JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2004, p. 106–114 Vol. 42, No. 1
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.1.106–114.2004

Expression and Immunogenicity of Proteins Encoded by Sequences Specific to *Mycobacterium avium* subsp. *paratuberculosis*

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Received 3 June 2003/Returned for modification 22 July 2003/Accepted 26 August 2003

The development of immunoassays specific for the diagnosis of Johne's disease in cattle requires antigens specific to *Mycobacterium avium* subsp. *paratuberculosis*. However, because of genetic similarity to other mycobacteria comprising the *M. avium* complex, no such antigens have been found. Through a comparative genomics approach, 21 potential coding sequences of *M. avium* subsp. *paratuberculosis* that are not represented in any other mycobacterial species tested ($n = 9$) were previously identified (J. P. Bannantine, E. Baechler, Q. Zhang, L. Li, and V. Kapur, J. Clin. Microbiol. 40:1303-1310, 2002). Here we describe the cloning, heterologous expression, and antigenic analysis of these *M. avium* subsp. *paratuberculosis*-specific sequences in *Escherichia coli*. Nucleotide sequences representing each unique predicted coding region were amplified and cloned into two different *E. coli* expression vectors encoding polyhistidine or maltose binding protein (MBP) affinity purification tags. All 21 of the MBP fusion proteins were successfully purified under denaturing conditions and were evaluated in immunoblotting studies with sera from rabbits and mice immunized with *M. avium* subsp. *paratuberculosis*. These studies showed that 5 of the 21 gene products are produced by *M. avium* subsp. *paratuberculosis* and are antigenic. Immunoblot analysis with a panel of sera from 9 healthy cattle and 10 cattle with clinical disease shows that the same five *M. avium* subsp. *paratuberculosis* proteins are also detected within the context of infection. Collectively, these studies have used a genomic approach to identify novel *M. avium* subsp. *paratuberculosis* antigens that are not present in any other mycobacteria. These findings may have a major impact on improved diagnostics for Johne's disease.

Johne's disease, caused by infection of ruminant animals with *Mycobacterium avium* subsp. *paratuberculosis*, has resulted in significant economic losses to the dairy and beef cattle industries (7, 29). A recent survey estimated that 21% of dairy herds in the United States are infected with *M. avium* subsp. *paratuberculosis* and that producers lose $227 annually for each infected animal, totaling more than $200 million annually (29). The control of Johne's disease has long been hampered by diagnostic tests that cannot consistently detect infected animals (17, 38). A major challenge in controlling Johne's disease is the ability to detect *M. avium* subsp. *paratuberculosis*-infected cattle prior to the appearance of disease signs, such as fecal shedding of *M. avium* subsp. *paratuberculosis* in the environment. In particular, subclinical infections are difficult to diagnose due to the lack of disease signs and intermittent shedding of the pathogen (34).

With this critical need for improved diagnostic tests to detect paratuberculosis infection, efforts need to be concentrated on the development of simple, rapid, noninvasive tests that can be performed by veterinarians or producers without expensive laboratory equipment. Bacteriologic culture is the most definitive method of diagnosis, since it can detect infection during both the subclinical and clinical stages of disease, but it is time-consuming, requiring as many as 12 weeks of incubation, and also labor-intensive (taking medium preparation into account). Contamination is an added problem when *M. avium* subsp. *paratuberculosis* is being cultured from fecal specimens. The intradermal skin test evaluates the delayed-type hypersensitivity reaction of an animal by injection of *M. avium* subsp. *paratuberculosis* extracts; however, problems involving antigenic cross-reactivity with other mycobacteria have limited its usefulness (12). Serologic tests for diagnosis of paratuberculosis such as agar gel immunodiffusion, enzyme-linked immunosorbent assay (ELISA), and complement-fixation are relatively easy to perform but suffer from a lack of specificity (8, 11). In tests that measure a cell-mediated host response, it has been demonstrated that young calves and uninfected cattle often respond to mycobacterial whole-cell antigen mixtures or secreted proteins in the gamma interferon test without showing any evidence of infection (22); that study also suggests that specificity could be improved by using defined, specific antigens. Currently, all antigen-based diagnostic tests for Johne's disease use a complex mixture of antigens, many of which are highly conserved among mycobacterial species. New approaches are needed to identify specific DNA sequences or antigens that will yield better diagnostic reagents for Johne's disease. However, no *M. avium* subsp. *paratuberculosis*-specific antigens have been described to date.

Although several antigens have been identified in mycobacteria, particularly *Mycobacterium tuberculosis*, fewer than 20 have been identified in *M. avium* subsp. *paratuberculosis*. Among these are the heat shock proteins GroES (9) and GroEL (14), 2 alkyl hydroperoxide reductases (25), a serine protease (6), superoxide dismutase (21), and 11 other proteins of unknown function that are named on the basis of their sizes

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in kilodaltons (3, 10, 13, 15, 16, 23, 24, 26–28, 33, 37). Some of these antigens appear to show promise, but none has been incorporated as a routine diagnostic tool according to the published literature and available commercial tests. This may be due, at least in part, to the presence of these antigens in other mycobacterial species. The close genetic relationship between *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* has hindered progress in the identification of specific antigens for *M. avium* subsp. *paratuberculosis*. Greater than 95% nucleotide similarity exists between most strains of *M. avium* subsp. *paratuberculosis* and *M. avium* (4, 18, 32). Their genome sizes are also similar at ~5 million bases. This has led to the proposal that the organism previously known as *Mycobacterium paratuberculosis* be considered a subspecies of *M. avium* subsp. *avium* (36). Work from our laboratories reveals substantial levels of genetic identity even above that previously described in the literature, further complicating efforts at developing a specific and sensitive diagnostic test (4).

The recent completion of the *M. avium* subsp. *paratuberculosis* genome project (L.-L. Li, J. P. Bannantine, Q. Zhang, D. Alt, A. Amonsin, and V. Kapur, unpublished data) will herald a dramatic acceleration in the understanding of the biology of this significant veterinary pathogen. Another critical step will be learning the functional roles of all the proteins encoded by the genome. Achievement of this objective depends on the rapid expression and purification of proteins on a large scale. A comparison between the recently completed genomes of *M. avium* subsp. *paratuberculosis* and *M. avium* strain 104 (sequenced at The Institute for Genomic Research) has already been performed (2). That study identified 21 predicted coding sequences that are specific to *M. avium* subsp. *paratuberculosis*. The specificity of these 21 sequences was maintained following analysis of 9 additional mycobacterial species by PCR amplification (2). In this study, we address the critical importance of identifying species-specific antigens for the serodiagnosis of Johne's disease through the cloning, heterologous expression, and analysis of those 21 unique predicted coding sequences found in the *M. avium* subsp. *paratuberculosis* genome.

MATERIALS AND METHODS

Bacterial strains. *M. avium* subsp. *paratuberculosis* K-10, a bovine clinical isolate from a dairy herd in Wisconsin, was cultured in Middlebrook 7H9 broth (pH 6.0) supplemented with oleic acid-albumin-dextrose-catalase (Becton Dickinson Microbiology, Sparks, Md.), 0.05% Tween 80, and ferric mycobactin J (2 mg/liter; Allied Monitor Inc., Fayette, Mo.). This strain served as the source of the template for all PCR amplifications. *Escherichia coli* DH5α was routinely grown in Luria-Bertani broth (LB) and maintained on LB agar plates. All maltose binding protein (MBP) fusions were constructed in this strain. *E. coli* TOP10F′ and *E. coli* BL21(DE3) (both from Invitrogen Life Technologies, Carlsbad, Calif.) cells were cultured in SOC medium prepared according to Sambrook et al. (31a). When appropriate, ampicillin (50 or 100 μg/ml) was added to the medium for selection. Frozen stocks were prepared by harvesting mid-log-phase cells in fresh LB supplemented with 20% glycerol and storing at −80°C.

Cloning of *M. avium* subsp. *paratuberculosis*-specific sequences. MBP fusions of 21 *M. avium* subsp. *paratuberculosis* predicted coding sequences were produced in *E. coli* by using the pMAL-c2 vector (New England Biolabs, Beverly, Mass.). These coding sequences and their nucleotide accession numbers have been described previously (2) and are designated genes 10, 11, 38, 56, 57, 135, 159, 217, 218, 219, 228, 240, 241, 250, 251, 252, 253, 254, 255, 256, and 257. Primers were designed from the reading frame of each coding sequence and contained an *Xba*I site in the 5′ primer and a *Hin*dIII site in the 3′ primer for cloning purposes. Amplifications were performed by using *Pwo* polymerase (Roche Diagnostics Corp., Indianapolis, Ind.) and *M. avium* subsp. *paratuberculosis* genomic DNA as the template under conditions described previously (2). The vector and amplification product were digested with *Xba*I and *Hin*dIII. Ligation of these restricted DNA fragments resulted in an in-frame fusion between the *malE* gene in the vector and the reading frame of interest. Following ligation, the products were transformed into *E. coli* DH5α and selected on LB agar plates containing 100 μg of ampicillin/ml. The pCRT7/CT-TOPO vector (Invitrogen Life Technologies) was used to produce polyhistidine (six-His)-tagged *M. avium* subsp. *paratuberculosis* proteins. No restriction sites were engineered in primers used in these amplifications, because each gel-purified PCR product was cloned into the vector by using topoisomerase. The construct was transformed into *E. coli* TOP10F′ cells and then subcloned into the BL21(DE3)/pLysS cells (Invitrogen Life Technologies). Transformed cells were plated on LB agar containing 50 μg of ampicillin/ml. Inserts in all plasmid constructions were confirmed by DNA sequencing. Table 1 lists the primers used to clone each gene in the *E. coli* expression vectors pMAL-c2 and pCRT7/CT-TOPO.

Protein expression. Each MBP fusion protein (e.g., MBP-gene 218) was overexpressed in *E. coli* by induction with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, Mo.) and purified by affinity chromatography using an amylose resin supplied by New England Biolabs. A detailed protocol has been published previously (1). Expression of mycobacterial fusion proteins was monitored by GelCode Blue (Pierce Biotechnology Inc., Rockford, Ill.)-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and by immunoblot analysis with a monoclonal antibody against MBP (α-MBP) developed at the National Animal Disease Center (NADC). The same approach was used for production and purification of all MBP fusion proteins. *E. coli* DH5α harboring the parental plasmid pMAL-c2 was expressed, purified, and used as a control in all experiments. Purified protein from this control strain consists of an MBP fusion of the LacZ alpha peptide. Expression of six-His-tagged *M. avium* subsp. *paratuberculosis* proteins was similarly induced with 0.3 mM IPTG. Three hours following induction, the cells were lysed by sonication and the recombinant protein was purified on TALON resin (Clontech, Palo Alto, Calif.) columns under denaturing conditions. Mycobacterial genes were expressed as soluble proteins with a C-terminal six-His tag downstream of the gene of interest. Expression of mycobacterial protein was monitored by immunoblot analysis with a horseradish peroxidase-conjugated monoclonal antibody to the six-His tag (Clontech). Purified fractions were pooled and dialyzed by using Slide-A-Lyzer cassettes (Pierce Biotechnology Inc.) in 1 liter of phosphate-buffered saline (PBS, comprising 150 mM NaCl and 10 mM $NaPO_4$ [pH 7.4]) with three exchanges at 4°C.

SDS-PAGE and immunoblotting. *E. coli* protein lysates were prepared as previously described (30). PAGE was performed using 12% (wt/vol) polyacrylamide gels. Proteins were electrophoretically transferred onto pure nitrocellulose filters (Schleicher & Schuell, Keene, N.H.) with the Trans Blot Cell (Bio-Rad Laboratories, Hercules, Calif.) in sodium phosphate buffer (25 mM; pH 7.8) at 0.8 amps for 90 min. After transfer, filters were blocked with PBS plus 2% bovine serum albumin (BSA) and 0.1% Tween 20 (PBS-BSA). Antisera were diluted as listed in Table 2 in PBS-BSA and were incubated on the blot at room temperature for 2 h. After three washes in PBS plus 0.1% Tween 20, blots were incubated for 1.5 h in either protein A-peroxidase (Pierce Biotechnology Inc.) or peroxidase-conjugated antibodies that detect mouse and cow antibodies (Pierce Biotechnology Inc.). All secondary antibodies were diluted 1:20,000 in PBS-BSA. The blots were again washed three times as described above and were developed for chemiluminescence by using Supersignal detection reagents (Pierce Biotechnology Inc.).

Sera from *M. avium* subsp. *paratuberculosis*-exposed rabbits and mice and from cattle with Johne's disease. New Zealand White rabbits were immunized with heat-killed ($n = 3$) or live ($n = 1$) preparations of *M. avium* subsp. *paratuberculosis* in earlier studies (35). BALB/c mice ($n = 2$) were immunized with a sonicated lysate of *M. avium* subsp. *paratuberculosis* K-10. This lysate was prepared by harvesting *M. avium* subsp. *paratuberculosis* at an optical density at 540 nm of 0.4, resuspending in cold PBS (1/100 of culture volume), and sonicating three times for 10 min each time, with a 10-min incubation on ice in between sonications. Cell debris was centrifuged out at 10,000 × *g*.

The NADC is a national leader in large animal biocontainment. There are currently more than 20 head of cattle (holsteins and Brown Swiss) housed at the NADC for Johne's disease research activities. Control cattle are allowed to graze on pastures, whereas clinical cows are held in BL2 animal barns at NADC. Sera from 10 well-characterized cattle in the clinical stage of Johne's disease (shedding more than 100 bacilli per g of feces as determined by plate counts on Middlebrook 7H9 slopes) and 9 control cattle were used in immunoblot analysis for detection of antibodies that bind unique *M. avium* subsp. *paratuberculosis* proteins. Table 2 lists all sera used in these studies. All cattle sera were diluted 1:500 in immunoblot assays.

TABLE 1. Primers used to amplify and clone *M. avium* subsp. *paratuberculosis*-specific sequences

Gene no.[a]	Vector	Primer[b]	Sequence[c]
10	pMal c-2	For	ATCCTCTAGACGGCGGATCAGCATCTAC
		Rev	GCGCAAGCTTCACCTCATCGTGGCCAGGTT
	pCRT7/CT	For	ATGCGCCCGCACACCGGCGGACGG
		Rev	CCTCATCGTGGCCAGGTTGTGCCA
11	pMal c-2	For	ATCCTCTAGAACCGAACACGAGTGGAGCA
		Rev	GCGCAAGCTTCAGACTCTGACCGACGTCAT
	pCRT7/CT	For	ATGGTGGCAACCGAACACGAG
		Rev	TCAGACTCTGACCGACGTCAT
38	pMal c-2	For	ATCCTCTAGAAGCATTTCGGCTCCCACGGTG
		Rev	GCGCAAGCTTCTACGTCGGTTCGGCGCGCAT
	pCRT7/CT	For	ATGGTGGTAAGCATTTCGGCTC
		Rev	CGTCGGTTCGGCGCGCATCAAAGC
56	pMal c-2	For	ATCCTCTAGAATGAACACTTCTTCCTCTCTA
		Rev	GCGCAAGCTTCATATCGCGGTGATCCTGAC
	pCRT7/CT	For	ATGAACACTTCTTCCTCTCTA
		Rev	TATCGCGGTGATCCTGACCGC
57	pMal c-2	For	ATCCTCTAGAATGGCCACCAACGACGACCA
		Rev	GCGCAAGCTTCACGCGGCCGTCGGGCCGGCTG
	pCRT7/CT	For	ATGGCCACCAACGACGACCAA
		Rev	CGCGCGGCCGTCGGGCCGGC
135	pMal c-2	For	ATCCTCTAGACGCATCGGCGCGCGCGAGCAG
		Rev	GCGCAAGCTTCATAGGACCTCCCGCGCTGC
	pCRT7/CT	For	ATGGCGGGGATGCCGGAGGAG
		Rev	TAGGACCTCCCGCGCTGCGCT
159	pMal c-2	For	ATCCTCTAGAATGCGTTTCGCCCTCCCG
		Rev	GCGCAAGCTTCACGCCTTGATTTCGTCCTG
	pCRT7/CT	For	ATGCGTTTCGCCCTCCCGACGCGC
		Rev	CGCCTTGATTTCGTCCTGCCGCGA
217	pMal c-2	For	ATCCTCTAGAATGGCCGAACGCGGACTGTTC
		Rev	GCGCAAGCTTCTAGGAATCCGCGTCGACGAT
	pCRT7/CT	For	ATGCGCAGGCTGAGTGCCGAAC
		Rev	GGAATCCGCGTCGACGATCTT
218-c	pMal c-2	For	CCTCTTCTAGACGCATGCGATCGAACAGT
		Rev	GCGCAAGCTTCACGAGTTGCGGCGGGCGAC
218-n	pMal c-2	For	AGGCTGTCTAGAACGCTGCGG
		Rev	GCGCAAGCTTCACAGCGGCCCGACCCCGGC
218 (full length)	pCRT7/CT	For	ATGCGCGGAAACCGCAGCGAGTTC
		Rev	ACGTTGCAGGCAGGCCGAGCACAG
219	pMal c-2	For	ATCCTCTAGAACATCTACTGAGCGCCGTTTG
		Rev	GCGCAAGCTTCACGCCGCCACCCCGTCCCG
	pCRT7/CT	For	GTGTCAACATCTACTGAGCGC
		Rev	CGCCGCCACCCCGTCCCGGCG
228	pMal c-2	For	ATCCTCTAGAGTGCCATACGCCGAATCG
		Rev	GCGCAAGCTTCTAGGAGCCTGCTGAATT
	pCRT7/CT	For	ATGCCATACGCCGAATCGCCCAGG
		Rev	GGAGCCTGCTGAATTAATTCCGCG
240	pMal c-2	For	ATCCTCTAGAGCTGCCTTGTGGAGCATCCG
		Rev	GCGCAAGCTTCTAGCGCTCACGAAAGTAAGC
	pCRT7/CT	For	ATGGTCATTGCGCTAGCTGCCTTG
		Rev	GCGTCCACGAAAGTAAGCCACT
241	pMal c-2	For	CCTCTTCTAGACCGTCGCTGAAGCTCACCGTCATC
		Rev	GCGCAAGCTTCACAGAGGTCGATCGGGTCG
	pCRT7/CT	For	ATGCCGTCGCTGAAGCTCACC
		Rev	CAGAGGTCGATCGGGTCGAGG

Continued on following page

TABLE 1—*Continued*

Gene no.[a]	Vector	Primer[b]	Sequence[c]
250	pMal c-2	For	ATCCTCTAGAGGCTCCTCGATGCTAACCGCT
		Rev	GCGCAAGCTTCAGTCGGCCGGCGAAACGCC
	pCRT7/CT	For	ATGGTTGCGGCGCAAGGCTCCTCG
		Rev	GTCGGCCGGCGAAACGCCCGCGGC
251	pMal c-2	For	ATCCTCTAGAATGGTGCGCGACGGCAGCCGC-
		Rev	GCGCAAGCTTCACGTGCTGTCCCCATCGGC
	pCRT7/CT	For	ATGGTGCGCGACGGCAGCCGCCGC
		Rev	CGTGCTGTCCCCATCGGCGTCCTC
252	pMal c-2	For	ATCCTCTAGAATGACCACCGACAACCCCACG
		Rev	GCGCAAGCTTCATGAGGGCTGTCCCTC
	pCRT7/CT	For	ATGACCACCGACAACCCCACGCCC
		Rev	TGAGGGCTGTCCCTCTCCGGCGGC
253	pMal c-2	For	ATCCTCTAGATTGACCGCGTTGACGGCGTTG
		Rev	GCGCAAGCTTCAGCGGTCCGCGCTCTTCGC
	pCRT7/CT	For	GCGGTCCGCGCTCTTCGCATCGGC
		Rev	TTGACCGCGTTGACGGCGTTGCGC
254	pMal c-2	For	ATCCTCTAGACGCCAGCGCTACAAGGTGATT
		Rev	GCGCAAGCTTCACGCGCTCCTTTCAGCCTT
	pCRT7/CT	For	ATGAGCGACGAGTTACGCCAGCG
		Rev	CGCGCTCCTTTCAGCCTTGCGGCG
255	pMal c-2	For	ATCCTCTAGACTTGCGCGGCTGGTGGGGGTT
		Rev	GCGCAAGCTTCAGTCACCCCGCGGCCGGTA
	pCRT7/CT	For	ATGGTCAACGTGCCGCGTGCG
		Rev	GTCACCCCGCGGCCGGTAGTC
256	pMal c-2	For	ATCCTCTAGAATGGCATACCGAATGAGT
		Rev	GCGCAAGCTTCATGACCCTGCCGGCGTCCC
	pCRT7/CT	For	ATGGCATACCGAATGAGTCCC
		Rev	TGACCCTGCCGGCGTCCCGGA
257	pMal c-2	For	ATCCTCTAGAGTGCCAGTCGGCACGACA
		Rev	GCGCAAGCTTCAACTGACCACCAGGGCCGG
	pCRT7/CT	For	GTGCCAGTCGGCACGACACC
		Rev	ACTGACCACCAGGGCCGGCCG

[a] Gene numbers are consistent with those presented in reference 2.
[b] For, forward; Rev, reverse.
[c] Listed 5′ to 3′.

RESULTS

Cloning, expression, and purification of *M. avium* subsp. *paratuberculosis* sequences in *E. coli*. Twenty-one predicted coding sequences that are present uniquely in *M. avium* subsp. *paratuberculosis* have been identified previously (2). The deduced polypeptides produced from these coding sequences have no known peptide motifs or sequence similarity to other proteins in public databases such as GenBank. Each of these coding sequences was amplified from purified *M. avium* subsp. *paratuberculosis* genomic DNA and cloned into an *E. coli* expression vector as described in Materials and Methods. Because affinity purification tags coexpressed with the protein of interest can profoundly influence stability, solubility, and expression levels of recombinant protein, we evaluated two different affinity tags with each protein of interest. Predicted coding sequences were expressed in conjunction with either a six-His tag (5) or the 42-kDa MBP tag (31) for affinity purification. Efforts to clone and express each of the coding regions yielded various degrees of success with each tag. All of the MBP fusion proteins were successfully expressed and purified, although protein yields were very different (Fig. 1A). Coding sequences designated genes 253 (Fig. 1A, lane 3), 57 (lane 11), 252 (lane 16), 135 (lane 19), 217 (lane 20), 257 (lane 21), and 254 (lane 23) and the C-terminal half of gene 218 (218-C) (lane 25) were all expressed to high levels in *E. coli*. Other coding sequences including genes 219 (Fig. 1, lane 8), 256 (lane 18), and 250 (lane 22) were only marginally produced by *E. coli*. Still other proteins showed multiple bands (gene 159 [lane 5] and gene 251 [lane 4]), possibly due to proteolytic cleavage. Conversely, only 5 of the 21 six-His tag clones produced recombinant *M. avium* subsp. *paratuberculosis* protein, and those that did, produced a relatively small yield that could be detected only by immunoblotting (Fig. 2). Therefore, this study focused only on the MBP fusion proteins that were expressed and purified from *E. coli*. Table 3 lists the number of deduced amino acids, predicted antigenicity, calculated molecular size, and purification yield for each MBP fusion protein.

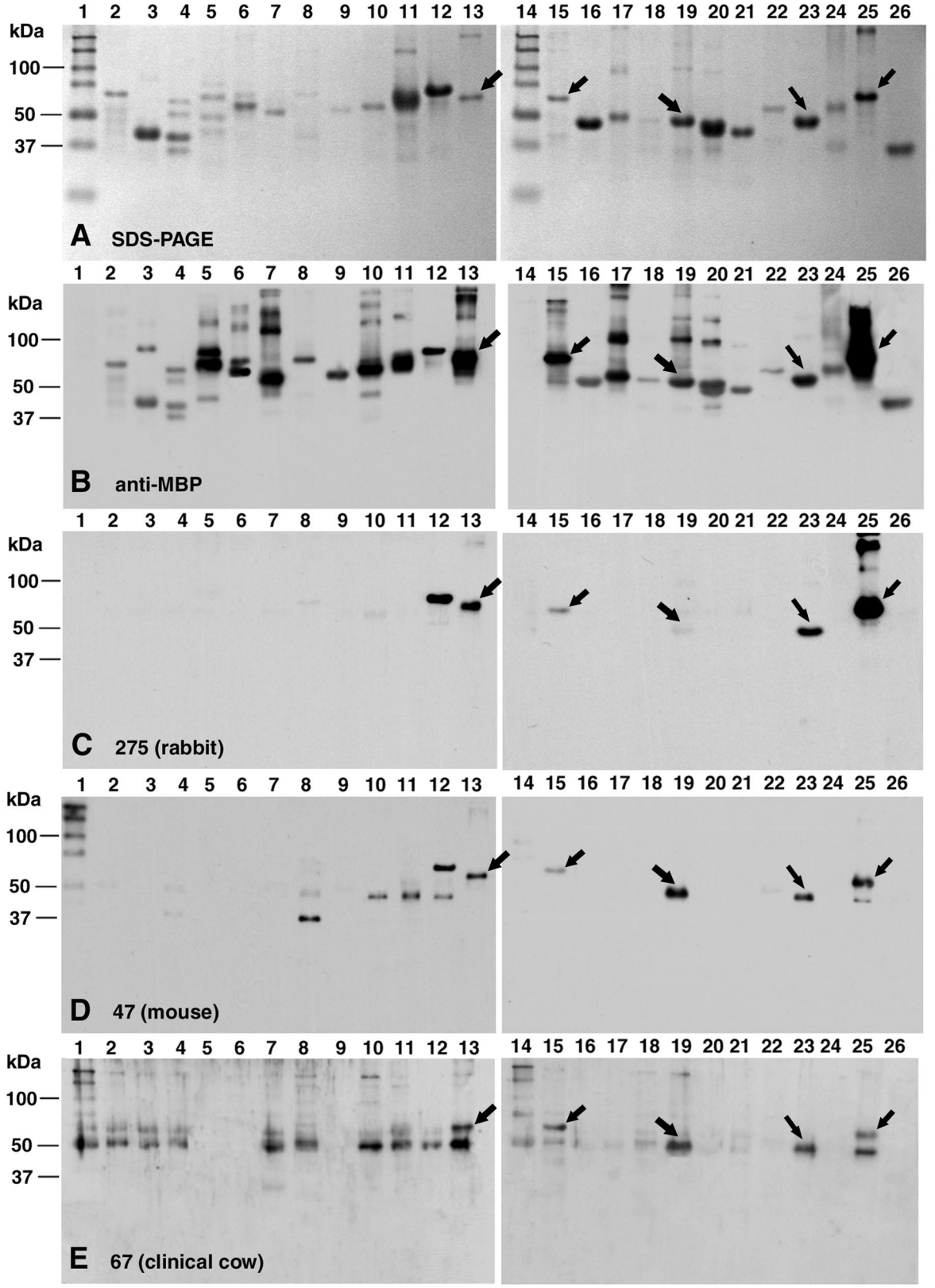

FIG. 1. SDS-PAGE and immunoblot analysis of affinity-purified *M. avium* subsp. *paratuberculosis* gene products. (A) Shown are two SDS–12% PAGE gels loaded with affinity-purified MBP fusion proteins that were stained with GelCode Blue. Size standards (in kilodaltons) are indicated on the left. (B through E) Corresponding immunoblots were probed with a monoclonal antibody to MBP (B) or with serum from rabbit 275 (C), mouse 47 (D), and clinical cow 67 (E). Note that MBP is not detected in panel C, D, or E. Lanes: 1, protein size standards; 2, gene 255; 3, gene 253; 4, gene 251; 5, gene 159; 6, gene 228; 7, gene 10; 8, gene 219; 9, gene 38; 10, gene 56; 11, gene 57; 12, MBP-MMP; 13, gene 218 (N-terminal half); 14, protein size standards; 15, gene 241; 16, gene 252; 17, gene 240; 18, gene 256; 19, gene 135; 20, gene 217; 21, gene 257; 22, gene 250; 23, gene 254; 24, gene 11; 25, gene 218 (C-terminal half); 26, MBP. Arrows point to the five subspecies-specific antigens identified in this study.

TABLE 2. Antisera used in this study

Animal	Immunizing antigen or animal status	Dilution[a]
Mouse 47	*M. avium* subsp. *paratuberculosis* K-10 sonicate	1:3,000
Mouse 48	*M. avium* subsp. *paratuberculosis* K-10 sonicate	1:3,000
Rabbit 270	Live *M. avium* subsp. *paratuberculosis*	1:1,000
Rabbit 275	Heat-killed *M. avium* subsp. *paratuberculosis*	1:1,500
Rabbit 273	Heat-killed *M. avium* subsp. *paratuberculosis*	1:1,000
Rabbit 274	Heat-killed *M. avium* subsp. *paratuberculosis*	1:1,000
Cow 67	Naturally infected clinical cow	1:500
Cow 598	Naturally infected clinical cow	1:500
Cow 159	Naturally infected clinical cow	1:500
Cow 167	Naturally infected clinical cow	1:500
Cow 14	Naturally infected clinical cow	1:500
Cow 144	Naturally infected clinical cow	1:500
Cow 47	Naturally infected clinical cow	1:500
Cow Kay	Naturally infected clinical cow	1:500
Cow 116	Naturally infected clinical cow	1:500
Cow 200	Naturally infected clinical cow	1:500
Cow 107	Control cow	1:500
Cow 108	Control cow	1:500
Cow 109	Control cow	1:500
Cow 110	Control cow	1:500
Cow 111	Control cow	1:500
Cow 181	Control cow	1:500
Cow 182	Control cow	1:500
Cow 183	Control cow	1:500
Cow 184	Control cow	1:500
α-MBP	Monoclonal antibody developed at NADC	1:3,000

[a] Working dilution used in immunoblotting experiments.

Serological analysis of recombinant *M. avium* subsp. *paratuberculosis* proteins with rabbit and mouse sera. Each protein was next examined by immunoblot analysis with rabbit and mouse sera containing α-*M. avium* subsp. *paratuberculosis* antibodies. Rabbits and mice that were immunized with various preparations of *M. avium* subsp. *paratuberculosis* are listed in Table 2. All fusion proteins were detected by a monoclonal antibody to the MBP tag (Fig. 1B); however, only five of the purified proteins were detected by α-*M. avium* subsp. *paratuberculosis* antibodies present in both rabbit and mouse sera (Fig. 1C and D, respectively). These antigenic proteins are MBP-gene 56 (Fig. 1C and D, lanes 10), MBP-gene 218 (lanes 13 and 25), MBP-gene 241 (lanes 15), MBP-gene 135 (lanes 19), and MBP-gene 254 (lanes 23). Serological reactivity associated with the two fusion proteins encoded by genes 56 (lanes 10) and 135 (lanes 19) was readily visible in mouse (Fig. 1D) but not rabbit (Fig. 1C) serum. Additional immunoblotting experiments showed that the same five proteins were also detected by sera from mouse 48 and rabbits 273 and 274 (data not shown). One MBP fusion protein detected by the mouse serum only (Fig. 1D, lane 8) was not of the expected size and therefore was not considered further. In addition, a known *M. avium* subsp. *paratuberculosis* protein, termed the major membrane protein (MMP) (3), included as a control, was detected by both the rabbit and mouse sera (Fig. 1C and D, lanes 12), whereas the MBP control protein was detected by neither (Fig. 1C and D, lanes 26). These data suggest that at least five subspecies-specific proteins are produced by *M. avium* subsp. *paratuberculosis* and are immunogenic.

TABLE 3. Characteristics of *M. avium* subsp. *paratuberculosis* MBP fusion proteins

Protein	Calculated mol size (Da)[a]	Amino acids[b]	Antigenic index[c]	Yield[d] (mg/ml)
MBP-gene 250	21,434	199/199	Hydrophilic	0.2
MBP-gene 251	19,500	179/179	Hydrophilic	0.7
MBP-gene 252	9,687	96/96	Hydrophobic	2.0
MBP-gene 253	7,881	74/74	50/50	0.9
MBP-gene 254	16,262	146/141	50/50	1.3
MBP-gene 255	25,851	241/241	Hydrophilic	0.3
MBP-gene 256	15,120	141/141	Hydrophilic	0.1
MBP-gene 257	8,837	87/87	Hydrophilic	1.0
MBP-gene 240	21,734	194/163	Hydrophobic	0.4
MBP-gene 241	30,724	280/280	Hydrophilic	0.4
MBP-gene 228	40,817	369/216	Hydrophilic	0.6
MBP-gene 217	11,567	106/106	50/50	2.1
MBP-gene 218-C	91,530	835/365	Hydrophilic	1.4
MBP-gene 218-N	91,530	835/353	Hydrophilic	1.8
MBP-gene 219	10,004	87/85	Hydrophilic	0.04
MBP-gene 159	20,655	185/185	Hydrophobic	0.2
MBP-gene 135	17,018	157/141	Hydrophobic	1.0
MBP-gene 56	21,116	193/193	Hydrophilic	0.7
MBP-gene 57	25,485	252/252	50/50	1.4
MBP-gene 38	18,730	173/170	Hydrophilic	0.06
MBP-gene 10	36,380	322/205	Hydrophilic	0.8
MBP-gene 11	21,826	191/191	Hydrophilic	0.7

[a] Represents the calculated size of the entire predicted coding sequence but does not represent the size of the fusion protein.

[b] Total number of amino acids in predicted coding sequence/number of amino acids represented in the MBP fusion. In some instances, the mycobacterial protein of interest was truncated to minimize toxicity and/or enhance expression in *E. coli.*

[c] The hydrophilic nature of a protein is considered a crude estimate of the protein's antigenicity. *M. paratuberculosis* proteins were analyzed by Kyte-Doolittle hydropathy plots (20) using MacVector sequence analysis software. Proteins that were more than 60% hydrophilic were termed "hydrophilic," proteins less than 40% hydrophilic were termed "hydrophobic," and proteins displaying 40 to 60% hydrophilicity were termed "50/50."

[d] The purification yield was determined by using a Bio-Rad protein assay on pooled fractions.

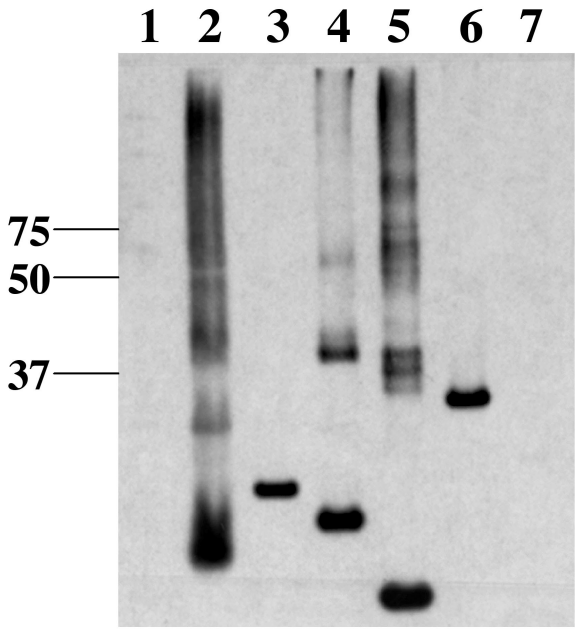

FIG. 2. Expression of *M. avium* subsp. *paratuberculosis* six-His tag clones in *E. coli*. An immunoblot containing IPTG-induced *E. coli* BL21(DE3) lysates was probed with a monoclonal antibody that detects the polyhistidine tag. Size standards (in kilodaltons) are indicated on the left. Lanes: 1, protein size standards; 2, gene 254; 3, gene 159; 4, gene 56; 5, gene 38; 6, gene 228; 7, *E. coli* BL21(DE3) cells.

TABLE 4. *M. avium* subsp. *paratuberculosis* reactivity with NADC cattle sera[a]

Cattle no.	Reactivity of serum to the following antigen:				
	56	135	218	241	254
Control					
107	−	−	−	−	+
108	−	+	−	+/−[b]	−
181	−	−	−	−	−
184	−	−	−	−	+
Clinical					
167	+	+	+	+	+
14	−	−	+	+	+
598	+	+	+	+	+
Kay	+	−	−	+	−
47	+	+	+	+	+
67	+/−	+	+	+	+

[a] MBP was detected with sera from control cattle 109, 110, 111, 182, and 183 and with sera from infected cattle 159, 116, 144, and 200.
[b] +/−, inconclusive result due to background in immunoblot experiments.

Reactivity to sera from control and Johne's disease cattle. To determine whether *M. avium* subsp. *paratuberculosis* fusion proteins will specifically react with antibodies from Johne's disease cattle, sera from 10 cattle in the clinical stage of Johne's disease were used in immunoblot experiments with the same set of purified proteins described above. Results of a typical experiment using serum from clinical cow 67 are shown in Fig. 1E. As was seen in the immunoblots with rabbit and mouse sera, both the N- and C-terminal halves of protein 218 were detected by serum from this clinical cow. In addition, products from genes 241 (lane 15), 135 (lane 19), and 254 (lane 23) were detected. The antigen produced from gene 56 (lane 10) may also be detected; however, this is not conclusive due to background present in that region of the immunoblot. We base this conclusion on the fact that the nonspecific binding occurs at a size range that is inconsistent with the predicted and observed size values of the recombinant purified proteins (as shown in Fig. 1A). However, this background (primarily a 50-kDa band) was not observed with the mouse or rabbit serum.

Serum from clinical cow 67 did not detect the MMP control protein (Fig. 1E, lane 12), although this protein was detected in sera from other clinical cattle (data not shown). Sera from nine additional cattle showing overt clinical disease were also evaluated against the five antigenic proteins listed in Table 4; however, four sera had antibodies to the MBP protein, making interpretation difficult (Table 4). Sera from cows 47, 167, and 598 detected all five immunogenic proteins, whereas sera from cows 14, 67, and Kay did not have antibodies to all five proteins. Only the MBP-gene 241 antigen was detected by all MBP-negative clinical sera tested. Control sera from five of nine healthy cows had antibodies to MBP, and only serum from animal 181 failed to react with any of the five proteins (Table 4).

DISCUSSION

Among the greatest needs for effectively combating Johne's disease are new diagnostic tools that more accurately identify animals infected with *M. avium* subsp. *paratuberculosis*. A highly specific test that could distinguish between infected and noninfected animals within herds might have a greater immediate impact on the U.S. dairy industry than any other current management approach, including vaccination. This is because accurate identification of infected animals would allow removal of only those animals from a herd. Therefore, developing an improved immunological assay for the detection of paratuberculosis-specific proteins is a critical research focus.

Unfortunately, the diagnosis of Johne's disease has long been hindered by the lack of sensitive and specific assays that detect *M. avium* subsp. *paratuberculosis* as well as a lack of antigens that distinguish it from other closely related mycobacteria, including *M. avium* subsp. *avium*. All antigen-based diagnostic tests for Johne's disease currently use a complex mixture of undefined antigens, such as purified protein derivative (PPD), that can exhibit cross-reactivity with other mycobacteria (17). In order to develop more-reliable antigen-based tests such as ELISAs, species-specific *M. avium* subsp. *paratuberculosis* antigens must first be identified. Investigators have long pursued the search for *M. avium* subsp. *paratuberculosis*-specific genes and antigens, with no progress until recently (2, 19). With the genome sequence for *M. avium* subsp. *paratuberculosis* now completed (Li et al., unpublished), a collection of 21 *M. avium* subsp. *paratuberculosis*-specific DNA sequences have been identified (2) and another 14 are still under evaluation (M. L. Paustian, V. Kapur, and J. P. Bannantine, unpublished data). The present study represents an initial effort to heterologously express these sequences and evaluate their antigenic character. These studies have shown that of the 21 predicted coding sequences analyzed, 5 (genes 56, 135, 218, 241, and 254) encode proteins that appear to be immunogenic. It is noteworthy that, except for gene 241, no single antigen was detected by all MBP-negative cattle sera tested. However, sera from subclinically infected cows have yet to be tested, and it is possible that all five proteins may not be detected at that stage of infection. While this finding highlights the gene 241 sequence as a potential diagnostic antigen, it also underscores the need for a "cocktail" consisting of more than a single specific antigen to expand the possibility that at least one of the antigens might be detected at all stages of the disease. This strategy of combining several *M. avium* subsp. *paratuberculosis*-specific proteins should theoretically result in greater sensitivity as well.

Despite the fact that each of the proteins is encoded by genes present only in *M. avium* subsp. *paratuberculosis*, there remains the possibility that epitopes may be conserved with proteins from other mycobacteria and may therefore cross-react with sera from animals exposed to other mycobacteria. However, none of these five immunogenic proteins have significant similarities to any protein in the public databases, and their functions remain unknown. Furthermore, there are no known protein motifs present. Hence, the likelihood of true cross-reactivity is diminished. Nonetheless, raising polyclonal antibodies against *M. avium* and evaluating their reactivity to these purified *M. avium* subsp. *paratuberculosis* proteins is one of the strategies that we are using to identify possible immunologic cross-reactivity of these proteins due to shared epitopes. The novelty and lack of similarity in these *M. avium* subsp. *paratuberculosis* sequences suggests that they would

have remained unknown if not discovered through a whole-genome sequencing effort. Research directed at elucidating the functions of these proteins will provide a better understanding of why *M. avium* subsp. *paratuberculosis* is so phenotypically different from other closely related mycobacteria.

By using denaturing conditions for protein purification, all of the MBP fusions were purified. In contrast to the MBP-*M. avium* subsp. *paratuberculosis* fusion proteins, only five mycobacterial proteins were successfully expressed by using the polyhistidine tag. Each affinity tag used in this study has distinct advantages and disadvantages. Generally, expression of recombinant proteins as a fusion with MBP can overcome problems such as toxicity in *E. coli*, low expression levels, inclusion body formation, and insolubility (31). Our data appear to substantiate this observation in that a higher percentage of recombinant protein was produced with the MBP system than with the polyhistidine system. The disadvantage of MBP is the size and immunogenicity of the affinity tag, which complicates any downstream immunoassays. The MBP tag must be included as a control in experiments where the protein of interest could not be separated from the MBP tag with a protease such as factor Xa. Conversely, the polyhistidine tag is small and does not react with most sera. However, in our hands, several mycobacterial proteins were not expressed with this system and purification to homogeneity was not achieved.

Unlike the rabbit and mouse antisera, several cattle sera detected MBP. This result severely limited the number of cattle that could be used in these analyses. The outbred nature of cattle is conducive to exposure to environmental antigens such as *E. coli* MBP or *M. avium* proteins. Therefore, in order for these novel antigens to be incorporated into a routine diagnostic test, the proteins must be expressed and purified without the MBP affinity tag. Nonetheless, these five novel *M. avium* subsp. *paratuberculosis* antigens are perhaps the most promising diagnostic antigens identified to date.

These data show that at least some of the coding predictions were correct for the novel *M. avium* subsp. *paratuberculosis* genes. However, many of the 21 gene products were not detected by any of the sera. Three possibilities could account for this result. The coding sequence prediction may be incorrect, or in the wrong reading frame. Alternatively, the mycobacterial protein may not be folded or modified correctly in *E. coli*. Another possibility is that the gene product may actually be produced by *M. avium* subsp. *paratuberculosis* but may simply not be immunogenic. A limitation of the experimental design used in this study is that successful identification of an antigen depends on two variables. First, the protein expressed from each predicted coding sequence must actually be produced by *M. avium* subsp. *paratuberculosis*, and second, that protein must be immunogenic. To eliminate the dependence on these two variables, a proteomic approach is needed to bypass the requirement of antigenicity by confirming coding sequence predictions solely on the basis of protein profiles in two-dimensional SDS-PAGE gels and subsequent protein sequence determination by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) spectrophotometry.

In conclusion, this study demonstrates that serological analysis of recombinant proteins produced from *M. avium* subsp. *paratuberculosis*-specific sequences can lead to the identification of novel antigens that may be useful for diagnosis. These antigens may be able to distinguish *M. avium* subsp. *paratuberculosis* from other closely related mycobacteria. In order to ensure that reactivity was reliable, we have tested these antigens with sera from three different species of hosts including mice, rabbits, and naturally infected cattle. The evaluation of these antigens as diagnostic reagents will be expanded with field studies on well-characterized dairy herds.

ACKNOWLEDGMENTS

This work was supported by the USDA's Agricultural Research Service and USDA-NRI grants to J.P.B. and V.K.

The expert technical assistance of Trudy L. Bosworth is gratefully acknowledged.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York, N.Y.
2. **Bannantine, J. P., E. Baechler, Q. Zhang, L. Li, and V. Kapur.** 2002. Genome scale comparison of *Mycobacterium avium* subsp. *paratuberculosis* with *Mycobacterium avium* subsp. *avium* reveals potential diagnostic sequences. J. Clin. Microbiol. **40:**1303–1310.
3. **Bannantine, J. P., and J. R. Stabel.** 2001. Identification of two *Mycobacterium avium* subspecies *paratuberculosis* gene products differentially recognised by sera from rabbits immunised with live mycobacteria but not heat-killed mycobacteria. J. Med. Microbiol. **50:**795–804.
4. **Bannantine, J. P., Q. Zhang, L. Li, and V. Kapur.** 2003. Genomic homogeneity between *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis* belies their divergent growth rates. BMC Microbiol. **3:**10.
5. **Bornhorst, J. A., and J. J. Falke.** 2000. Purification of proteins using polyhistidine affinity tags. Methods Enzymol. **326:**245–254.
6. **Cameron, R. M., K. Stevenson, N. F. Inglis, J. Klausen, and J. M. Sharp.** 1994. Identification and characterization of a putative serine protease expressed in vivo by *Mycobacterium avium* subsp. *paratuberculosis.* Microbiology **140:**1977–1982.
7. **Chi, J., J. VanLeeuwen, A. Weersink, and G. Keefe.** 2002. Direct production losses and treatment costs from bovine viral diarrhea virus, bovine leukosis virus, *Mycobacterium avium* subspecies *paratuberculosis*, and *Neospora caninum*. Prev. Vet. Med. **55:**137.
8. **Clarke, C. J., I. A. Patterson, K. E. Armstrong, and J. C. Low.** 1996. Comparison of the absorbed ELISA and agar gel immunodiffusion test with clinicopathological findings in ovine clinical paratuberculosis. Vet. Rec. **139:** 618–621.
9. **Cobb, A. J., and R. Frothingham.** 1999. The GroES antigens of *Mycobacterium avium* and *Mycobacterium paratuberculosis.* Vet. Microbiol. **67:**31–35.
10. **Coetsier, C., X. Havaux, F. Mattelard, S. Sadatte, F. Cormont, K. Buergelt, B. Limbourg, D. Latinne, H. Bazin, J. F. Denef, and C. Cocito.** 1998. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in infected tissues by new species-specific immunohistological procedures. Clin. Diagn. Lab. Immunol. **5:**446–451.
11. **Colgrove, G. S., C. O. Thoen, B. O. Blackburn, and C. D. Murphy.** 1989. Paratuberculosis in cattle: a comparison of three serologic tests with results of fecal culture. Vet. Microbiol. **19:**183–187.
12. **Collins, M. T.** 1996. Diagnosis of paratuberculosis. Vet. Clin. N. Am. Food Anim. Pract. **12:**357–371.
13. **De Kesel, M., P. Gilot, M. Coene, and C. Cocito.** 1992. Composition and immunological properties of the protein fraction of A36, a major antigen complex of *Mycobacterium paratuberculosis.* Scand. J. Immunol. **36:**201–212.
14. **el-Zaatari, F. A., S. A. Naser, L. Engstrand, P. E. Burch, C. Y. Hachem, D. L. Whipple, and D. Y. Graham.** 1995. Nucleotide sequence analysis and seroreactivities of the 65K heat shock protein from *Mycobacterium paratuberculosis.* Clin. Diagn. Lab. Immunol. **2:**657–664.
15. **el-Zaatari, F. A., S. A. Naser, L. Engstrand, C. Y. Hachem, and D. Y. Graham.** 1994. Identification and characterization of *Mycobacterium paratuberculosis* recombinant proteins expressed in *E. coli.* Curr. Microbiol. **29:** 177–184.
16. **Gilot, P., M. De Kesel, L. Machtelinckx, M. Coene, and C. Cocito.** 1993. Isolation and sequencing of the gene coding for an antigenic 34-kilodalton protein of *Mycobacterium paratuberculosis.* J. Bacteriol. **175:**4930–4935.
17. **Harris, N. B., and R. G. Barletta.** 2001. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin. Microbiol. Rev. **14:**489–512.
18. **Hurley, S. S., G. A. Splitter, and R. A. Welch.** 1989. Development of a diagnostic test for Johne's disease using a DNA hybridization probe. J. Clin. Microbiol. **27:**1582–1587.
19. **Klitgaard Nielsen, K., and P. Ahrens.** 2002. Putative in vitro expressed gene fragments unique to *Mycobacterium avium* subspecies *paratuberculosis.* FEMS Microbiol. Lett. **214:**199.

20. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**1005–1032.
21. **Liu, X., Z. Feng, N. B. Harris, J. D. Cirillo, H. Bercovier, and R. G. Barletta.** 2001. Identification of a secreted superoxide dismutase in *Mycobacterium avium* ssp. *paratuberculosis.* FEMS Microbiol. Lett. **202:**233–238.
22. **McDonald, W. L., S. E. Ridge, A. F. Hope, and R. J. Condron.** 1999. Evaluation of diagnostic tests for Johne's disease in young cattle. Aust. Vet. J. **77:**113–119.
23. **Mullerad, J., A. H. Hovav, R. Nahary, Y. Fishman, and H. Bercovier.** 2003. Immunogenicity of a 16.7 kDa *Mycobacterium paratuberculosis* antigen. Microb. Pathog. **34:**81–90.
24. **Mutharia, L. M., W. Moreno, and M. Raymond.** 1997. Analysis of culture filtrate and cell wall-associated antigens of *Mycobacterium paratuberculosis* with monoclonal antibodies. Infect. Immun. **65:**387–394.
25. **Olsen, I., L. J. Reitan, G. Holstad, and H. G. Wiker.** 2000. Alkyl hydroperoxide reductases C and D are major antigens constitutively expressed by *Mycobacterium avium* subsp. *paratuberculosis*. Infect. Immun. **68:**801–808.
26. **Olsen, I., and A. K. Storset.** 2001. Innate IFN-γ production in cattle in response to MPP14, a secreted protein from *Mycobacterium avium* subsp. Paratuberculosis. Scand. J. Immunol. **54:**306–313.
27. **Olsen, I., M. Tryland, H. G. Wiker, and L. J. Reitan.** 2001. AhpC, AhpD, and a secreted 14-kilodalton antigen from *Mycobacterium avium* subsp. *paratuberculosis* distinguish between paratuberculosis and bovine tuberculosis in an enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. **8:**797–801.
28. **Olsen, I., H. G. Wiker, E. Johnson, H. Langeggen, and L. J. Reitan.** 2001. Elevated antibody responses in patients with Crohn's disease against a 14-kDa secreted protein purified from *Mycobacterium avium* subsp. *paratuberculosis*. Scand. J. Immunol. **53:**198–203.
29. **Ott, S. L., S. J. Wells, and B. A. Wagner.** 1999. Herd-level economic losses associated with Johne's disease on US dairy operations. Prev. Vet. Med. **40:**179–192.
30. **Rockey, D. D., and J. L. Rosquist.** 1994. Protein antigens of *Chlamydia psittaci* present in infected cells but not detected in the infectious elementary body. Infect. Immun. **62:**106–112.
31. **Sachdev, D., and J. M. Chirgwin.** 2000. Fusions to maltose-binding protein: control of folding and solubility in protein purification. Methods Enzymol. **326:**312–321.
31a. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Saxegaard, F., and I. Baess.** 1988. Relationship between *Mycobacterium avium*, Mycobacterium paratuberculosis and "wood pigeon mycobacteria." Determinations by DNA-DNA hybridization. APMIS **96:**37–42.
33. **Silbaq, F. S., S. N. Cho, S. T. Cole, and P. J. Brennan.** 1998. Characterization of a 34-kilodalton protein of *Mycobacterium leprae* that is isologous to the immunodominant 34-kilodalton antigen of *Mycobacterium paratuberculosis.* Infect. Immun. **66:**5576–5579.
34. **Stabel, J. R.** 1998. Johne's disease: a hidden threat. J. Dairy Sci. **81:**283–288.
35. **Stabel, J. R., M. R. Ackermann, and J. P. Goff.** 1996. Comparison of polyclonal antibodies to three different preparations of *Mycobacterium paratuberculosis* in immunohistochemical diagnosis of Johne's disease in cattle. J. Vet. Diagn. Investig. **8:**469–473.
36. **Thorel, M. F., M. Krichevsky, and V. V. Levy-Frebault.** 1990. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. Int. J. Syst. Bacteriol. **40:**254–260.
37. **White, W. B., D. L. Whipple, J. R. Stabel, and C. A. Bolin.** 1994. Comparison of cellular and extracellular proteins expressed by various isolates of *Mycobacterium paratuberculosis* and other mycobacterial species. Am. J. Vet. Res. **55:**1399–1405.
38. **Whitlock, R. H., and C. Buergelt.** 1996. Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet. Clin. N. Am. Food Anim. Pract. **12:**345–356.