using serum samples from M. avium subsp. paratuberculosis-infected and uninfected sheep. Briefly, 50 μl of serum was added to 96-well flat-bottom microplates (Nunc MaxiSorp; Nunc) and incubated overnight at 4°C. The plates were washed five times with purified reverse-osmosis (RO) water with Tween 20. ELISA plates coated with MBP fusion proteins and MBP-LacZ were blocked with 4% skim milk, and plates coated with cleaved M. avium subsp. paratuberculosis proteins and His-tagged M. avium subsp. paratuberculosis proteins were blocked with 1% (vol/vol) fetal calf serum (FCS) for 30 min each. The serum samples were diluted (1:100) in a diluent (0.1% [vol/vol] FCS in PBS with Tween 20 [PBST]) containing 1.3 mg/ml heat-killed M. phlei protein (Elizabeth Macarthur Agricultural Institute, New South Wales, Australia) and were absorbed overnight at 4°C, with constant end-to-end shaking. The absorbed serum was centrifuged at 2,500 x g for 10 min at RT to separate the supernatant from the particulate M. phlei. The absorbed serum supernatant (50 μl) was added to the required wells and incubated for 1 h at RT. The plate was machine washed 5 times with 3% H2O2 followed by 3 washes of PBST containing 0.05% Tween 20. ELISA plates coated with MBP fusion proteins were washed with 3% H2O2 followed by 3 washes of PBST containing 0.05% Tween 20. The plates were coated with MBP fusion proteins, using serum samples from M. avium subsp. paratuberculosis-infected and uninfected sheep. Briefly, 50 μl of serum was added to 96-well flat-bottom microplates (Nunc MaxiSorp; Nunc) and incubated overnight at 4°C. The plates were washed five times with purified reverse-osmosis (RO) water with Tween 20. ELISA plates coated with MBP fusion proteins and MBP-LacZ were blocked with 4% skim milk, and plates coated with cleaved M. avium subsp. paratuberculosis proteins and His-tagged M. avium subsp. paratuberculosis proteins were blocked with 1% (vol/vol) fetal calf serum (FCS) for 30 min each. The serum samples were diluted (1:100) in a diluent (0.1% [vol/vol] FCS in PBS with Tween 20 [PBST]) containing 1.3 mg/ml heat-killed M. phlei protein (Elizabeth Macarthur Agricultural Institute, New South Wales, Australia) and were absorbed overnight at 4°C, with constant end-to-end shaking. The absorbed serum was centrifuged at 2,500 x g for 10 min at RT to separate the supernatant from the particulate M. phlei. The absorbed serum supernatant (50 μl) was added to the required wells and incubated for 1 h at RT. The plate was machine washed 5 times with 3% H2O2 followed by 3 washes of PBST containing 0.05% Tween 20. ELISA plates coated with MBP fusion proteins were washed with 3% H2O2 followed by 3 washes of PBST containing 0.05% Tween 20. The plates were washed five times with 3% H2O2 followed by 3 washes of PBST containing 0.05% Tween 20.

Data analysis. Statistical analysis was performed using GenStat 12.1 (VSN International Ltd., United Kingdom) and GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA) software. The seroreactivities of MBP fusion antigens, factor Xa-cleaved M. avium subsp. paratuberculosis antigens, and His-tagged M. avium subsp. paratuberculosis antigens between groups were compared by analysis of variance (ANOVA) with a Bonferroni correction for multiple comparisons, as described previously (35). The area under the receiver operating characteristic curve (AUCROC) was calculated for the ability of an assay using each antigen to discriminate between uninfected and M. avium subsp. paratuberculosis-infected sheep. An assay with an AUCROC value of 1.0 is considered to be perfect, and one with a value of 0.5 is considered worthless (36).

RESULTS

Cleavage of MBP fusion proteins on a pilot scale. Three MBP fusion proteins were cleaved by factor Xa protease in a pilot study and analyzed by SDS-PAGE (Fig. 1A to C). Factor Xa was able to cleave MBP-MAP0435c and MBP-MAP1017c after 16 h of incubation (Table 2). The SDS-PAGE analyses of cleaved products are shown in Fig. 2A to F. For each MBP fusion protein, the MBP tag was separated from the M. avium subsp. paratuberculosis protein of expected molecular mass. Four pro-

**TABLE 2** Expected molecular masses of MBP fusion and cleaved proteins

| Cluster                         | Protein   | CTb (h) | Fusion proteina | Molecular mass (kDa) of: |
|---------------------------------|-----------|---------|-----------------|--------------------------|
| Amino acid metabolism           | MAP1297   | 40      | 67.8            | 25.3                     |
|                                 | MAP1846c  | 40      | 73              | 30.5                     |
| Antioxidant enzymes             | MAP1588c  | 20      | 61.3            | 18.8                     |
|                                 | MAP1589c  | 16      | 64.1            | 21.6                     |
|                                 | MAP1653   | 16      | 58.9            | 16.4                     |
| ATP biosynthesis                | MAP3393c  | 20      | 60              | 17.5                     |
| Cell division                   | MAP0068   | 40      | 60              | 17.5                     |
| Cellular processes              | MAP1560   | 16      | 57.7            | 15.2                     |
|                                 | MAP1885c  | 16      | 60.9            | 18.4                     |
|                                 | MAP2411   | 16      | 58              | 15.5                     |
|                                 | MAP2705c  | 20      | 56.4            | 13.9                     |
|                                 | MAP3538   | 16      | 58.4            | 15.9                     |
| Fatty acid metabolism           | MAP1017c  | 16      | 70.3            | 27.8                     |
|                                 | MAP2698c  | 20      | 73.9            | 31.4                     |
|                                 | MAP3190   | 16      | 75.8            | 33.3                     |
| Heat shock protein              | MAP3268   | 16      | 58.9            | 16.4                     |
|                                 | MAP3701c  | 40      | 58.7            | 16.2                     |
| Hypothetical protein            | MAP0593c  | 40      | 57.3            | 14.8                     |
|                                 | MAP3555   | 16      | 61.3            | 18.8                     |
| Phosphate metabolism            | MAP0435c  | 16      | 61.1            | 18.6                     |
| Proteolysis                     | MAP2280c  | 20      | 65.7            | 23.2                     |
| Universal stress proteins       | MAP1339   | 20      | 57.9            | 15.4                     |

a Fusion protein mass is the total mass of MBP (42.5 kDa) and the M. avium subsp. paratuberculosis protein.

b Cleavage time.
proteins, i.e., MBP-MAP0593c, MBP-MAP3701c, MBP-MAP1297, and MBP-MAP0068c, showed weak bands of undigested fusion proteins even with 40 h of incubation. Fusion proteins such as MBP-MAP0435c, MBP-MAP3393c, MBP-MAP3268, MBP-MAP1653, MBP-MAP1846, MBP-MAP2280c, MBP-MAP1885c, and MBP-MAP0068c were found to be cleaved, but there were multiple bands of \textit{M. avium} subsp. \textit{paratuberculosis} proteins.

**SDS-PAGE analysis of cleaved and purified proteins.** Due to the limited volumes of MBP fusion proteins received from the USDA Agricultural Research Service (500 µl each), only 22 proteins were used for factor Xa cleavage experiments. Of 22 cleaved proteins, only 10 proteins had adequate volumes for affinity liquid chromatography purification.

The flowthrough (cleaved \textit{M. avium} subsp. \textit{paratuberculosis} proteins) and eluate (cleaved MBP tag) fractions were verified by SDS-PAGE analysis (Fig. 3A to E). The expected molecular masses are shown in Table 2 and Fig. 3A to E. All of the cleaved \textit{M. avium} subsp. \textit{paratuberculosis} proteins were obtained with >95% purity, based on the band appearance on SDS-PAGE analysis. The yields of purified and concentrated \textit{M. avium} subsp. \textit{paratuberculosis} proteins were approximately 20 µg/ml (MAP2698c and MAP3555), 30 µg/ml (MAP1846, MAP1885c, and MAP2280c), 40 µg/ml (MAP1017c and MAP0593c), 60 µg/ml (MAP3538), or 70 µg/ml (MAP1588c and MAP3190). Separated bands for cleaved \textit{M. avium} subsp. \textit{paratuberculosis} proteins MAP2698c and MAP3555 were faint, due to the low protein concentrations.

**Antigenicity of MBP fusion proteins.** Fifty MBP fusion proteins were evaluated, and 29 (58%) were found to be detected by antibodies in sera obtained from \textit{M. avium} subsp. \textit{paratuberculosis}-infected sheep (Tables 3 and 4). The remaining 42% (21/50) of the proteins were not able to differentiate infected from uninfected animals. The seroreactivity of the control MBP-LacZ was not significantly different in the infected and uninfected groups ($P > 0.05$). The greatest ability to differentiate the infected group from the uninfected group was observed for the MAP0516c protein, which is encoded by \textit{echA20} and is involved in fatty acid metabolism (AUC$_{ROC} = 0.758$, $P = 0.001$), MAP2872c, which is encoded by \textit{fabG5} and is a 3-ketoacylreductase (AUC$_{ROC} = 0.765$, $P = 0.001$), and MAP1834c, which is encoded by \textit{prcA} and is a proteome subunit protein (AUC$_{ROC} = 0.765$, $P = 0.002$).

Among the MBP fusion proteins evaluated, 19/50 (38%) were able to produce significantly higher OD values in serum samples obtained from sheep with paucibacillary infections, compared with sera from unexposed sheep ($P < 0.05$). Simi-
larly, 26/50 proteins (52%) were able to produce significantly higher OD values in serum samples that were low reactors in the Institut Pourquier ELISA, in comparison with sera from unexposed sheep (P < 0.05) (Tables 3 and 4). However, none of the proteins was able to detect infections that were associated with no or low-grade lesions.

Antigenicity of cleaved and MBP fusion proteins. Successfully cleaved and purified M. avium subsp. paratuberculosis proteins were evaluated and ELISA results were compared with those for fused forms (Table 4). The OD values of infected sheep sera were significantly higher than those of the uninfected sheep sera for all factor Xa-cleaved proteins except MAP1885c. The MBP fusion proteins MAP0593c and MAP2698c were not able to differentiate between infected and uninfected sheep, but when they were evaluated as cleaved proteins purified from the MBP fusion proteins, the OD values of the infected group were significantly higher (P < 0.05) than those of the uninfected group. Protein MAP1885c was not able to differentiate between the infected and uninfected groups in either the fused or cleaved forms. Based on AUCROC values, factor Xa cleavage did not enhance the immunoreactivity of four proteins (MAP1846, MAP1588c, MAP1885c, and MAP3555). The M. avium subsp. paratuberculosis-specific immunoreactivity of two proteins (MAP2698c and MAP0593c) was enhanced while that of three proteins (MAP3538, MAP1017c, and MAP2280c) was marginally enhanced by cleavage of MBP. In general, the ability of cleaved proteins to differentiate the infected and uninfected groups was enhanced in only 50% of the proteins (5/10 proteins).

Antigenicity evaluation of His-tagged recombinant M. avium subsp. paratuberculosis proteins. All four His-tagged recombinant M. avium subsp. paratuberculosis proteins were able to differentiate between the infected and uninfected sheep sera (P < 0.05) (Table 5). The order of most antigenic to least antigenic proteins was MAP1272c > MAP3567 > MAP2487c > MAP2698c, with AUCROC values of 0.90, 0.75, 0.70, and 0.69, respectively.

The seroreactivity of two different protein expression systems (His tag and MBP fusion) were compared for MAP2698c, MAP1272c, MAP2487c, and MAP3567. All four proteins were able to differentiate between the infected and uninfected groups of sheep in both expression systems. The OD values obtained from an infected group of sheep using His-tagged recombinant proteins were significantly higher (P < 0.05) than those obtained using the corresponding MBP fusion proteins (Table 6).

The ELISA results obtained from the infected sheep using MAP2698c protein as a MBP fusion, factor Xa-cleaved, or His-tagged protein were analyzed by one-way ANOVA with a Bonferroni correction for multiple comparisons. The OD values obtained using factor Xa-cleaved M. avium subsp. paratuberculosis proteins and His-tagged M. avium subsp. paratuberculosis proteins were significantly higher than those obtained with MBP fu-
TABLE 3 Antigenicity evaluation of MBP fusion proteins

| Protein | Protein conc (µg/ml) | Unexposed samples (23) |Exposed samples (23) | P | AUCROC |
|---------|---------------------|------------------------|---------------------|---|---------|
| MAP0068^a | 5 | 0.133 | 0.218 | 0.002 | 0.71 |
| MAP0184c,b | 10 | 0.215 | 0.355 | 0.002 | 0.745 |
| MAP0187c | 5 | 0.236 | 0.31 | 0.118 |
| MAP0435c | 20 | 0.278 | 0.291 | 0.797 |
| MAP0508b,c | 6 | 0.3 | 0.501 | 0.004 | 0.713 |
| MAP0516c,b | 5 | 0.257 | 0.439 | <0.001 | 0.758 |
| MAP0540 | 7 | 0.298 | 0.36 | 0.238 |
| MAP0810 | 10 | 0.187 | 0.241 | 0.111 |
| MAP0834c,b | 5 | 0.141 | 0.216 | 0.002 | 0.742 |
| MAP1027c | 10 | 0.176 | 0.234 | 0.083 |
| MAP1272c,b | 10 | 0.26 | 0.451 | 0.005 | 0.703 |
| MAP1293c,b | 5 | 0.186 | 0.292 | 0.012 | 0.717 |
| MAP1297c,b | 10 | 0.172 | 0.321 | 0.002 | 0.744 |
| MAP1339 | 10 | 0.204 | 0.274 | 0.067 |
| MAP1560 | 7 | 0.252 | 0.273 | 0.51 |
| MAP1586 | 5 | 0.285 | 0.311 | 0.515 |
| MAP1589c | 10 | 0.183 | 0.269 | 0.02 | 0.677 |
| MAP1653c | 5 | 0.195 | 0.263 | 0.045 | 0.683 |
| MAP1834c,b | 9 | 0.262 | 0.44 | 0.002 | 0.765 |
| MAP1889c | 5 | 0.215 | 0.203 | 0.652 |
| MAP2058c,b | 6 | 0.265 | 0.442 | 0.005 | 0.703 |
| MAP2281c | 7 | 0.29 | 0.281 | 0.773 |
| MAP2440c,b | 8 | 0.202 | 0.293 | 0.045 | 0.677 |
| MAP2487c | 8 | 0.307 | 0.331 | 0.576 |
| MAP2705c | 10 | 0.208 | 0.228 | 0.541 |
| MAP2864c,b | 8 | 0.336 | 0.455 | 0.35 | 0.658 |
| MAP2872c | 8 | 0.166 | 0.289 | <0.001 | 0.765 |
| MAP3007b,c | 7 | 0.211 | 0.357 | 0.003 | 0.74 |
| MAP3200c | 10 | 0.216 | 0.339 | 0.008 | 0.699 |
| MAP3268 | 10 | 0.272 | 0.229 | 0.386 |
| MAP3393c | 10 | 0.261 | 0.291 | 0.497 |
| MAP3567c,b | 1 | 0.137 | 0.234 | 0.002 | 0.728 |
| MAP3577 | 7 | 0.194 | 0.215 | 0.465 |
| MAP3651c,b | 5 | 0.208 | 0.323 | 0.01 | 0.695 |
| MAP3701c | 10 | 0.166 | 0.198 | 0.274 |
| MAP3864 | 10 | 0.208 | 0.289 | 0.048 | 0.642 |
| MAP3968b,c | 7 | 0.154 | 0.277 | 0.002 | 0.719 |
| MAP4125 | 10 | 0.284 | 0.292 | 0.847 |
| MAP4340c | 10 | 0.194 | 0.296 | 0.006 | 0.732 |

^a Proteins able to produce significantly higher OD values for serum samples obtained from sheep with paratuberculosis than for serum samples from uninfected sheep.

^b Proteins able to produce significantly higher OD values for serum samples that were low reactors in the Institut Pourquier ELISA than for serum samples from uninfected sheep.

^c Mean OD values for the exposed group are significantly higher than those for the unexposed group (P < 0.05).

Discussion proteins (P < 0.001). The OD values obtained with factor Xa-cleaved M. avium subsp. paratuberculosis proteins and His-tagged M. avium subsp. paratuberculosis proteins were similar (P > 0.05).

**DISCUSSION**

This study investigated the antigenicity of M. avium subsp. paratuberculosis proteins in either MBP-fused, factor Xa-cleaved, or His-tagged forms, using sera that were known to have M. avium subsp. paratuberculosis-specific antibodies, as detected by the commercial Institut Pourquier ELISA (32). The proportion of antigenic proteins increased from 58% to 62% after factor Xa cleavage. Sufficient amounts of MBP fusion proteins were available for 22 proteins to be cleaved using factor Xa. Ten of 22 proteins were successfully purified and evaluated in both MBP fusion and factor Xa-cleaved forms. Four proteins were evaluated in the His-tagged form to compare the expression systems.

Factor Xa cleaves proteins after the arginine residue in its preferred cleavage site Ile-(Glu or Asp)-Gly-Arg in the fusion between MBP and the target protein. However, reports suggest that it quite often cleaves at secondary sites, depending on the conformation of the protein substrate (37, 38). The most common secondary site is Gly-Arg, usually in a partially unfolded protein (39). The probable cause of the appearance of multiple bands on SDS-PAGE gels in this study may be proteolysis or cleavage of target proteins at such secondary sites. Furthermore, on screening of the amino acid sequences of proteins that showed multiple bands on SDS-PAGE gels, several such sites were identified in proteins MAP0435c, MAP1846, and MAP1017c. MAP0435c had two Gly-Arg sites (amino acid positions 24-25 and 142-143), MAP1846 had three Gly-Arg sites (amino acid positions 68-69, 99-100, and 159-160), and MAP1017c had one Gly-Arg site (amino acid positions 168-169).

The protein cleavage experiment showed that 1 unit of factor Xa was sufficient to cleave 100 µg of MBP fusion protein after as little as 18 h of incubation at RT (22 to 23°C), and this finding was consistent with the findings from another study (40). However, factor Xa cleavage was not complete even after 40 h of incubation for proteins MBP-MAP0068c, MBP-MAP0593c, MBP-MAP1297, MBP-MAP1846c, and MBP-MAP3701c. The probable causes of incomplete cleavage may be an inaccessible factor Xa recognition site or alteration of the factor Xa recognition site during cloning (41). This process of protein cleavage, and particularly the need for such long cleavage times, is not well understood, and further investigation may be useful.

Factor Xa is a heterodimer protease composed of two disulfide-linked polypeptide chains with apparent molecular masses of 17 and 42 kDa (42). In this study, no obvious bands of these sizes were observed, which may be due to too low a concentration to be detected in SDS-PAGE analysis. Thus, removal of factor Xa using p-aminobenzamidine resin was not necessary. ELISA results for MBP fusion proteins were analyzed using OD values obtained for MBP fusion proteins without subtracting the OD values obtained for MBP-LacZ to allow for a comparative analysis between the MBP fusion proteins and the factor Xa-cleaved forms. This was justified, as levels of seroreactivity to MBP-LacZ were not significantly different between infected and uninfected samples.

The MBP fusion proteins evaluated in this study were previously reported to be differentially regulated under different in vitro stress conditions (8–10). More than one-half of the 50 MBP fusion proteins evaluated were found to be antigenic, suggesting that the majority of these proteins are expressed by M. avium subsp. paratuberculosis in vivo and are recognized by the host immune response. A recent study on the antigenicity of some of these proteins expressed as His-tagged recombinant proteins showed that 66% of the proteins (18/27 proteins) were detected by sera from M. avium subsp. paratuberculosis-infected sheep (13). Of those 27 proteins, 22 were included in this study. The proteins that were found to be antigenic in both studies are MAP0516c, MAP0435c, MAP1293c, MAP1560, MAP1586, MAP1589c, and MAP2411.
The proteins found to be antigenic were from clusters such as amino acid metabolism (four proteins), antioxidant enzymes (four proteins), fatty acid metabolism (six proteins), hypothetical proteins (three proteins), proteolysis (two proteins), two-component response regulators (two proteins), and cellular processes (three proteins), as well as cell wall synthesis, cell division, ATP biosynthesis, signal recognition, and a putative virulence factor (one protein each). These clusters of *M. avium* subsp. *paratuberculosis* proteins recognized by the host immune system may be involved in the ability of *M. avium* subsp. *paratuberculosis* to evade host defense mechanisms, which may be augmented by *in vivo* expression. Antioxidant enzymes are important for protecting *M. avium* subsp. *paratuberculosis* from the oxidative stress response mounted by the host (10, 44, 45); similarly, proteins involved in fatty acid metabolism play important roles in intracellular survival, growth of mycobacteria, and pathogenicity in mycobacterial infections (46). Proteins and lipoproteins associated with cell wall synthesis and lipid membranes are critical for protecting mycobacteria from damage (47).

In conclusion, this study has identified several stress-regulated *M. avium* subsp. *paratuberculosis* proteins as being antigenic in infected sheep. Proteins that were found to be able to detect paucibacillary infections in Institut Pourquier ELISA low reactors may be of potential use in early diagnosis. The proteins that were identified as antigenic in this study (MBP fusion or cleaved) and

### TABLE 4 Antigenicity evaluation of MBP fusion and factor Xa-cleaved proteins

| Protein      | OD<sub>450</sub> (μg/ml) | OD<sub>450</sub> (μg/ml) | OD<sub>450</sub> (μg/ml) | OD<sub>450</sub> (μg/ml) | AUCROC | AUCROC |
|--------------|---------------------------|---------------------------|---------------------------|---------------------------|--------|--------|
| MAP0593c     | 0.150 NE 0.003 0.685      | 0.153 0.206 0.033 0.662   | 0.178 0.23 0.029 0.708    | 0.166 0.169 0.864         | 0.239 0.344 0.002 0.786 |
| MAP1017c     | 0.172 0.247 0.022 0.703   | 0.188 0.264 0.001 0.792   | 0.203 0.311 0.002 0.738   |
| MAP1846c     | 0.207 0.268 0.15 0.702    | 0.197 0.239 0.007 0.7 8    |
| MAP1885c     | 0.158 0.239 0.002 0.702   | 0.166 0.169 0.864         |
| MAP2280c     | 0.237 0.261 0.659 0.702   | 0.239 0.344 0.002 0.786   |
| MAP2487c     | 0.331 NE 0.004 0.697      | 0.004 0.685 0.007 0.70    | 0.005 0.703 0.009 0.751   |
| MAP2698c     | 0.196 0.279 0.027 0.702   | 0.188 0.264 0.001 0.792   | 0.203 0.311 0.002 0.738   |
| MAP3538c     | 4 0.207 0.268 0.15 0.702  | 0.197 0.239 0.007 0.7 8    |
| MAP3555c     | 10 0.222 0.327 0.007 0.708 | 0.165 0.224 0.03 0.668   |
| MAP3567c     | 10 0.172 0.247 0.022 0.703 | 0.188 0.264 0.001 0.792   | 0.203 0.311 0.002 0.738   |

### TABLE 5 Antigenicity evaluation of His-tagged proteins

| Protein      | OD<sub>450</sub> | OD<sub>450</sub> | OD<sub>450</sub> | OD<sub>450</sub> | AUCROC | AUCROC |
|--------------|-------------------|-------------------|-------------------|-------------------|--------|--------|
| MAP1272c     | 0.150 NE 0.003 0.685 | 0.153 0.206 0.033 0.662 | 0.178 0.23 0.029 0.708 | 0.166 0.169 0.864 | 0.239 0.344 0.002 0.786 |
| MAP2487c     | 0.331 NE 0.004 0.697 | 0.004 0.685 0.007 0.70 | 0.005 0.703 0.009 0.751 | 0.150 NE 0.003 0.685 | 0.003 0.685 0.007 0.70 |
| MAP2698c     | 0.196 0.279 0.027 0.702 | 0.188 0.264 0.001 0.792 | 0.203 0.311 0.002 0.738 |
| MAP3567c     | 4 0.207 0.268 0.15 0.702 | 0.197 0.239 0.007 0.7 8 | 0.165 0.224 0.03 0.668 | 0.239 0.344 0.002 0.786 | 0.203 0.311 0.002 0.738 |

### TABLE 6 Comparison of antigenicity of MBP fusion, His-tagged, and factor Xa-cleaved proteins

| Protein      | Data by protein type |
|--------------|---------------------|
|              | MBP fusion | His tagged | Factor Xa cleaved |
|              | AUCROC | AUCROC | AUCROC |
| MAP1272c     | 0.002 0.728 | <0.0001 0.90 | NEb NE |
| MAP2487c     | 0.331 NE | 0.004 0.697 | NE NE |
| MAP2698c     | 0.150 NE | 0.003 0.685 | 0.007 0.70 | 0.005 0.703 0.009 0.751 |

a P and AUCROC values are for comparisons between infected and uninfected groups of sheep. Mean OD values for the exposed group are significantly higher than those for the unexposed group (P < 0.05).
b NE, not examined.
by Kawaji et al. (His-tagged) (13) may be of potential use in the diagnosis and control of JD. The antigenicity results for cleaved or His-tagged M. avium subsp. paratuberculosis proteins were marginally superior to those for MBP fusion forms. Further evaluation of these proteins using a larger panel of serum samples without the bias of prior positive ELISA results is required to select potentially useful proteins for diagnosis.

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