Profiling Bovine Antibody Responses to *Mycobacterium avium* subsp. *paratuberculosis* Infection by Using Protein Arrays^{∇†}

John P. Bannantine,^{1*} Michael L. Paustian,¹ W. Ray Waters,¹ Judith R. Stabel,¹
Mitchell V. Palmer,¹ Lingling Li,² and Vivek Kapur²

National Animal Disease Center, USDA-ARS, Ames, Iowa,¹ and University of Minnesota, St. Paul, Minnesota²

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With the genome sequence of *Mycobacterium avium* subsp. *paratuberculosis* determined, technologies are now being developed for construction of protein arrays to detect the presence of antibodies against *M. avium* subsp. *paratuberculosis* in host serum. The power of this approach is that it enables a direct comparison of *M. avium* subsp. *paratuberculosis* proteins to each other in relation to their immunostimulatory capabilities. In this study, 93 recombinant proteins, produced in *Escherichia coli*, were arrayed and spotted onto nitrocellulose. These proteins include unknown hypothetical proteins and cell surface proteins as well as proteins encoded by large sequence polymorphisms present uniquely in *M. avium* subsp. *paratuberculosis*. Also included were previously reported or known *M. avium* subsp. *paratuberculosis* antigens to serve as a frame of reference. Sera from healthy control cattle ($n = 3$) and cattle infected with either *M. avium* subsp. *avium* and *Mycobacterium bovis* were exposed to the array to identify nonspecific or cross-reactive epitopes. These data demonstrated a degree of cross-reactivity with the *M. avium* subsp. *avium* proteins that was higher than the degree of cross-reactivity with the more distantly related *M. bovis* proteins. Finally, sera from naturally infected cattle ($n = 3$) as well as cattle experimentally infected with *M. avium* subsp. *paratuberculosis* ($n = 3$) were used to probe the array to identify antigens in the context of Johne's disease. Three membrane proteins were the most strongly detected in all serum samples, and they included an invasion protein, an ABC peptide transport permease, and a putative GTPase protein. This powerful combination of genomic information, molecular tools, and immunological assays has enabled the identification of previously unknown antigens of *M. avium* subsp. *paratuberculosis*.

Johne's disease is responsible for severe economic losses to the U.S. dairy industry totaling over \$200 million annually (21). The causative agent of this significant animal disease is *Mycobacterium avium* subsp. *paratuberculosis*. This bacterium can infect domestic ruminants, including cattle, sheep (27, 37), and goats (54, 55). Wildlife such as deer (22, 32, 33), antelope (9, 14), bison (59), and rabbits (7, 25) can also be colonized and may serve as a reservoir for this pathogen. Initial infection of young calves is followed by a lengthy subclinical stage of Johne's disease during which the animals are generally asymptomatic but may be shedding low numbers of organisms in feces or milk. The late stage of Johne's disease in cattle involves shedding high numbers of the pathogen in feces, along with cachexia and diarrhea as hallmark signs. Transmission of *M. avium* subsp. *paratuberculosis* is by ingestion of bacilli during grazing on contaminated pastures or through the milk of an infected cow.

Understanding the host immunity to *M. avium* subsp. *paratuberculosis* infection is critical to controlling the spread of this disease, as it is central to the development of better diagnostic tests and the identification of protective immunogens for use as vaccine candidates. During the early subclinical stage of infection, a cell-mediated response predominates in the host and can be characterized by strong delayed-type IV hypersensitivity

reactions, lymphocyte-proliferative responses to select mycobacterial antigens, and production of cytokines stimulated by T cells (50). Through some unknown signal, the cell-mediated immune response wanes with progression of Johne's disease and a humoral immune response becomes measurable (49). However, there is recent evidence that suggests antibody production in cattle does occur early postinfection (26, 57).

Since the completion of the *M. avium* subsp. *paratuberculosis* genome sequencing project (29), this organism has been characterized for genomic diversity (34, 42) and unique diagnostic (2, 3, 11, 30) and subtyping (1, 36) targets as well as preliminary antigen screens (4, 11, 30, 41). In particular, the genetic diversity among *M. avium* subsp. *paratuberculosis* isolates has been extensively studied. By use of techniques from repetitive DNA sequences (1, 8) to amplified fragment length polymorphism and pulsed-field gel electrophoresis analysis (13, 39), differences on *M. avium* subsp. *paratuberculosis* chromosomes have been identified and utilized for discriminatory subtyping of isolates. Many of these studies have used the genome sequence of *M. avium* subsp. *paratuberculosis* to aid in the identification of genetic regions of variability (1, 40, 42, 46). Over 30 proteins encoded within these unique genetic regions, termed large sequence polymorphisms (LSPs), were produced and analyzed in this study.

Currently, all antigen-based tests that detect *M. avium* subsp. *paratuberculosis* use a complex, ill-defined mixture of proteins, such as a whole-cell sonicated extract (35), surface antigen extract (16), or purified protein derivative (51). These antigen preparations show variability in potency (52) and cross-react with closely related mycobacteria such as *M. avium* subsp. *avium*. Recognizing that these tests could potentially be improved if a few well-defined

* Corresponding author. Mailing address: National Animal Disease Center, ARS-USDA, 2300 North Dayton Avenue, Ames, IA 50010. Phone: (515) 663-7340. Fax: (515) 663-7458. E-mail: john.bannantine@ars.usda.gov.

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antigens were identified, our laboratories used genomic data to identify novel *M. avium* subsp. *paratuberculosis* antigens as candidates to be used to improve diagnosis of Johne's disease in antigen-based immunoassays, such as the enzyme-linked immunosorbent assay (ELISA), an immunoblot, or a gamma interferon (IFN- γ) release assay. From these studies, we have identified at least four novel antigens (30, 41) but are not certain how these antigens compare with other proteins produced by *M. avium* subsp. *paratuberculosis*.

Clearly, a proteomic approach is necessary to define the most antigenic components. One method is to fractionate proteins from the whole-cell, membrane, or secreted fraction and resolve them via two-dimensional (2-D) gels. The secreted fraction of *M. avium* subsp. *paratuberculosis* has recently been analyzed by using this methodology (10, 24, 28). Another way is to express recombinant proteins from cloned *M. avium* subsp. *paratuberculosis* coding sequences and use them to construct a protein array. This array can then be used to probe sera from animals with Johne's disease and healthy controls. We pursued the second approach to develop a 96-dot protein array from *M. avium* subsp. *paratuberculosis*, because of the ease in identifying reactive proteins. Furthermore, this array would enable the direct comparison of known antigens with newly identified antigens. This systematic approach will lead most directly to new diagnostic tools for improved management of Johne's disease. Finally, the results described herein provide a unique examination of the humoral immune responses in cattle exposed to *M. avium* subsp. *paratuberculosis* as well as open new frontiers in vaccine and diagnostic development.

MATERIALS AND METHODS

Mycobacterial antigen preparation. A whole-cell sonicated lysate of *M. avium* subsp. *paratuberculosis* K-10 was prepared as described previously (57).

Recombinant protein production and purification. The cloning, protein production, and purification is described in detail previously (5). Briefly, maltose binding protein (MBP) fusions of *M. avium* subsp. *paratuberculosis* predicted coding sequences listed in Table 1 were constructed in *Escherichia coli* by using the pMAL-c2 vector (New England Biolabs, Beverly, MA). Primers were designed from the reading frame of each coding sequence and contained an XbaI site in the 5' primer and a HindIII site in the 3' primer for cloning purposes. The vector and amplification product were digested with XbaI and HindIII. 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* DH5 α and selected on LB agar plates containing 100 μ g/ml ampicillin. Each MBP fusion protein (e.g., MBP-MAP 4025) was overexpressed in 1-liter *E. coli* LB cultures by induction with 0.3 mM isopropyl- β -D-thiogalactopyranoside (Sigma, St. Louis, MO) and purified by affinity chromatography using an amylose resin supplied by New England Biolabs. A similar approach was used for production and purification of all MBP fusion proteins. *E. coli* DH5 α 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 (MBP-LacZ). Selected fractions eluted from the amylose resin column were pooled and dialyzed using Slide-A-Lyzer cassettes (Pierce Biotechnology Inc.) in 1.5-liter phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM NaPO $_4$, pH 7.4) with three exchanges at 4°C. Purified protein aliquots were stored at -20°C.

Quality control assessment of purified proteins. All expression clones were sequenced to confirm that the cloned insert both matched the native *M. avium* subsp. *paratuberculosis* gene and was in frame with expression signals built into the pMAL-c2 expression vector. Furthermore, final protein concentrations of pooled fractions postdialysis were determined by NanoDrop spectrometry measurements at 280 nm. All recombinant proteins were further evaluated by GelCode Blue (Pierce Biotechnology Inc., Rockford, IL) stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels to assess purity and to ensure that they migrated at the expected size.

Animals, antibodies, and host serum. Sera from a rabbit that was exposed to live *M. avium* subsp. *paratuberculosis* ATCC 19698 by intraperitoneal injection was used in these studies. Sera from three clinical cattle (509, 3235, and 3494) and three uninfected control cattle (109, 110, and 111) were obtained from a herd dedicated to Johne's disease research activities at the National Animal Disease Center. The infection statuses and health histories of these animals were monitored quarterly by fecal culture, ELISA, and IFN- γ release assays (Table 2). All sera from these animals were diluted 1:500. Animals 5902, 5903, and 5904 were experimentally infected by intratonsillar inoculation by using four weekly doses of 4×10^6 CFU (strain K-10) in two tonsillar crypts as described in a previous study (57). Sera from all three animals experimentally infected with *M. avium* subsp. *paratuberculosis* were collected at day 321 post-intratonsillar infection and diluted 1:500 in these experiments. Sera from cow 202, experimentally infected with *M. bovis* (10^4 CFU of strain 95-1315 in each tonsillar crypt), was diluted 1:500 and sera from *M. avium* subsp. *avium*-infected cow 6137 (10^{10} CFU of strain 3988 split between the tonsillar crypts) were also diluted 1:500 (Table 2). A monoclonal antibody to the MBP affinity tag (diluted 1:3,000) was developed at the National Animal Disease Center and has been used in previous studies (6).

Protein array production. PBS was used as the spotting buffer and diluent for all *M. avium* subsp. *paratuberculosis* proteins. The protein array was constructed on a nitrocellulose membrane using a Bio-Dot 96-well manifold apparatus (Bio-Rad, Hercules, CA). Diluted working stocks of purified proteins were stored in deep 96-well plates with a 1-ml capacity per round-bottom well (Nalgene Nunc International). Each well of the assembled 96-well dot blot apparatus was loaded with 100 μ l of PBS containing the appropriate protein approximated to a final concentration of 0.5 mg/ml. The PBS-diluted samples were allowed to flow through the membrane by gravity flow. Each well was then washed in 200 μ l of PBS with 0.1% Tween-20 (TPBS). A vacuum was engaged to pull the wash solution through, and the nitrocellulose membrane was removed from the apparatus and placed in a petri dish containing a blocking solution (PBS with 2% bovine serum albumin and 0.1% Tween 20). After 30 min in the blocking solution, the immunoblotting assay was performed. PBS was also included on the dot blot array as a spotting buffer control. MBP-LacZ was also added as a control to assess immunoreactivity to the affinity tag. Protein arrays were produced in groups of four with one of the arrays used as a control with the monoclonal antibody to MBP. This enabled tracking of spotting efficiency between experiments.

Immunoblot analysis of the protein array. Dot blot arrays were incubated with sera from several hosts exposed to *M. avium* subsp. *paratuberculosis*. Sera were diluted in blocking buffer and exposed to spotted arrays for 2 h at room temperature on a rocker platform. The rabbit sera were diluted 1:1,000. After three washes in TPBS, the nitrocellulose array was incubated with the appropriate horseradish peroxidase-conjugated anti-immunoglobulin G antibody diluted at 1:20,000 in TPBS-bovine serum albumin for 1.5 h. This was followed by a final three washes in TPBS. Assay development utilized SuperSignal detection reagents (Pierce) and Kodak BioMax MR film. A control strip containing unconjugated bovine antibody (heavy plus light chains; Pierce) was processed in parallel with arrays exposed to uninfected cow sera. The top spot (A) on the control strip contained 0.11 mg/ml bovine antibody, the B spot contained 0.02 mg/ml, the C spot contained 0.002 mg/ml, and so on.

Measurement of spot intensities. Quantitative spot intensities were obtained by performing a densitometric scan on autoradiograms containing antibody-probed arrays. LabWorks 4.5 software (UVP Bioimaging System; UVP Inc., Upland, CA) was used to obtain the integrated optical density values for each spot. This quantification software processes spot intensities on the array and determines the mean intensities of pixels within a spot as well as those of the background pixels around the spot. These local-background intensities are subtracted from the raw signals to obtain the local-background-corrected levels. The measured diameter that was selected for scans of different arrays was consistent for all arrays in this study. Adjusted intensities were obtained following normalization of each spot with the corresponding spot on the MBP array produced from that set of experiments. Statistical analysis included the binomial test in which the null hypothesis *P* value was set to 0.5. Under this null hypothesis, 46 proteins were predicted to show a stronger intensity in *M. avium* subsp. *paratuberculosis*-infected animals than in non-*M. avium* subsp. *paratuberculosis*-infected animals and 46 proteins would have a weaker signal in *M. avium* subsp. *paratuberculosis*-infected animals than in non-*M. avium* subsp. *paratuberculosis*-infected animals. Any deviance from this null model was considered nonrandom.

RESULTS

Protein array content. All recombinant proteins represented on the *M. avium* subsp. *paratuberculosis* protein array were

TABLE 1. *Mycobacterium avium* subsp. *paratuberculosis* proteins used in this study

Protein ^a	Yield (mg) ^b	Gene size (bp) ^c	No. of amino acids ^d	Predicted function/description ^e
MAP sonicate	3.0	NA ^e	NA	NA
MAP0075	0.54	423	140	Conserved small membrane protein
MAP0087-His	3.0	717	238	Probable secreted protein
MAP0105c	3.72	1,058	352/889	Site-specific recombinase
MAP0182c	2.01	411	136	Conserved hypothetical protein
MAP0216	4.98	1,028	342/347	Antigen 85A, mycolyltransferase
MAP0261c	1.47	465	154/161	19-kDa lipoprotein
MAP0389	6.12	947	315/337	Diarylpropane peroxidase (EC 1.11.1.14)
MAP0736	17.19	744	247	Hypothetical protein
MAP0852	31.2	546	181	Unknown, no similarity
MAP0853	12.12	660	219	Unknown, no similarity
MAP0855	1.02	926	308/314	Possible DNA repair protein
MAP0857c	2.10	318	105	Unknown, no similarity
MAP0858	1.32	537	178/182	Unknown, no similarity
MAP0859c	1.98	609	202	Putative phosphoesterase
MAP0860c	0.12	885	294/296	Unknown, no similarity
MAP0861	1.29	342	113	Unknown, no similarity
MAP0862	1.44	989	329/360	Unknown, no similarity
MAP0863	1.98	675	224	Unknown, no similarity
MAP0864	22.26	426	141	Unknown, no similarity
MAP0865	0.24	1,272	423	Contains an FtsK domain
MAP0866	9.21	804	267	Phage-related integrase
MAP0904	2.43	371	124/246	Hypothetical protein, contains an LGFP 54-amino-acid repeat
MAP0961c	0.54	719	239/352	Contains a glycosyltransferase domain
MAP1087	1.29	441	146	Probable ABC peptide transport system, permease
MAP1121c	0.96	498	165	Putative MerR-like transcriptional regulator
MAP1174c	4.92	756	251	6-Phosphogluconolactonase
MAP1204	7.83	735	244	Putative invasin; contains an Nlpc_P60 domain
MAP1233	0.12	723	240	Putative methyltransferase
MAP1345	2.07	600	199	Putative tellurite resistance protein
MAP1388	0.24	396	131	Hypothetical protein
MAP1416c	1.35	666	221	Hypothetical protein
MAP1417c	1.62	435	144	Hypothetical protein
MAP1609c	0.15	987	328/330	Antigen 85B, mycolyltransferase
MAP1636c	3.36	425	141/157	Hypothetical protein
MAP1643	1.35	2,277	759/762	Isocitrate lyase, [beta] module
MAP1655c	2.73	381	126	Hypothetical protein
MAP1730c	0.78	1,023	340	Putative GTPases, G3E family/cobalamin synthesis
MAP1931c	0.96	1,104	367	Anthranilate phosphoribosyltransferase
MAP2077c	4.98	330	109	STAS domain containing protein
MAP2116c	0.81	1,269	422	ABC-type transport system, resistance to solvents
MAP2121c	21.42	887	295/307	Major membrane protein I
MAP2121c-His	2.97	924	307	Major membrane protein I
MAP2151	8.37	438	145	Unknown, no similarity
MAP2155	35.7	312	103	Similar to IS6110
MAP2156	1.77	963	320	Contains an integrase core domain
MAP2157	1.14	1,221	406	IS900 transposase
MAP2158	7.20	582	193	Unknown, no similarity
MAP2182c	0.78	435	144	Conserved hypothetical protein
MAP2231	0.93	1,422	473	Pap3A_1 protein, contains condensation domain
MAP2360c	0.39	534	177	Hypothetical protein
MAP2380	6.00	600	200/550	CaiC, acyl-CoA synthetases (AMP-forming)
MAP2657	0.78	651	216	Nitro-FMN-reductase
MAP2663c	0.09	510	169	Putative integral membrane protein
MAP2676c	3.90	354	117	Hypothetical protein
MAP2734	1.11	1,341	446	Nonheme iron oxygenase (RO) family
MAP2740	0.72	666	221	Uncharacterized membrane-associated protein
MAP2751	2.10	582	193	Unknown, no similarity
MAP2753	0.51	309	103/252	Unknown, no similarity
MAP2761c	0.09	692	230/238	Unknown, no similarity
MAP2762c	4.35	425	141/146	Unknown, no similarity
MAP2764c	0.30	290	96/149	Unknown, no similarity
MAP2765c	0.18	539	179/396	Hypothetical protein
MAP2767c	1.26	473	157/183	Unknown, no similarity
MAP2963c	2.64	1,499	499/874	Conserved hypothetical protein
MAP3084c-His	3.00	684	227	Probable secreted protein
MAP3121	1.14	855	284	Enoyl-CoA hydratase

Continued on following page

TABLE 1—Continued

Protein ^a	Yield (mg) ^b	Gene size (bp) ^c	No. of amino acids ^d	Predicted function/description ^f
MAP3129	0.27	404	134/141	Hypothetical protein
MAP3131	0.90	1,096	365/942	Large membrane protein, drug exporter
MAP3155c	3.66	273	90	Hypothetical protein
MAP3434	0.54	690	230/330	Probable membrane protein
MAP3437c	0.87	843	279/280	Helix-turn-helix motif, DNA binding protein
MAP3531c	0.84	1,034	344/352	Antigen 85C, probable esterase
MAP3734c	0.57	1,559	519/593	ABC transporter, ATP-binding protein
MAP3735c	1.26	1,181	393/429	ABC transporter, integral membrane
MAP3743	2.55	998	332/348	Saccharopine dehydrogenase
MAP3751	0.33	879	293/979	Conserved membrane protein, drug exporter
MAP3753	1.92	1,353	450	Hypothetical protein, contains condensation domain
MAP3761c	0.21	729	242	Conserved membrane protein
MAP3771	0.06	294	97	50S ribosomal protein L31
MAP3817c	1.95	939	312	Unknown, no similarity
MAP3833c	0.39	626	208/260	Hypothetical protein
MAP3840	0.30	1,872	623	70-kDa heat shock protein, DnaK
MAP3902c	0.45	522	173	Hypothetical protein
MAP3903c	1.26	531	176	Hypothetical protein
MAP3954	0.15	279	92	Conserved hypothetical protein
MAP4014	0.63	951	316	Amino acid transporter
MAP4025	0.96	854	284/325	Cytochrome <i>c</i> biogenesis protein
MAP4129	0.78	965	321/336	ABC transporter, organic solvent resistance
MAP4198	0.39	1,283	427/441	Preprotein translocase, SecY subunit
MAP4199	0.09	540/546	179/181	Adenylate kinase
MAP4207c	15.84	686	228/231	ABC-type transporter, ATPase component
MAP4228	0.15	222	73	Translation initiation factor IF-1

^a Protein names corresponds to the genome project designations (29). His indicates polyhistidine tag.

^b Protein yield is reported in total milligrams obtained from 1-liter culture as measured by Nanodrop spectrometry at 280 nm.

^c Gene size is reported in base pairs (bp). In cases in which only a portion of the *M. avium* subsp. *paratuberculosis* gene was cloned, the number of bp present in the clone is reported first, followed by the number of bp in the full-length *M. avium* subsp. *paratuberculosis* gene.

^d Numbers of amino acids present in the *M. avium* subsp. *paratuberculosis* fusion proteins. In cases in which only a portion of the *M. avium* subsp. *paratuberculosis* protein is represented, the number of amino acids present is listed first, followed by the total number of amino acids in the full-length *M. avium* subsp. *paratuberculosis* protein.

^e NA, not applicable.

^f Acyl-CoA, acyl coenzyme A; FMN, flavin mononucleotide.

initially evaluated for purity by SDS-PAGE analysis. A selection of 25 proteins, representing a typical experiment, is shown in Fig. 1. The largest fusion protein purified was MAP1643 at 127 kDa (Fig. 1), and the smallest protein was MAP4228 at

50.4 kDa (data not shown). The protein with the highest yield obtained from all the purifications was MAP2155, which represents a putative transposase similar to IS6110. A total of 35 mg of this protein was purified from a 1-liter culture at a

TABLE 2. Cultures, ages, and immunological statuses of cattle used in this study

Cow no.	Infection status ^a	Age (yr)	Fecal culture (CFU) ^b	IFN- γ (ng/ml)			ELISA S/P ratio ^d
				NS	PPDa	MPS	
109	Control	2	0, 0, 0, 0	0.054	0.062	0.117	0.006
110	Control	3	0, 0, 0, 0	0.057	0.095	0.077	0.023
111	Control	2	0, 0, 0, 0	0.101	0.142	0.089	0.004
5902	MAP-Exp ^c	>1	0, 2, 0, 1	0.053	0.143	0.097	0.70
5903	MAP-Exp	>1	0, 0, 1, 0	0.084	0.241	0.145	1.47
5904	MAP-Exp	>1	1, 1, 0, 0	0.068	0.362	0.114	1.29
509	Clinical	4	TMTC	0.133	0.936	0.742	1.87
3235	Clinical	6	TMTC	0.037	0.761	1.054	1.11
3494	Clinical	6	TMTC	0.167	1.30	0.924	1.22
202	<i>M. bovis</i> -Exp	>1	ND	0.377	0.949	0.412	0.89
6137	<i>M. avium</i> -Exp	>1	0, 0, 0, 0	0.177	1.99	0.417	0.33

^a Each naturally infected animal was surveyed quarterly to monitor progression of disease. Infection status was classified based on fecal culture, IFN- γ assay, and ELISA results. Experimentally infected animals were monitored at 2-week intervals and classified as "Exp" regardless of results.

^b Fecal culture is reported as numbers of CFU present on each Herold's egg yolk medium slant (platings are in quadruplicate). TMTC, too many colonies to count on that slant; ND, not determined.

^c The gamma interferon (IFN- γ) assay was performed on peripheral blood mononuclear cells isolated as previously described (55). NS, no stimulation; PPDa, *M. avium* subsp. *avium* purified protein derivative; MPS, *M. avium* subsp. *paratuberculosis* sonicate.

^d The ELISA sample-to-positive (S/P) ratio is calculated from the optical density reading as described previously (55). A whole-cell sonicate preparation (1 mg/ml) served as the antigen used to coat the plates.

^e Animal experimentally infected with *M. avium* subsp. *paratuberculosis*.

FIG. 1. SDS-PAGE analysis of purified recombinant fusion proteins. Two gels stained with GelCode Blue demonstrate the purity of 25 *M. avium* subsp. *paratuberculosis* recombinant fusion proteins plus the MBP-LacZ control. Most proteins show a single discrete band with limited contamination of *E. coli* host proteins. The MBP affinity tag is 42 kDa in size and the largest *M. avium* subsp. *paratuberculosis* protein, MAP1643, is 85 kDa in size. Therefore, this fusion protein migrates at the expected size of 127 kDa (lane 19). Protein size standards are indicated in kilodaltons in the left margin, and the migration position of the MBP-LacZ control is indicated in the right margin. Lane assignments: 1, protein size standards; 2, MBP-MAP1174c; 3, MBP-MAP1121c; 4, MBP-MAP3131; 5, MBP-MAP4198; 6, MBP-MAP2657; 7, MBP-MAP3121; 8, MBP-MAP1204; 9, MBP-MAP0961c; 10, MBP-MAP0853; 11, MBP-MAP3734c; 12, MBP-MAP2360; 13, MBP-MAP1388; 14, MBP-MAP0075; 15, MBP-LacZ; 16 and 17, protein size standards; 18, MBP-MAP3743; 19, MBP-MAP1643; 20, MBP-MAP3735c; 21, MBP-MAP3902c; 22, MBP-MAP0736; 23, MBP-MAP1609c; 24, MBP-MAP1087; 25, MBP-MAP1233; 26, MBP-MAP3761c; 27, MBP-MAP2740; 28, MBP-MAP0855; 29, MBP-MAP3751; 30, MBP-LacZ.

concentration of 9.57 mg/ml. The protein with the lowest yield obtained was also the smallest protein, MAP4228, which represents an IF-1 initiation factor. A complete listing of the proteins present on the array along with their sizes, purification yield and predicted function are listed in Table 1. Additional information surrounding each protein, including their similarity in other mycobacterial genomes, IPR number, and clusters of orthologous groups, etc., is listed in Table S1 in the supplemental material.

The 96-dot protein array was constructed on nitrocellulose filters. Represented among these spots are 89 MBP fusion proteins, 3 polyhistidine-tagged proteins, and 4 controls, including *M. avium* subsp. *paratuberculosis* sonicate (two spots; positive control), spotting buffer (one spot; negative control), and a LacZ alpha peptide fused to MBP (specificity control). The dot assignments for the protein array are shown in Fig. 2A. The three polyhistidine-tagged proteins (MAP0087, MAP2121c, and MAP3084c) are present on the upper right corner of the array (Fig. 2A, column 12). Note that MAP2121c,

which represents a widely conserved and antigenic membrane protein (56), is represented twice on the array, once as an MBP fusion (spot 4F) and also a polyhistidine-tagged protein (spot 12B). Coding sequences that are present uniquely in LSPs previously reported by our laboratories (42) and others (45) are also represented on the array. In fact, nearly all the coding sequences in the initial two columns comprise the *M. avium* subsp. *paratuberculosis*-specific LSP4 segment (MAP0851 to MAP0866) reported by Behr and colleagues (45).

Antibody response in healthy control cattle. Because most proteins spotted on the dot array were produced as a fusion with the MBP affinity purification tag, probing with a monoclonal antibody to this affinity tag was performed to determine relative amounts of each protein spotted (Fig. 2B). The three uninfected cows (109, 110, and 111) showed weak reactivity or no reactivity to recombinant proteins or the whole-cell sonicate antigen (see Table S2 in the supplemental material). Of the three uninfected cows, cow 111 showed the most antibody reactivity to recombinant proteins on the array and is shown in Fig. 2B along with an array exposed to the MBP monoclonal antibody. These data demonstrate that all MBP-tagged fusion proteins are detectable by using the monoclonal antibody and that nonspecific reactivity is low for uninfected cattle.

Antibody profiles for *M. bovis*- and *M. avium* subsp. *avium*-infected cattle. In order to identify potentially cross-reactive epitopes, protein arrays were used to probe sera from *M. bovis*- and *M. avium* subsp. *avium*-infected cattle (Fig. 3). In general, there was more reactivity observed with *M. avium* subsp. *avium*-infected cow serum than with *M. bovis*-infected cow serum. These data agree with the fact that *M. avium* subsp. *avium* is more phylogenetically similar to *M. avium* subsp. *paratuberculosis* than it is to *M. bovis* and hence shows more antibody cross-reactivity than *M. bovis*. Five proteins were detected with sera from both types of infected animals (Fig. 3). The majority of *M. avium* subsp. *paratuberculosis*-specific coding sequences present within LSPs and spotted in columns 1 to 3 and elsewhere on the array were not detected with sera from either infected cow (Fig. 3 and Table 3). MAP1087 (11A) showed more reactivity in both the *M. avium* subsp. *avium*-infected and *M. bovis*-infected cows than any other protein present on the dot array. TBLASTn analysis of this protein with *M. avium* subsp. *avium* 104 showed 99% similarity over the full length of the protein but only 29% similarity over a partial length in the *M. bovis* protein (see Table S1 in the supplemental material). In conclusion, while sera from healthy, uninfected cows show little or no reactivity with the spotted proteins, some proteins do cross-react with sera from cattle exposed to other mycobacteria.

Analysis of the humoral immune response in hosts exposed to *M. avium* subsp. *paratuberculosis*. The dot arrays were initially tested using sera from a rabbit infected with live *M. avium* subsp. *paratuberculosis* (Fig. 4A). MAP1204, a homolog of Rv1478 in *Mycobacterium tuberculosis*, which represents a putative exported p60 homolog possibly involved in virulence (18), was among the antigens most reactive with the rabbit sera. Likewise, the major membrane protein, MAP2121c, was also strongly detected in this host. The protein arrays were next used to obtain antibody profiles for cows with clinical signs of Johne's disease as well as for experimentally infected cows (Fig. 4B). The antibody profiles for all cattle were generally

FIG. 2. Controls to test the performance of the 96-dot array. Shown are the dot assignments for the protein array (A) and two dot array (B) experiments. The dot assignments are consistent for all experiments in this study. The monoclonal antibody or serum sample used is indicated beneath each array. All MBP fusion proteins were detected by the MBP monoclonal antibody (MBP mAb), whereas the serum from uninfected cow 111 showed little to no reactivity with most of the proteins. Because background reactivity was so low with all three uninfected cow sera, a control strip (shown on the right side of the array) containing serial dilutions of unconjugated bovine antibody was processed in parallel with all arrays exposed to uninfected cow sera to ensure the experiment worked correctly. A whole-cell lysate representing a majority of the proteins produced by *M. avium* subsp. *paratuberculosis* is spotted in positions 12E and 12H for all dot arrays. Note that the MBP monoclonal antibody does not detect the three His-tagged proteins spotted in positions 12A, 12B, and 12C.

similar regardless of the infection mode or health status. In addition, all experiments using cattle sera were subjected to densitometry analysis to provide quantitation for reactivity at each spot, reported as spot intensities. The mean intensity measurements for the experimentally infected cattle ($n = 3$) as well as the naturally infected clinical cattle ($n = 3$) are listed in Table 3. The strongest antigens that were consistently reactive with all the cattle serum included MAP1087 and MAP1730c (Table 3), which represent an ABC peptide transport permease and a putative GTPase protein, respectively (29). MAP1087 showed the strongest intensity values in three of the six *M. avium* subsp. *paratuberculosis*-infected cows and showed the second-strongest intensities in the remaining three cows

(see Table S2 in the supplemental material). The protein showing the third-strongest intensity, MAP1204, had the highest intensity value among the proteins from the rabbit (data not shown). The MAP1204 gene codes for a putative invasion protein that possesses an Nlpc_P60 domain of unknown function that is found in several lipoproteins. PSORTb analysis software predicts that MAP1730c is located in the cytoplasm, MAP1087 is in the membrane, and MAP1204 is extracellular (19).

To test the significance of these findings, a binomial test was performed on all proteins and the null hypothesis P value was set to 0.5. These calculations showed that rather than an equal 50/50 distribution of proteins showing a stronger signal with

FIG. 3. Cross-reactive antibodies from cattle infected with mycobacteria other than *M. avium* subsp. *paratuberculosis*. Each array was exposed to serum from an infected cow as indicated beneath the image. Antibodies from the *M. avium* subsp. *avium* (*M. avium*)-infected cow were observed to be more cross-reactive than those from the *M. bovis*-infected cow. Dot assignments are as shown in Fig. 2A.

TABLE 3. Spot intensities for protein arrays exposed to cattle sera in this study^a

Mean spot intensity for cattle infected with:						Mean spot intensity for cattle infected with:					
Spot ^b	Protein	<i>M. avium</i> subsp. <i>avium</i> (cow 6137)	<i>M. bovis</i> (cow 202)	<i>M. avium</i> subsp. <i>paratuberculosis</i>		Spot ^b	Protein	<i>M. avium</i> subsp. <i>avium</i> (cow 6137)	<i>M. bovis</i> (cow 202)	<i>M. avium</i> subsp. <i>paratuberculosis</i>	
				Natural infection ^c	Exptl infection ^d					Natural infection ^c	Exptl infection ^d
A11	MAP1087	283.71 ^e	98.71	462.32	825.16	F5	MAP1417c	12.36	9.81	12.51	18.34
G6	MAP1730c	146.74	80.10	427.09	746.49	B7	MAP2663c	10.57	10.99	12.14	45.70
C11	MAP1204	266.55	26.60	298.77	739.38	E10	MAP3121	11.69	15.40	12.12	12.28
H3	MAP3817c	21.71	10.05	268.71	168.57	B8	MAP4129	70.30	11.54	11.89	52.08
D11	MAP2116c	75.99	23.27	257.29	588.02	A10	MAP1174c	12.04	4.55	11.87	57.40
F10	MAP4025	276.63	60.80	177.80	639.06	G10	MAP4199	10.24	14.63	11.79	11.18
G11	MAP3155c	13.13	7.43	168.94	31.10	F9	MAP3902c	32.94	15.03	11.76	21.04
D2	MAP0865	8.82	38.03	142.66	543.65	B3	MAP2764c	69.82	11.80	11.61	48.07
D1	MAP0857c	8.83	11.95	120.22	314.57	E7	MAP3131	61.93	13.40	11.60	34.95
D7	MAP2734	175.47	9.50	106.79	232.89	G4	MAP0261c	51.64	12.33	11.31	50.40
A8	MAP3761c	27.95	8.35	89.78	273.10	B9	MAP2360c	8.44	6.90	11.07	7.28
F2	MAP1233	92.24	6.88	80.21	348.88	G9	MAP3903c	9.85	8.34	11.02	22.54
G5	MAP0961c	122.41	11.61	75.79	525.79	D6	MAP2761c	33.34	9.58	10.93	10.72
A7	MAP0075	48.16	7.39	74.30	258.46	B11	MAP1121c	12.08	13.89	10.70	8.32
G7	MAP3734c	54.09	10.43	60.36	199.25	B5	MAP0182c	142.09	10.83	10.41	101.63
H5	MAP2077c	10.45	9.31	59.38	79.22	G12	MAP2156	14.56	10.67	10.34	46.96
D9	MAP3753	36.80	5.41	59.16	178.81	B2	MAP0863	8.00	4.84	10.01	31.20
C7	MAP2740	186.78	10.17	58.18	174.40	H10	MAP4228	13.04	12.47	10.01	13.05
E11	MAP2231	56.12	15.11	54.40	235.10	F6	MAP2963c	9.10	13.16	9.92	13.35
G2	MAP1636c	92.91	12.03	52.52	121.85	A6	MAP2676c	10.27	6.78	9.91	10.70
A5	MAP0105c	10.38	8.72	46.81	46.32	E6	MAP2762c	38.31	12.56	9.90	18.00
C8	MAP4198	15.56	10.40	45.70	108.19	C12	MAP3084c-His	8.35	9.80	9.85	10.07
G1	MAP0860c	16.49	8.19	43.06	62.07	C3	MAP2767c	14.03	8.33	9.73	54.48
H1	MAP0861	12.02	13.29	41.23	16.62	D4	MAP1609c	5.99	9.61	9.72	11.62
F7	MAP3434	70.95	12.95	40.08	263.92	A3	MAP2158	9.36	8.69	9.64	35.07
F11	MAP4014	11.50	14.90	34.07	111.36	E1	MAP0858	10.00	14.32	9.50	16.12
H12	MAP cells ^f	11.27	8.49	30.63	17.79	F4	MAP2121c	33.59	12.19	9.23	22.46
H9	MAP3954	10.12	11.67	26.37	35.80	G8	MAP0904	11.51	13.18	9.16	10.72
E3	MAP3735c	11.63	12.00	26.04	96.37	H7	MAP3751	11.63	11.38	9.14	17.13
E12	MAP cells	6.83	10.67	25.97	14.57	H8	MAP1655c	9.93	10.42	9.04	14.47
A9	MAP2182c	93.98	8.05	24.25	151.90	C5	MAP1345	13.60	11.17	9.02	27.06
F3	MAP3743	71.46	45.15	24.18	188.16	D5	MAP1388	7.76	9.31	8.95	9.80
A2	MAP0862	64.74	9.35	24.12	130.29	B12	MAP2121c-His	10.68	12.10	8.69	17.65
C10	MAP2380	16.89	14.06	23.77	36.15	B1	MAP0853	10.03	7.36	8.39	66.78
A4	MAP3840	11.45	8.17	23.36	41.05	D12	PBS buffer ^g	6.84	10.46	8.27	8.88
E8	MAP0389	42.73	9.75	22.85	71.01	F12	MAP2155	12.07	10.17	8.16	9.36
C9	MAP3129	21.37	14.16	18.30	35.46	C2	MAP0864	13.27	14.21	8.14	18.46
C1	MAP0855	11.86	12.71	17.70	66.44	F1	MAP0859c	11.55	10.43	8.03	16.00
E9	MAP3833c	44.88	10.62	17.63	64.47	A12	MAP0087-His	7.63	7.07	7.76	5.79
E2	MAP0866	9.34	14.31	16.66	71.19	C4	MAP0216	5.79	7.34	7.72	18.99
B10	MAP1931c	18.87	7.69	16.53	35.05	G3	MAP3771	21.40	9.17	7.72	15.53
D10	MAP2657	20.43	21.32	15.72	163.75	H6	MAP2157	11.49	10.19	7.61	17.76
B6	MAP2751	23.29	8.91	15.54	38.99	E5	MAP1416c	31.35	14.01	7.24	20.19
C6	MAP2753	62.02	6.99	14.46	34.16	H11	MAP2765c	10.79	11.35	6.99	19.83
E4	MAP3531c	13.63	9.08	14.10	43.32	B4	MAP1643	15.42	8.35	5.82	11.57
D8	MAP4207c	72.21	11.06	13.57	82.51	D3	MAP3437c	7.57	12.44	5.54	16.27
H2	MAP2151	38.57	10.71	13.42	167.00	A1	MAP0852	12.44	6.57	4.84	10.93
F8	MAP0736	48.76	6.99	12.84	27.62	H4	MBP-LacZ	8.43	12.42	4.76	18.10

^a Shown are the spot intensity values for each spot arranged in descending order by mean spot intensity for the naturally *M. avium* subsp. *paratuberculosis*-infected cattle.

^b Location on the dot blot array.

^c The average intensity of spot measurements from sera of cattle naturally infected with *M. avium* subsp. *paratuberculosis* ($n = 3$).

^d The average intensity of spot measurements from sera of cattle experimentally infected *M. avium* subsp. *paratuberculosis* ($n = 3$).

^e The strongest intensity in each column is shown in bold.

^f MAP cells, a whole-cell sonicated lysate of *M. avium* subsp. *paratuberculosis*.

^g PBS buffer in spot D12 represents the spotting-buffer-only control.

the sera from *M. avium* subsp. *paratuberculosis*-infected cows than with non-*M. avium* subsp. *paratuberculosis*-infected cows, as the null hypothesis would suggest, it showed a nonrandom distribution of 72 proteins that had higher intensity values with the *M. avium* subsp. *paratuberculosis*-infected cattle sera than

with the non-*M. avium* subsp. *paratuberculosis*-infected cattle sera and 24 proteins for which the reverse was observed.

Immunogenicity of proteins encoded within LSPs. A total of 40 proteins on the array are produced by coding sequences present within the 17 LSPs identified in the *M. avium* subsp.

FIG. 4. Protein arrays used to assess the humoral immune response to *M. avium* subsp. *paratuberculosis*. The protein arrays were processed by immunoblot analysis with various primary sera, as indicated in the margins. (A) Arrays probed with sera from a rabbit exposed to *M. avium* subsp. *paratuberculosis* and anti-MBP antibody. mAb, monoclonal antibody. (B) Antibody profiles from one experimentally infected cow (5903) and three clinical cattle (509, 3235, and 3494). Sera from cow 5903 were collected at 321 days post-intratracheal infection with *M. avium* subsp. *paratuberculosis* and represent an early stage of disease. Sera were collected from the other three naturally infected cows during the appearance of clinical Johne's disease. Antibody profiles are generally similar among all four cattle despite the representation of distinct infection routes and disease stages. Dot assignments are as shown in Fig. 2A.

paratuberculosis K-10 genome. These LSPs comprise 187 total coding sequences and span over 226 kb of genomic DNA (45). Seven of the LSPs are not present in any of the 96 *M. avium* complex isolates tested (45). Overall, MAP1730c, encoded within LSP9, had the most consistently strong intensities among all six sera from the infected cattle tested (Table 3; see Table S2 in the supplemental material). However, LSP9 did show some cross-reactivity with *M. avium* complex isolates in PCR screens (45). The three most-immunogenic proteins encoded within LSPs that were observed to be 100% specific for *M. avium* subsp. *paratuberculosis* isolates included MAP0865 and MAP0857c (LSP4) and MAP3817c (LSP16). These proteins had the sixth (MAP0865)-, eighth (MAP3817c)-, and ninth (MAP0857c)-strongest mean intensities (Table 3) and thus are excellent candidates for an antigen-based diagnostic test.

DISCUSSION

Although previous studies have correlated bovine immune responses to the 22-kDa protein (43), heat shock proteins (17), and others encoded by the MAP1272c (30), MAP1138c (15), or MAP3968 (44) gene, all these *M. avium* subsp. *paratuberculosis* proteins have been evaluated singly, not in comparison to other proteins. More recently, investigators have addressed the need to evaluate multiple mycobacterial antigens in parallel by recombinant expression and immunoreactivity studies

through multiantigen print immunoassays (31, 58), immunoblot analysis (41), or ELISA (11, 38, 47, 60). However, these studies are limited by the printing capabilities, the number of wells on a polyacrylamide gel or microtiter plate, and therefore, only 15 or fewer proteins were analyzed. It would be more informative if dozens of proteins were compared not only with each other but also with known mycobacterial antigens and other *M. avium* subsp. *paratuberculosis* proteins. Therefore, a comprehensive analysis of all *M. avium* subsp. *paratuberculosis* antigens at a whole-genome level in an unbiased assay system is ideal in order to fully determine the antigenic basis of host protective and pathogenic responses to *M. avium* subsp. *paratuberculosis* infection. The available *M. avium* subsp. *paratuberculosis* genome sequence (29) has made it possible to perform a comprehensive analysis of the antigenicities of all proteins encoded by this bacterium.

Microbial proteomics typically involves performing 2-D SDS-PAGE separation followed by excision of spots either stained or identified in a corresponding immunoblot. The excised protein(s) is then identified by a mass spectrometry (MS) method, such as matrix-assisted laser desorption/ionization-time of flight MS or tandem MS. Using this approach, as many as 715 *E. coli* proteins of the total of approximately 5,000 predicted from the genome sequence have been identified in the *E. coli* proteome (23). However, with a complete genome sequence, the potential to construct a complete protein array representing all proteins encoded in the genome exists. The

experiments herein represent the most comprehensive parallel analysis of multiple antigens of *M. avium* subsp. *paratuberculosis*. Novel findings emerge when many proteins are analyzed in this way. Investigators can unequivocally determine the strongest and weakest antigens in the set of proteins under analysis. The strongest antigens identified from this set of recombinant proteins included MAP1087, MAP1730c, and MAP1204. All three of these proteins have not been previously evaluated or reported in the literature. Furthermore, unlike with spot identification within 2-D gels, with this research tool, proteins that do not react with infected animals are also readily identified.

Three naturally infected cattle, all in the clinical stage of Johne's disease, were analyzed in conjunction with three experimentally infected animals. Sera collected at 321 days postinfection from the experimentally infected animals were used. The lengths of exposure to *M. avium* subsp. *paratuberculosis* of the naturally infected animals are not known; however, animals can have a lengthy subclinical phase for two to five years or more before showing clinical signs of Johne's disease (48). Despite the differences in disease introduction and length of exposure, the antibody profiles obtained from all cattle were strikingly similar. These data suggest that the humoral immune response to *M. avium* subsp. *paratuberculosis* in cattle is established relatively quickly after exposure and may remain consistent throughout the disease stages. This conclusion may seem to counter current hypotheses, which suggest that a strong cell-mediated immune response occurs initially while the humoral immune response begins in the later stages of disease in cattle. However, the more likely explanation is that a strong cell-mediated immune response occurs initially in addition to a developing humoral immune response that has historically been more difficult to measure. Data from this study show that the whole-cell sonicate preparation was poorly detected by cattle sera (Fig. 4B, spots 12E and 12H). This preparation is likely similar to other complex protein preparations used in ELISAs and underscores the need to identify alternate antigen preps to better detect the antibody response following infection.

The cattle and rabbit sera showed remarkably similar antibody response profiles. Notably, 6 of the top 10 strongest intensities in the rabbit experiment were also among the top 10 most-reactive antigens in the cattle experiments. For example, MAP1087 showed the strongest intensity in cattle and was the third strongest in the rabbit. Likewise, MAP1204 was the third-strongest antigen in cattle and the strongest in the rabbit. The consistency with which these proteins were detected by sera from all animals tested gives added strength to these results. Furthermore, those proteins that had weak reactivities in cattle also showed weak reactivities in the rabbit. The use of sera from the two different animal hosts was a tool to ensure that observed antibody responses were consistent and not the result of spurious cross-reactivity. Further work should be done to examine the use of rabbits as a model of humoral immunity for Johne's disease in cattle.

Second-generation protein arrays containing many additional *M. avium* subsp. *paratuberculosis* proteins, as well as proteins detected by *M. bovis* and *M. avium* subsp. *avium* only, will be used in future studies to better demonstrate the humoral immune responses in a panel of sera from cattle with

well-characterized Johne's disease in comparison to those in sera from cattle infected with *M. bovis* or *M. avium* subsp. *avium*. In addition, the protein array can be used in studies to compare the antibody profiles for cattle to those for sheep, another ruminant animal of commercial interest that is affected by Johne's disease. It will be interesting to determine if specific and reproducible sets of antigens are associated with each host or disease state, as that information may provide clues to the pathogenesis of Johne's disease.

Data obtained from this study have even broader implications among the genus *Mycobacterium*. In silico analysis of the *M. avium* subsp. *paratuberculosis* genome identified >3,000 genes with homologs to genes of the human pathogen *M. tuberculosis* (29). One of these homolog products that has been identified as the strongest antigen in this study is MAP1087, a putative membrane protein with a possible role in peptide transport (29). The corresponding coding sequence in *M. tuberculosis* is designated Rv3665c (12), and that in *M. bovis* is designated Mb3698c (20). This antigen can now be immediately tested for diagnostic potential or perhaps as a subunit vaccine candidate against tuberculosis. The cross-reactivity observed with this protein is a benefit for subunit vaccine development, as it may protect against multiple mycobacterial diseases. The second-strongest antigen identified from these studies, MAP1730c, which represents a putative GTPase protein, has no homolog in either *M. tuberculosis* or *M. bovis*. Further studies are necessary to evaluate cell-mediated responses to these promising antigens.

Our previous work has shown that MAP0862 and MAP2963c are detected by sera from infected cattle (41). Leroy et al. also found MAP2963c to be weakly immunogenic in cattle (28). This study suggests that while both MAP2963c and MAP0862 are detected by sera from infected cattle, they are only weakly detected (Table 3), and that other proteins, identified from this more-comprehensive study, appear to be better antigens. The *M. avium* subsp. *paratuberculosis*-specific proteins, present in columns 1 and 2 on the array, were generally not detected by sera from cows infected with either *M. bovis* or *M. avium* subsp. *avium*. The exceptions include MAP0865, which was weakly detected by sera from the *M. bovis*-infected cow, and MAP0863, which was weakly detected by sera from the *M. avium* subsp. *avium*-infected cow. This suggests that not only are the coding sequences uniquely present in *M. avium* subsp. *paratuberculosis*, but most of the gene products also do not contain cross-reactive epitopes.

Among the proteins strongly detected in the experimentally infected cows were MAP0961c, MAP0865, MAP3743, MAP2151, and MAP3734c. MAP0961c is especially interesting. Annotated as a hypothetical protein, it contains a glycosyltransferase domain, and an ortholog of this protein was found in the recently completed *Mycobacterium ulcerans* genome sequence (53). Findings from this study show that it is not only among the 10 most-antigenic proteins, but it may also be detected early postinfection. This protein was strongly detected in the experimentally infected cows but was detected only weakly in the naturally infected cows (Table 3). It also showed some degree of cross-reactivity in serum from *M. avium* subsp. *avium*-infected cattle.

Data obtained from comparative genomic studies have uncovered 17 large genetic polymorphisms in *M. avium* subsp.

paratuberculosis, termed LSPs. We produced 40 recombinant proteins that represent coding sequences contained within most of these LSPs. We specifically focused on LSP4, a 15.3-kb region containing 16 coding sequences that are not present in any of 276 non-*M. avium* subsp. *paratuberculosis* isolates tested (45). Furthermore, the genes contain no significant similarity with any sequences in public databases. Thirteen of the 16 coding sequences that comprise LSP4 are fully represented on the protein array. Data from this study suggest that at least some of these coding sequences, notably those coding for MAP0865 and MAP0857c, are produced during infection of cattle. MAP3817c, whose encoding gene is located on the *M. avium* subsp. *paratuberculosis*-specific LSP16 genomic island, is a putative membrane protein and also appears to be antigenic. These three promising proteins need to be tested further with additional cattle sera.

In summary, novel antigens have been identified by using a newly developed 96-dot protein array. These studies yield solid hints at candidates for a subunit vaccine as well as proteins that might be incorporated into an antigen-based diagnostic test. Furthermore, they have revealed novel immune-reactive *paratuberculosis* antigens, not only indicating that the hypothetical open reading frame-encoded proteins are expressed during infection in cattle and other hosts but also providing the proof of principle that the fusion protein-based approach can be used to profile humoral immune responses to infection at the whole-genome scale.

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REFERENCES

- Amons, A., L. L. Li, Q. Zhang, J. P. Bannantine, A. S. Motiwala, S. Sreevatsan, and V. Kapur. 2004. Multilocus short sequence repeat sequencing approach for differentiating among *Mycobacterium avium* subsp. *paratuberculosis* strains. *J. Clin. Microbiol.* **42**:1694–1702.
- 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.
- Bannantine, J. P., R. G. Barletta, J. R. Stabel, M. L. Paustian, and V. Kapur. 2004. Application of the genome sequence to address concerns that *Mycobacterium avium* subspecies *paratuberculosis* might be a foodborne pathogen. *Foodborne Pathog. Dis.* **1**:3–15.
- Bannantine, J. P., J. K. Hansen, M. L. Paustian, A. Amons, L. L. Li, J. R. Stabel, and V. Kapur. 2004. Expression and immunogenicity of proteins encoded by sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*. *J. Clin. Microbiol.* **42**:106–114.
- Bannantine, J. P., and M. L. Paustian. 2006. Identification of diagnostic proteins in *Mycobacterium avium* subspecies *paratuberculosis* by a whole genome analysis approach, p. 185–196. In L. O'Connor (ed.), *Diagnostic bacteriology protocols*, 2nd ed. Humana Press, Totowa, NJ.
- Bannantine, J. P., T. J. Radosevich, J. R. Stabel, S. Berger, J. F. Griffin, and M. L. Paustian. 2007. Production and characterization of monoclonal antibodies against a major membrane protein of *Mycobacterium avium* subsp. *paratuberculosis*. *Clin. Vaccine Immunol.* **14**:312–317.
- Beard, P. M., M. J. Daniels, D. Henderson, A. Pirie, K. Rudge, D. Buxton, S. Rhind, A. Greig, M. R. Hutchings, I. McKendrick, K. Stevenson, and J. M. Sharp. 2001. *Paratuberculosis* infection of nonruminant wildlife in Scotland. *J. Clin. Microbiol.* **39**:1517–1521.
- Bull, T. J., K. Sidi-Boumedine, E. J. McMinn, K. Stevenson, R. Pickup, and J. Hermon-Taylor. 2003. *Mycobacterial interspersed repetitive units (MIRU)* differentiate *Mycobacterium avium* subspecies *paratuberculosis* from other species of the *Mycobacterium avium* complex. *Mol. Cell. Probes* **17**:157–164.
- Burton, M. S., J. H. Olsen, R. L. Ball, and G. A. Dumonceaux. 2001. *Mycobacterium avium* subsp. *paratuberculosis* infection in an addax (*Addax nasomaculatus*). *J. Zoo Wildl. Med.* **32**:242–244.
- Cho, D., and M. T. Collins. 2006. Comparison of the proteomes and antigenicities of secreted and cellular proteins produced by *Mycobacterium paratuberculosis*. *Clin. Vaccine Immunol.* **13**:1155–1161.
- Cho, D., S. J. Shin, A. M. Talaat, and M. T. Collins. 2007. Cloning, expression, purification and serodiagnostic evaluation of fourteen *Mycobacterium paratuberculosis* proteins. *Protein Expr. Purif.* **53**:411–420.
- Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekle, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, et al. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**:537–544.
- de Juan, L., A. Mateos, L. Dominguez, J. M. Sharp, and K. Stevenson. 2005. Genetic diversity of *Mycobacterium avium* subspecies *paratuberculosis* isolates from goats detected by pulsed-field gel electrophoresis. *Vet. Microbiol.* **106**:249–257.
- Dukes, T. W., G. J. Glover, B. W. Brooks, J. R. Duncan, and M. Swendrowski. 1992. *Paratuberculosis* in saiga antelope (*Saiga tatarica*) and experimental transmission to domestic sheep. *J. Wildl. Dis.* **28**:161–170.
- Dupont, C., K. Thompson, C. Heuer, B. Gicquel, and A. Murray. 2005. Identification and characterization of an immunogenic 22 kDa exported protein of *Mycobacterium avium* subspecies *paratuberculosis*. *J. Med. Microbiol.* **54**:1083–1092.
- Eda, S., J. P. Bannantine, W. R. Waters, Y. Mori, R. H. Whitlock, M. C. Scott, and C. A. Speer. 2006. A highly sensitive and subspecies-specific surface antigen enzyme-linked immunosorbent assay for diagnosis of Johne's disease. *Clin. Vaccine Immunol.* **13**:837–844.
- 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.
- Gao, L. Y., M. Pak, R. Kish, K. Kajihara, and E. J. Brown. 2006. A mycobacterial operon essential for virulence in vivo and invasion and intracellular persistence in macrophages. *Infect. Immun.* **74**:1757–1767.
- Gardy, J. L., M. R. Laird, F. Chen, S. Rey, C. J. Walsh, M. Ester, and F. S. Brinkman. 2005. PSORTb v. 2.0: expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis. *Bioinformatics* **21**:617–623.
- Garnier, T., K. Eiglmeier, J. C. Camus, N. Medina, H. Mansoor, M. Pryor, S. Duthoy, S. Grondin, C. Lacroix, C. Monsemp, S. Simon, B. Harris, R. Atkin, J. Doggett, R. Mayes, L. Keating, P. R. Wheeler, J. Parkhill, B. G. Barrell, S. T. Cole, S. V. Gordon, and R. G. Hewinson. 2003. The complete genome sequence of *Mycobacterium bovis*. *Proc. Natl. Acad. Sci. USA* **100**:7877–7882.
- Garry, F., S. Wells, S. Ott, and D. Hansen. March 1999. Animal and Plant Health Inspection Service info sheet: who can afford a \$200 loss per cow? or Johne's disease—what do I need to know? USDA, Washington, DC. <http://www.aphis.usda.gov/vs/ceah/ncahs/naahms/dairy/dairy96/johnsart.htm>.
- Godfroid, J., C. Delcorps, L. M. Irenge, K. Walravens, S. Marche, and J. L. Gala. 2005. Definitive differentiation between single and mixed mycobacterial infections in red deer (*Cervus elaphus*) by a combination of duplex amplification of p34 and f57 sequences and Hpy188I enzymatic restriction of duplex amplicons. *J. Clin. Microbiol.* **43**:4640–4648.
- Han, M. J., and S. Y. Lee. 2006. The *Escherichia coli* proteome: past, present, and future prospects. *Microbiol. Mol. Biol. Rev.* **70**:362–439.
- Hughes, V., S. Smith, A. Garcia-Sanchez, J. Sales, and K. Stevenson. 2007. Proteomic comparison of *Mycobacterium avium* subspecies *paratuberculosis* grown in vitro and isolated from clinical cases of ovine *paratuberculosis*. *Microbiology* **153**:196–205.
- Judge, J., I. Kyriazakis, A. Greig, D. J. Allcroft, and M. R. Hutchings. 2005. Clustering of *Mycobacterium avium* subsp. *paratuberculosis* in rabbits and the environment: how hot is a hot spot? *Appl. Environ. Microbiol.* **71**:6033–6038.
- Koets, A. P., V. P. Rutten, M. de Boer, D. Bakker, P. Valentin-Weigand, and W. van Eden. 2001. Differential changes in heat shock protein-, lipooligosaccharide-, and purified protein derivative-specific immunoglobulin G1 and G2 isotype responses during bovine *Mycobacterium avium* subsp. *paratuberculosis* infection. *Infect. Immun.* **69**:1492–1498.
- Lambeth, C., L. A. Reddacliff, P. Windsor, K. A. Abbott, H. McGregor, and R. J. Whittington. 2004. Intrauterine and transmammary transmission of *Mycobacterium avium* subsp. *paratuberculosis* in sheep. *Aust. Vet. J.* **82**:504–508.
- Leroy, B., V. Roupie, I. Noel-Georis, V. Rosseels, K. Walravens, M. Govaerts, K. Huygen, and P. Wattiez. 2007. Antigen discovery: a postgenomic approach to *paratuberculosis* diagnosis. *Proteomics* **7**:1164–1176.
- Li, L., J. P. Bannantine, Q. Zhang, A. Amons, B. J. May, D. Alt, N. Banerji, S. Kanjilal, and V. Kapur. 2005. The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proc. Natl. Acad. Sci. USA* **102**:12344–12349.
- Li, L., S. Munir, J. P. Bannantine, S. Sreevatsan, S. Kanjilal, and V. Kapur. 2007. Rapid expression of *Mycobacterium avium* subsp. *paratuberculosis* recombinant proteins for antigen discovery. *Clin. Vaccine Immunol.* **14**:102–105.

31. Lyashchenko, K. P., M. Singh, R. Colangeli, and M. L. Gennaro. 2000. A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. *J. Immunol. Methods* **242**:91–100.
32. Machackova-Kopečna, M., M. Bartos, M. Straka, V. Ludvik, P. Svastova, J. Alvarez, J. Lamka, I. Trcka, F. Treml, I. Parmova, and I. Pavlik. 2005. Paratuberculosis and avian tuberculosis infections in one red deer farm studied by IS900 and IS901 RFLP analysis. *Vet. Microbiol.* **105**:261–268.
33. Mackintosh, C. G., G. W. de Lisle, D. M. Collins, and J. F. Griffin. 2004. Mycobacterial diseases of deer. *N. Z. Vet. J.* **52**:163–174.
34. Marsh, I. B., J. P. Bannantine, M. L. Paustian, M. L. Tizard, V. Kapur, and R. J. Whittington. 2006. Genomic comparison of *Mycobacterium avium* subsp. *paratuberculosis* sheep and cattle strains by microarray hybridization. *J. Bacteriol.* **188**:2290–2293.
35. McKenna, S. L., G. P. Keefe, H. W. Barkema, and D. C. Sockett. 2005. Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. *Vet. Microbiol.* **110**:105–111.
36. Motiwala, A. S., M. Strother, A. Amonsin, B. Byrum, S. A. Naser, J. R. Stabel, W. P. Shulaw, J. P. Bannantine, V. Kapur, and S. Sreevatsan. 2003. Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: evidence for limited strain diversity, strain sharing, and identification of unique targets for diagnosis. *J. Clin. Microbiol.* **41**:2015–2026.
37. Muskens, J., D. Bakker, J. de Boer, and L. van Keulen. 2001. Paratuberculosis in sheep: its possible role in the epidemiology of paratuberculosis in cattle. *Vet. Microbiol.* **78**:101–109.
38. 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.
39. O'Shea, B., S. Khare, K. Bliss, P. Klein, T. A. Ficht, L. G. Adams, and A. C. Rice-Ficht. 2004. Amplified fragment length polymorphism reveals genomic variability among *Mycobacterium avium* subsp. *paratuberculosis* isolates. *J. Clin. Microbiol.* **42**:3600–3606.
40. Overduin, P., L. Schouls, P. Roholl, A. van der Zanden, N. Mahmmoud, A. Herrewegh, and D. van Soelingen. 2004. Use of multilocus variable-number tandem-repeat analysis for typing *Mycobacterium avium* subsp. *paratuberculosis*. *J. Clin. Microbiol.* **42**:5022–5028.
41. Paustian, M. L., A. Amonsin, V. Kapur, and J. P. Bannantine. 2004. Characterization of novel coding sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*: implications for diagnosis of Johne's disease. *J. Clin. Microbiol.* **42**:2675–2681.
42. Paustian, M. L., V. Kapur, and J. P. Bannantine. 2005. Comparative genomic hybridizations reveal genetic regions within the *Mycobacterium avium* complex that are divergent from *Mycobacterium avium* subsp. *paratuberculosis* isolates. *J. Bacteriol.* **187**:2406–2415.
43. Rigden, R. C., D. M. Jandhyala, C. Dupont, D. Crosbie-Caird, N. Lopez-Villalobos, N. Maeda, B. Gicquel, and A. Murray. 2006. Humoral and cellular immune responses in sheep immunized with a 22 kilodalton exported protein of *Mycobacterium avium* subspecies *paratuberculosis*. *J. Med. Microbiol.* **55**:1735–1740.
44. Sechi, L. A., N. Ahmed, G. E. Felis, I. Dupre, S. Cannas, G. Fadda, A. Bua, and S. Zanetti. 2006. Immunogenicity and cytoadherence of recombinant heparin binding haemagglutinin (HBHA) of *Mycobacterium avium* subsp. *paratuberculosis*: functional promiscuity or a role in virulence? *Vaccine* **24**:236–243.
45. Semret, M., D. C. Alexander, C. Y. Turenne, P. de Haas, P. Overduin, D. van Soelingen, D. Cousins, and M. A. Behr. 2005. Genomic polymorphisms for *Mycobacterium avium* subsp. *paratuberculosis* diagnostics. *J. Clin. Microbiol.* **43**:3704–3712.
46. Semret, M., G. Zhai, S. Mostowy, C. Cleto, D. Alexander, G. Cangelosi, D. Cousins, D. M. Collins, D. van Soelingen, and M. A. Behr. 2004. Extensive genomic polymorphism within *Mycobacterium avium*. *J. Bacteriol.* **186**:6332–6334.
47. Shin, S. J., H. S. Yoo, S. P. McDonough, and Y. F. Chang. 2004. Comparative antibody response of five recombinant antigens in related to bacterial shedding levels and development of serological diagnosis based on 35 kDa antigen for *Mycobacterium avium* subsp. *paratuberculosis*. *J. Vet. Sci.* **5**:111–117.
48. Stabel, J. R. 2006. Host responses to *Mycobacterium avium* subsp. *paratuberculosis*: a complex arsenal. *Anim. Health Res. Rev.* **7**:61–70.
49. Stabel, J. R. 1998. Johne's disease: a hidden threat. *J. Dairy Sci.* **81**:283–288.
50. Stabel, J. R. 2000. Transitions in immune responses to *Mycobacterium paratuberculosis*. *Vet. Microbiol.* **77**:465–473.
51. Stabel, J. R., and R. H. Whitlock. 2001. An evaluation of a modified interferon-gamma assay for the detection of paratuberculosis in dairy herds. *Vet. Immunol. Immunopathol.* **79**:69–81.
52. Steadham, E. M., B. M. Martin, and C. O. Thoen. 2002. Production of a *Mycobacterium avium* ssp. *paratuberculosis* purified protein derivative (PPD) and evaluation of potency in guinea pigs. *Biologicals* **30**:93–95.
53. Stinear, T. P., T. Seemann, S. Pidot, W. Frigui, G. Reyssset, T. Garnier, G. Maurice, D. Simon, C. Bouchier, L. Ma, M. Tichit, J. L. Porter, J. Ryan, P. D. Johnson, J. K. Davies, G. A. Jenkin, P. L. Small, L. M. Jones, F. Tekai, F. Laval, M. Daffe, J. Parkhill, and S. T. Cole. 2007. Reductive evolution and niche adaptation inferred from the genome of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. *Genome Res.* **17**:192–200.
54. Storset, A. K., I. Berg, and B. Djonne. 2005. Evaluation of the gamma interferon test for diagnosis of paratuberculosis in goats. *Vet. Immunol. Immunopathol.* **107**:87–94.
55. Storset, A. K., H. J. Hasvold, M. Valheim, H. Brun-Hansen, G. Berntsen, S. K. Whist, B. Djonne, C. M. Press, G. Holstad, and H. J. Larsen. 2001. Subclinical paratuberculosis in goats following experimental infection. An immunological and microbiological study. *Vet. Immunol. Immunopathol.* **80**:271–287.
56. Triccas, J. A., N. Winter, P. W. Roche, A. Gilpin, K. E. Kendrick, and W. J. Britton. 1998. Molecular and immunological analyses of the *Mycobacterium avium* homolog of the immunodominant *Mycobacterium leprae* 35-kilodalton protein. *Infect. Immun.* **66**:2684–2690.
57. Waters, W. R., J. M. Miller, M. V. Palmer, J. R. Stabel, D. E. Jones, K. A. Koistinen, E. M. Steadham, M. J. Hamilton, W. C. Davis, and J. P. Bannantine. 2003. Early induction of humoral and cellular immune responses during experimental *Mycobacterium avium* subsp. *paratuberculosis* infection of calves. *Infect. Immun.* **71**:5130–5138.
58. Waters, W. R., M. V. Palmer, J. P. Bannantine, D. L. Whipple, R. Greenwald, J. Esfandiari, P. Andersen, J. McNair, J. M. Pollock, and K. P. Lyashchenko. 2004. Antigen recognition by serum antibodies in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*. *Clin. Diagn. Lab. Immunol.* **11**:849–855.
59. Whittington, R. J., I. B. Marsh, and R. H. Whitlock. 2001. Typing of IS 1311 polymorphisms confirms that bison (*Bison bison*) with paratuberculosis in Montana are infected with a strain of *Mycobacterium avium* subsp. *paratuberculosis* distinct from that occurring in cattle and other domesticated livestock. *Mol. Cell. Probes* **15**:139–145.
60. Willemsen, P. T., J. Westerveen, A. Dinkla, D. Bakker, F. G. van Zijderveld, and J. E. Thole. 2006. Secreted antigens of *Mycobacterium avium* subspecies *paratuberculosis* as prominent immune targets. *Vet. Microbiol.* **114**:337–344.