Novel Secreted Antigens of \textit{Mycobacterium paratuberculosis} as Serodiagnostic Biomarkers for Johne’s Disease in Cattle

Antonio Facciolo,a David F. Kelton,b Lucy M. Muthariaa

Department of Molecular and Cellular Biologya and Department of Population Medicine, Ontario Veterinary College,b University of Guelph, Guelph, Ontario, Canada

Johne’s disease is a chronic gastroenteritis of cattle caused by \textit{Mycobacterium avium} subsp. \textit{paratuberculosis} that afflicts 40% of dairy herds worldwide. \textit{M. avium} subsp. \textit{paratuberculosis}-infected cattle can remain asymptomatic for years while transmitting the pathogen via fecal contamination and milk. Current serodiagnosis with enzyme-linked immunosorbent assays (ELISAs) fails to detect asymptomatic \textit{M. avium} subsp. \textit{paratuberculosis}-infected cattle due to the use of poorly defined antigens and knowledge gaps in our understanding of \textit{M. avium} subsp. \textit{paratuberculosis} components eliciting pathogen-specific immune responses. We set out to (i) define a subset of proteins that contain putative antigenic targets and (ii) screen these antigen pools for immunogens relevant in detecting infection. To accomplish our first objective, we captured and resolved \textit{M. avium} subsp. \textit{paratuberculosis}-secreted proteins using a 2-step fractionation method and reverse-phase liquid chromatography to identify 162 unique proteins, of which 66 had not been previously observed in \textit{M. avium} subsp. \textit{paratuberculosis} culture filtrates. Subsequent screening of \textit{M. avium} subsp. \textit{paratuberculosis}-secreted proteins showed four antigens, of which one or more reacted on immunoblotting with individual serum samples from 35 \textit{M. avium} subsp. \textit{paratuberculosis}-infected cows. Moreover, these novel antigens reacted with sera from 6 low \textit{M. avium} subsp. \textit{paratuberculosis} shedders and 3 fecal-culture-positive cows labeled as ELISA seronegative. The specificity of these antigens was demonstrated using negative-control sera from uninfected calves ($n = 5$) and uninfected cows ($n = 5$), which did not react to any of these antigens in immunoblotting. As three of the four antigens are novel, their characterization and incorporation into an ELISA-based format will aid in detecting asymptomatic cattle in early or subclinical stages of disease.

Enzyme-linked immunosorbent assays (ELISAs) are simple, rapid, and cost-effective tests that have been used for decades for determination of infection status. One of the major challenges in the development of an effective ELISA is the selection of antigens that are pathogen specific and permit sensitive detection. Antibodies against shared epitopes in closely related species can contribute to cross-reactivity (resulting in false-positive identification), and fluctuations in antibody titers and antibody compositions in chronic diseases hinder the development of sensitive tests. These factors have been problematic for the development of ELISAs for all mycobacterial diseases, including human tuberculosis (\textit{Mycobacterium tuberculosis}), leprosy (\textit{Mycobacterium leprae}), bovine tuberculosis (\textit{Mycobacterium bovis}), and Johne’s disease (\textit{Mycobacterium avium} subsp. \textit{paratuberculosis}).

Johne’s disease is a chronic gastroenteritis affecting ruminant animals worldwide, with national prevalence rates of 32% in Canada and 68% in the United States (1, 2). Calves are most susceptible and, once colonized, they remain asymptomatic for 2 to 5 years while shedding \textit{M. avium} subsp. \textit{paratuberculosis} in feces, colostrum, and milk (3). As there is no effective or approved treatment for Johne’s disease, control of \textit{M. avium} subsp. \textit{paratuberculosis} at the herd level requires identification of infected animals, specifically \textit{M. avium} subsp. \textit{paratuberculosis} shedders, and their removal from the herd (4). In addition, certain calf-rearing, cleaning, and animal husbandry practices have shown promise for reducing \textit{M. avium} subsp. \textit{paratuberculosis} prevalence (5). To accurately detect \textit{M. avium} subsp. \textit{paratuberculosis}-infected animals, a number of methodologies have been employed, including serodiagnosis by ELISA, detection by PCR amplification of \textit{M. avium} subsp. \textit{paratuberculosis}-specific sequences in feces and milk, measurement of gamma interferon secretion by T cells, and bacterial culture of fecal and milk samples. However, variables such as antigen specificity and sensitivity, reproducibility, time to colony detection, PCR inhibitors in feces and milk, and cross-reactivity are all factors that currently limit the efficacy of these methods (6). Serodiagnosis by ELISA has been recommended as the primary methodology, as this platform is relatively less laborious, more rapid, and more cost effective and the results are simple to interpret (7).

Commercially available ELISAs for Johne’s disease have high specificity (90 to 99%), but all suffer from low sensitivity (13.5 to 42%) (8). The antigen compositions of these ELISAs are mostly proprietary in nature but have been shown to contain a mixture of cellular proteins and/or purified protein derivative (9). The sensitivity of these assays was reported to be lowest (5 to 7%) in cattle shedding low levels of \textit{M. avium} subsp. \textit{paratuberculosis} and reached sensitivity values of 70 to 80% only when high levels of \textit{M. avium} subsp. \textit{paratuberculosis} were detected in feces (10). Moreover, preabsorption of serum with \textit{Mycobacterium phlei} crude protein lysates has improved the specificity of commercial ELISAs by removing cross-reactive antibodies (11).

The sensitivity of serodiagnostics improved with the use of \textit{M. avium} subsp. \textit{paratuberculosis} culture filtrate (CF) proteins and...
similarly for other mycobacterial pathogens, including *M. bovis* and *M. tuberculosis* (6, 12, 13). Compared with cellular proteins, *M. avium* subsp. *paratuberculosis* CF proteins showed greater reactivity with serum from *M. avium* subsp. *paratuberculosis*-infected cattle, with respect to both the number of antigens detected and the intensity of reactions on immunoblots (14). The use of *M. avium* subsp. *paratuberculosis* CF antigens in ELISAs increased assay sensitivity by 25% over commercial ELISAs for low-*M. avium* subsp. *paratuberculosis*-shedding cattle (15). However, antigen selection remains a challenge, as there is no single *M. avium* subsp. *paratuberculosis*-specific antigen that is recognized by all infected cattle, especially those in early and subclinical stages of disease. Recently, early serodiagnosis was addressed using experimentally infected calves and screening for antibody responses to a panel of 96 recombinant *M. avium* subsp. *paratuberculosis* antigens (16). Antibody responses were detected as early as 70 days postinfection; however, fluctuations in antibody responses and epitope specificity were observed over 321 days (16). These data suggest the need for a standardized cocktail of antigens for incorporation into a single ELISA for detection at all stages of disease in infected cattle.

The aim of this study was to identify *M. avium* subsp. *paratuberculosis*-specific immunodominant antigens within the *M. avium* subsp. *paratuberculosis* CF proteome. Our results revealed 66 proteins not previously reported as being secreted in *M. avium* subsp. *paratuberculosis* CF. We fractionated *M. avium* subsp. *paratuberculosis* CF using reverse-phase liquid chromatography (RPLC) and identified four antigens that reacted with 35 serum samples from *M. avium* subsp. *paratuberculosis*-infected cows, ranging from low to high shedders. No evidence of cross-reactivity was seen with negative-control sera obtained from fecal-culture- and ELISA-negative cows from a Johne’s disease-free herd or 2-month-old calves. These four antigens are promising candidates for the development of an *M. avium* subsp. *paratuberculosis* ELISA with improved sensitivity.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *Mycobacterium avium* subsp. *hominisuis* strain 104 was obtained from Luiz Bermudez (Oregon State University). *M. avium* subsp. *paratuberculosis* strains Madonna, gc86, and gD30 were isolated in our laboratory (in December 2001) from the feces of different cows from different dairy herds in southern Ontario. All three *M. avium* subsp. *paratuberculosis* strains were mycobactin J dependent and PCR (IS900, hspX, ISMAP02, hsp65) positive and were identified as cow type using IS1311. *M. avium* subsp. *hominisuis* and *M. avium* subsp. *paratuberculosis* were cultured as static cultures at 37°C for 4 or 8 weeks, respectively, in Watson-Reid medium (pH 6.0) supplemented with 2 mg/liter mycobactin J, 4.1 g/liter sodium pyruvate, and 0.075 g/liter ferric ammonium citrate (17).

*M. avium* subsp. *paratuberculosis* cultures were initiated by inoculating a 1-ml frozen seedlot containing 10⁶ CFU/ml into 50 ml of Middlebrook 7H9 medium (Difco) supplemented with 5 g/liter glycerol, 1 g/liter Casitone, OADC (oleic acid-albumin-dextrose-catalase) enrichment, and 2 mg/liter mycobactin J. At 4 weeks, cells were harvested by centrifugation, washed with 10 mM phosphate-buffered saline (PBS) (pH 7.2), suspended in 60 ml of Watson-Reid medium, and cultured as mentioned earlier.

**Preparation of culture filtrate proteins and cell lysates.** For harvesting of bacterial cells, cultures were supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM EDTA (pH 8.0) and chilled on ice for 15 min. Cells were separated from the CF by centrifugation (3,000 × g for 25 min), and the supernatant was passed through a 0.22-μm polyethersulfone (PES) filter. CF proteins were size fractionated by sequential ultrafiltration using Amicon Ultra-15 centrifugal filter units with molecular mass cutoffs of 50 and 3 kDa (Millipore). The filtered volumes retained on the 50- and 3-kDa membranes, labeled supernatant filtrate 1 (SF1) and SF2, respectively, were dialyzed against 10 mM PBS (pH 7.2).

To obtain cellular proteins, the harvested cells were suspended in lysis solution (10 mM PBS [pH 7.2], 1% [vol/vol] Tween 20) and placed in screw-cap microcentrifuge tubes containing 0.1-mm zirconia/silica. Tubes were shaken in a Mini-Beadbeater cell disrupter for eight 20-s pulses, with 3-min rests on ice. Cellular debris and beads were pelleted by centrifugation at 10,000 × g for 10 min, and the whole-cell lysate was stored at −20°C. Protein concentrations were quantified using a bicinchoninic acid kit (Sigma-Aldrich).

**One- and two-dimensional SDS-PAGE analysis.** For one-dimensional (1D) SDS-PAGE, protein samples were diluted in Laemmli sample buffer, incubated at 95°C for 7 min, and separated at 90 V in a 12% (wt/vol) polyacrylamide gel. For two-dimensional (2D) PAGE, 70 μg each of SF1 and SF2 protein preparations were precipitated according to the ReadyPrep 2-D cleanup kit instructions (Bio-Rad), and the pellet was dissolved in 125 μl of ReadyPrep sequential extraction reagent 3 (Bio-Rad) containing 50 mM dithiothreitol (DTT) and 0.05% (wt/vol) bromophenol blue. The sample was used to passively rehydrate a 7-cm immobilized pH gradient (IPG) strip (pH 4 to 7, ReadyStrip; Bio-Rad) for 1 h before overlaying with mineral oil and active rehydration for 12 h at 50 V. Isoelectric focusing was conducted in a Bio-Rad Protein IEF cell at 250 V for 1 h, 500 V for 2 h, and 4,000 V to 25,000 V-h, with the current set at 50 μA per gel. The IPG strips were stored at −70°C until needed or were immediately equilibrated in SDS equilibration buffer (6 M urea, 375 mM Tris-HCl [pH 8.8], 2% [vol/wt] SDS, 20% [vol/wt] glycerol) containing 65 mM DTT in the first 15-min wash and 68 mM iodoacetamide in the second 15-min wash. The IPG strips were finally equilibrated in Laemmli running buffer, sealed onto 10% or 15% (wt/vol) polyacrylamide gel using agarose sealing solution (0.05% [wt/vol] agarose and 0.002% [wt/vol] bromophenol blue in Laemmli buffer), and electrophoresed at 90 V. Gels were silver stained as described previously (18), with the following changes: step 2, the gel was washed with water for 1.5 h; step 5, formaldehyde was omitted; step 8, the reaction was stopped and the mixture was stored in 1% (vol/vol) acetic acid.

**In-gel trypsin digestion.** Protein gel slices from 1D or 2D SDS-PAGE were excised using a sterile scalpel blade and were destained with 15 mM potassium ferricyanide and 50 mM sodium thiosulfate in water. Cystine residues were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide. Gel slices were dried by vacuum centrifugation before excising and combining with 50 μl of 25 mM ammonium bicarbonate buffer and 20 μl of 100 mM Tris-HCl (pH 8.8). The gel was incubated with 10 μg of trypsin at 37°C overnight in 0.1 μg of sequencing-grade modified porcine trypsin (Promega) in 50 mM ammonium bicarbonate buffer. Tryptic peptides were extracted from gel slices by one aqueous wash (50 μl and bath sonication for 10 min) followed by two washes with 5% formic acid in 50% acetonitrile (ACN) (75 μl and bath sonication for 5 min). Samples were reduced to dryness by vacuum centrifugation.

**LC-MS/MS.** Protein samples were subjected to liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis using the service provided by the Advanced Protein Technology Center (The Hospital for Sick Children, Toronto, Canada). The digested peptides were loaded onto a 150-μm inner diameter (i.d.) precolumn (Magic C₁₁₆; Michrom Biosciences) at 4 μl/min and separated over a 75-μm i.d. analytical column packed into an emitter tip containing the same packing material. The peptides were eluted over 60 min at 300 nl/min with a 0% to 40% ACN gradient in 0.1% trifluoroacetic acid (TFA), using an EASY nL nanochromatography pump operated at room temperature (Proxeon Biosystems, Odense, Denmark). The peptides were eluted into an LTQ-Orbitrap hybrid mass spectrometer (Thermo-Fisher, Bremen, Germany) operated in a data-dependent mode. The mass spectra were acquired at a resolution of 60,000 full width at half-maximum in the Fourier transform mass spectrometer, and MS/MS was carried out in the linear ion trap. Six MS/MS scans were obtained per mass spectrometry (MS) cycle.
Tandem MS/MS spectra were extracted, charge state deconvoluted, and deisotoped using BioWorks version 3.3 software. All MS/MS samples were analyzed using Mascot (version 2.3.02; Matrix Science, London, United Kingdom) and X! Tandem (version 2007.01.01.1) (www.thegpm.org). X! Tandem was set up to search a subset of the NCBI Inr_20110515 database, and Mascot was set up to search the NCBI Inr_20110515 database (selected for paratuberculosis, unknown version; 4,553 entries), assuming the digestion enzyme trypsin. Mascot and X! Tandem were searched with Mascot identity scores of >45. Peptides that contained similar peptide and proteins could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins were subjected to bioinformatic analyses for putative secretory signal peptides using SignalP (version 4.1), PRED-TAT, and SecretomeP (version 2.0) programs.

Reverse-phase LC analysis. RPLC was performed on a Beckman Coulter ProteomeLab PF2D system using a C18-reverse-phase column (3.6 µm, 150 mm by 4.6 mm; Aeris Phenomenex), with a SecurityGuard Ultra cartridge (Phenomenex), operated at 23°C. The mobile phase consisted of gradient elution with solvent B (0.1% [vol/vol] TFA in ACN) and solvent A (0.1% [vol/vol] TFA in water). The gradient profile was as follows, with a flow rate of 1.0 ml min⁻¹: 2 to 4 min, 0% solvent B to 20% solvent B; 24 to 34 min, 20% solvent B to 80% solvent B; 34 to 36 min, 80% solvent B to 100% solvent B; 36 to 37 min, 100% solvent B to 0% solvent B. The injection volume was 200 µl, and the detection wavelength was 214 nm. Eluate was collected using an FC204 fraction collector (Gilson), and samples were concentrated by vacuum centrifugation and reconstituted in 10 mM PBS (pH 7.2).

Animal sera. Thirty-five serum samples from M. avium subsp. paratuberculosis-infected cows were used in this study. Fourteen serum samples collected from culled M. avium subsp. paratuberculosis ELISA- and fecal-culture-positive animals were obtained from the USDA National Veterinary Services Laboratory. These 14 sera were pooled, generating JD-14 serum, and were used in immunoblots. Twenty-five serum samples were matched to fecal culture and serum ELISA (IDEXX Laboratories, Inc.) results. The negative-control sera included uninfected calf and ELISA-negative cow sera (a kind gift from Niel Karrow, University of Guelph). The calf sera were collected from 2-month-old Holstein calves or paratuberculosis inactivated cells were prepared by harvesting 1 ml of an M. avium subsp. paratuberculosis or M. avium subsp. hominis suis cell suspension that had been removed within 24 h after birth and maintained in an animal isolation facility. Sera from uninfected cattle were collected from a herd with no reported cases of Johne’s disease and were confirmed seronegative by ELISA (IDEXX Laboratories, Inc.).

A 1:1 emulsion of 100 µg of M. avium subsp. paratuberculosis SF1 and SF2 proteins or M. avium subsp. hominis suis SF1 and SF2 proteins in 10 mM PBS (pH 7.2) and TiterMax gold adjuvant (Sigma-Aldrich) was injected intramuscularly into eight Sprague-Dawley rats. Four subsequent immunizations consisting of 50 µg of protein and heat-inactivated M. avium subsp. paratuberculosis or M. avium subsp. hominis suis cells emulsified in Freund’s incomplete adjuvant were administered biweekly. Heat-inactivated cells were prepared by harvesting 1 ml of an M. avium subsp. paratuberculosis or M. avium subsp. hominis suis log-phase culture by centrifugation and heat-killing the cells at 80°C for 30 min. Four days following the last immunization, rats were euthanized and whole blood was collected for serum preparation. The immunization protocols and the use and care of all animals in this study were approved by the University of Guelph Animal Care and Use Committee.

Immunoblotting. Proteins electrophoresed by either 1D or 2D SDS-PAGE, as described above, were transferred to nitrocellulose membranes (BioTrace NC; Pall) using a semidyblotting device (Trans-Blot Turbo transfer system; Bio-Rad) for 30 min at 10 V and 100 mA. When stated, membranes were subjected to periodate oxidation as described previously (19). Membranes were blocked for 2 h at room temperature in PBS containing 0.05% (vol/vol) Tween 20 (PBS-T) and 5% (wt/vol) milk powder (Oxoid) and were incubated overnight at 15°C with primary antibody diluted in PBS-T-1% milk powder (1:1000 for rat antisera or 1:100 for bovine serum). After the primary and secondary antibody incubations, membranes were rinsed twice and washed 3 times for 10 min in PBS-T. Membranes were incubated for 1.5 h at room temperature with secondary antibody (either 1:3,500 rabbit anti-bovine IgG, horseradish peroxidase [HRP] conjugated, or 1:7,500 rabbit anti-rat IgG, HRP conjugated; Sigma-Aldrich) diluted in PBS-T-1% milk powder. Visualization of protein bands that were reactive with various antisera was performed as described previously (20).

RESULTS

M. avium subsp. paratuberculosis CF secretome analysis. LC-MS/MS analysis was used to characterize the protein profiles of the SF1 and SF2 preparations of M. avium subsp. paratuberculosis CF. A total of 162 unique proteins were identified in the three M. avium subsp. paratuberculosis strains, with 81 in SF1, 89 in SF2, and 8 in both fractions (see Table S1 in the supplemental material). Among these, 66 proteins (27 in SF1 and 39 in SF2) were deemed novel. Interestingly, the distributions of hypothetical and annotated proteins varied greatly, with 19.7% and 60% hypothetical proteins in SF1 and SF2, respectively. Live-dead staining with fluorescein diacetate and ethidium bromide revealed no dead cells after 8 weeks of incubation (data not shown). Using bioinformatic tools, 28% of the 162 CF proteins and 34% of the novel proteins were predicted to contain a canonical secretory peptide for protein export via the general secretion pathway or the twin-arginine translocation system (see Fig. S1A and B in the supplemental material). The 2D PAGE analysis using various IPGs revealed the majority of proteins as having isoelectric points between 4 and 7 (data not shown). This result is consistent with previous findings showing that mycobacterial secreted proteins are more acidic than cellular proteins (14, 21).

Immunoblotting screening of SF1 and SF2 preparations. Based on the protein concentrations and 1D SDS-PAGE analysis, M. avium subsp. paratuberculosis strains secrete a greater abundance and heterogeneity of proteins than does M. avium subsp. hominis suis strain 104 (Fig. 1A). This observation is consistent with yields obtained for M. avium subsp. paratuberculosis and M. avium subsp. hominis suis for purified protein derivatives (22). Two complementary approaches were used to screen for immunogenic proteins in the SF1 and SF2 preparations. The first approach used polyclonal rat antisera and the second the JD-14 serum. Polyclonal rat anti-M. avium subsp. paratuberculosis antibodies exhibited cross-reactivity against antigens in the whole-cell lysates and SF1 and SF2 preparations of both M. avium subsp. paratuberculosis and M. avium subsp. hominis suis (Fig. 1B). Interestingly, the sera showed relatively higher reactivity with homologous low-molecular-mass (<26-kDa) antigens in the M. avium subsp. paratuberculosis SF1 and SF2 preparations. In comparison, the polyclonal rat anti-M. avium subsp. hominis suis antibodies recognized fewer antigens in both M. avium subsp. hominis suis and M. avium subsp. paratuberculosis whole-cell lysates and SF1 preparations and only a single antigen in the M. avium subsp. paratuberculosis
Facciuolo et al.

A Silver-stained gel

B Rat anti-MAP

C Rat anti-MAH

D JD-14 serum

FIG 1 Comparative 1D SDS-PAGE and immunoblots of M. avium subsp. paratuberculosis (MAP) and M. avium subsp. hominis suis (MAH) whole-cell lysates (L) and SF1 and SF2 preparations. (A) Silver-stained 12% (wt/vol) polyacrylamide gel. (B to D) Immunoblots showing reactions with polyclonal rat anti-M. avium subsp. paratuberculosis sera (B), polyclonal rat anti-M. avium subsp. hominis suis sera (C), and JD-14 serum (D). Each sample lane contained 20 μg of protein. The blots were subjected to periodate oxidation immediately after electrotransfer and before incubation with blocker and serum. The brackets in panels B and D denote the <26-kDa proteins detected in M. avium subsp. paratuberculosis but not M. avium subsp. hominis suis. CF. Molecular mass standards are listed.

SF2 preparation (Fig. 1C). To identify antigens relevant to M. avium subsp. paratuberculosis infection, JD-14 serum was used to screen the same set of antigen pools. Whole-cell lysates and the SF1 preparations of both M. avium subsp. paratuberculosis and M. avium subsp. hominis suis showed similar antigenic profiles (Fig. 1D). In contrast, the M. avium subsp. paratuberculosis SF2 preparation contained a number of immunoreactive proteins that were absent in M. avium subsp. hominis suis SF2. The M. avium subsp. paratuberculosis SF2 preparation was chosen for subsequent analysis and epitope screening.

A comparison of M. avium subsp. hominis suis SF2 and M. avium subsp. paratuberculosis SF2 proteins prepared by 2D PAGE showed subspecies-distinct secreted protein profiles (Fig. 2A and B). In agreement with the 1D immunoblotting results, 2D immunoblotting with polyclonal rat anti-M. avium subsp. paratuberculosis sera (Fig. 2C and D) and the JD-14 serum (Fig. 2E and F) reacted strongly with M. avium subsp. paratuberculosis SF2 antigens (Fig. 2D and F), in comparison with the M. avium subsp. hominis suis antigens (Fig. 2C and E). The JD-14 serum had significantly lower staining intensity. Both sera detected a few cross-reactive antigens present in M. avium subsp. hominis suis SF2 (Fig. 2C and E). Nearly identical reactivity profiles were observed for polyclonal rat anti-M. avium subsp. paratuberculosis and JD-14 serum toward M. avium subsp. paratuberculosis SF1; however, the number of antigens and the intensity of reaction were significantly less than for M. avium subsp. paratuberculosis SF2 (see Fig. S2 in the supplemental material). Taken together, these data suggest that antibodies in sera from naturally M. avium subsp. paratuberculosis-infected cattle and immunized rats are detecting M. avium subsp. paratuberculosis-specific antigens in the M. avium subsp. paratuberculosis SF2 preparation.

In-gel digestion of 16 reactive spots from 2D PAGE gave rise to multiple identifications per spot in MS/MS analysis. To better identify the immunoreactive proteins, the M. avium subsp. paratuberculosis SF2 preparation was subjected to RPLC, 1-ml eluate fractions were collected, and proteins were concentrated and resolved by 1D SDS-PAGE (Fig. 3A). At least 10 antigen bands were identified by immunoblotting with JD-14 serum, 1 each in fractions 12, 13, and 14 and 7 in fraction 15 (F15) (Fig. 3B). Immunoblotting was performed with each of the 14 serum samples constituting the JD-14 serum (Fig. 3C). All 14 serum samples reacted with one or more of the F15 proteins, of which three antigens with apparent molecular sizes of 52 kDa, 47 kDa, and 28 kDa were the most frequently recognized (Fig. 3C).

To test the potential of F15 proteins as diagnostic reagents, we separated the cow sera (n = 25) into 4 groups based on the matched IDEXX ELISA reading and M. avium subsp. paratuberculosis CFU per gram of feces (see Table S2 in the supplemental material). Sera from ELISA-positive/high-fecal-level, ELISA-positive/low-fecal-level, ELISA-negative/high-fecal-level, and ELISA-negative/low-fecal-level animals were pooled. The pooled sera, along with positive- and negative-control sera, were reacted with F15 antigens (Fig. 4A). Unlike commercial ELISAs, the cow sera in this study were not preabsorbed with environmental Mycobacterium or Escherichia coli whole cells or lysates to remove antibodies to shared antigenic epitopes. The order of seroreactivity to F15 proteins determined by immunoblotting, ranked from highest to lowest, was ELISA positive/high fecal level, ELISA negative/high fecal level, and ELISA negative/low fecal level (Fig. 4A, lanes a, c, b, and d, respectively). The individual serum samples in each pool were reacted with F15 proteins. Antibodies against at least 1 of the 3 most frequently recognized antigens were detected (Fig. 4C). Based on these data, reactivity against F15 proteins correlates better with fecal shedding than the test ELISA values.

To address the specificity of these antigens, the unabsorbed pooled sera from 2-month-old calves (n = 5) and M. avium subsp. paratuberculosis fecal-culture- and ELISA-negative cows (n = 5) from a Johne’s disease-free herd were reacted against the F15 proteins. No antibodies against these proteins were detected in either of these serum samples (Fig. 4A, lanes e and f). In contrast, the positive-control JD-14 serum reacted with at least 6 proteins (Fig. 4A, lane g). Polyclonal rat anti-M. avium subsp. paratuberculosis antibodies reacted with only two F15 proteins; polyclonal rat anti-M. avium subsp. hominis suis antibodies weakly reacted with only one (Fig. 4A, lanes h and i). With the exception of sera from the neonatal calves, all cow sera reacted with multiple antigens in M. avium subsp. paratuberculosis whole-cell lysates (Fig. 4B). These data demonstrate the lack of specificity of M. avium subsp. paratuberculosis whole-cell lysates and the specificity of the F15 antigens.

In-gel digestion of the three most frequently recognized antigens from 1D and 2D gels revealed the 52-kDa antigen band as...
MAP0196c. The 42-kDa antigen band consisted of 2 comigratory proteins, i.e., MAP0196c and ModD/Apa (MAP1569). The 28-kDa antigen band consisted of two comigratory proteins, i.e., MAP0471 and MAP1981c. BLASTP analyses of these four antigens identified similar proteins in *M. avium*, *M. tuberculosis*, *M. bovis*, and *M. leprae* (Table 1). FASTA sequence comparison of the *M. avium* subsp. *paratuberculosis* and *M. bovis* proteins revealed amino acid identities ranging from 74 to 94%. However, comparison of these proteins from *M. avium* subsp. *paratuberculosis* and *M. bovis* revealed that very few shared antigenic epitopes were predicted by Bepipred B-cell epitope prediction (Table 1). MAP0196c, MAP0471, and MAP1981c have been annotated as hypothetical proteins in the *M. avium* subsp. *paratuberculosis* genome. MAP1569/ModD is a fibronectin attachment protein and has previously been reported as a B-cell antigen (23).

**DISCUSSION**

Using a novel approach of 2-step size fractionation to capture and concentrate *M. avium* subsp. *paratuberculosis* CF proteins, 162 unique proteins were identified; 66 of these have not been previously reported in *M. avium* subsp. *paratuberculosis* CF. The use of this methodology was compared to trichloroacetic acid precipitation and single size fractionation (data not shown). 1D SDS-PAGE analyses revealed distinct protein profiles for each method, with the 2-step size fractionation method yielding higher concentrations and a heterogeneous protein profile. We believe this two-step methodology contributed to the identification of these previously unreported proteins.

Of the 162 unique proteins identified in this study, only 28% were predicted to contain a canonical secretory peptide. This low value is consistent with extracytoplasmic proteomic analyses of *M. avium* subsp. *paratuberculosis* and other mycobacterial species. Previous analysis of *M. avium* subsp. *paratuberculosis* CF using a polyacrylamide gel-based proteomic approach identified 125 unique proteins, of which 28% contained a putative secretory peptide (24). Analysis of the *M. avium* subsp. *paratuberculosis* cell wall proteome by He and De Buck identified 309 unique proteins where 6% and 50% of cell-wall-localized and surface-exposed proteins, respectively, were predicted to contain a secretory signal peptide (25). In other studies, bioinformatic analysis of the *M. avium* subsp. *paratuberculosis* proteome identified 373 unique proteins, of which 14% and 28% contained a secretory signal peptide and a transmembrane signal peptide, respectively (26). In contrast, proteomic analysis of *M. avium* subsp. *hominissuis* CF using a polyacrylamide gel-based approach identified 354 unique proteins, of which 22% contained a secretory signal peptide (27). This low percentage of secretory proteins may reflect the low overall yield of secreted proteins in this bacterial species. In summary, this study demonstrates the potential of a two-step size fractionation approach for identifying novel secreted antigens in *M. avium* subsp. *paratuberculosis* and highlights the need for further characterization of these proteins as potential biomarkers for Johne’s disease.

**FIG 2** Comparative 2D PAGE and immunoblots of *M. avium* subsp. *hominissuis* (MAH) SF2 (A, C, and E) and *M. avium* subsp. *paratuberculosis* (MAP) SF2 (B, D, and F) proteins. (A and B) Silver-stained 2D PAGE of *M. avium* subsp. *hominissuis* SF2 (A) and *M. avium* subsp. *paratuberculosis* SF2 (B) focused over pH 4 to 7 and resolved on 15% (wt/vol) polyacrylamide gels. (C to F) Corresponding immunoblots of *M. avium* subsp. *hominissuis* SF2 (C and E) and *M. avium* subsp. *paratuberculosis* SF2 (D and F) proteins reacted with polyclonal rat anti-*M. avium* subsp. *paratuberculosis* sera (C and D) or JD-14 serum (E and F). Boxes in panels E and F denote immunoreactive protein spots common to *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis*, respectively. Molecular mass standards are listed.
tuberculosis genome predicted that only 52 of the 3,924 open reading frames (ORFs) would contain a secretory signal peptide, in contrast to the >250 proteins identified in CF studies (26, 27).

Comparative analyses of whole-cell lysates and CF proteins using 2D PAGE found significant overlap (45%) between these subproteomes, and only 31% of the 137 CF proteins were predicted to contain a secretory signal peptide (21). One group reported purifying M. tuberculosis CF proteins, with 62% predicted to contain a secretory signal peptide, but attributed strain differences as the source of disparity between their observations and those reported previously (27).

There are two possible explanations for why such a small percentage of mycobacterial proteins are assigned a predictable secretory peptide. The first results from the lack of data regarding signaling components necessary to orchestrate the translocation of proteins across the cell wall, and the second can be attributed to the phenomenon of moonlighting proteins. To date, three secretion systems have been identified in mycobacteria, i.e., SecA1/SecA2, Tat, and the type VII secretion system (28). Canonical signals are well characterized for SecA1 and Tat, but conserved motifs for the type VII secretion system have been identified only recently (29). With limited understanding of secretion signals in mycobacteria being further confounded by the complexity of mycobacterial cells walls, it may be speculated that unidentified signal peptides in combination with additional translocation mechanisms may explain the export of these unaccounted-for secreted proteins not falling within the Sec/Tat/type VII pathways. The second confounding factor in defining a secreted protein arises from dual localization. A significant amount of data has accumulated to support dual localization of prokaryotic (and eukaryotic) cytoplasmic proteins termed “moonlighting proteins” for their ability to coexist in two subcellular compartments, with diverse roles in each location (30). This phenomenon has been identified in Mycobacteria, Listeria, Neisseria, Staphylococcus, Streptococcus, Helicobacter, and Franciscella (31). Some of the functions assigned to these moonlighting proteins, to date, include their capacity to mediate host cell adhesion, modulate macrophage cytokine secretion, and control gene expression through secretion of transcriptional regulators (32–34). Taken together, extracellular protein localization is still limited to empirical methods of detection, as we currently lack an understanding of noncanonical translocation systems, especially in mycobacteria. Moreover, the localization of a protein based on its prior localization or annotation cannot always be determined. Our results, and those of other groups, suggest that secretion of these unusual proteins is a true phenomenon and illustrates the extraordinary plasticity of proteins.

The 162-protein secretome defined in this study was compared with three M. avium subsp. paratuberculosis cellular proteomes containing both cytoplasmic and membrane-enriched protein fractions (35–37). A total of 130 proteins overlapped with the cytoplasmic protein fractions from M. avium subsp. paratuberculosis cells cultured in vitro (35, 37); 54 proteins overlapped with the membrane-enriched protein fractions (35, 36). The proteins overlapping in these studies include both previously described moonlighting proteins and proteins observed in the CF of mycobacteria (i.e., DnaK, Antigen-85, eIF-Tu, malate synthase G, and isocitrate lyase) (31, 38). Interestingly, 117 proteins from our in vitro secretome were also identified in M. avium subsp. paratuberculosis cytoplasmic (total of 938 proteins) and membrane-enriched (total of 325 proteins) protein fractions obtained from mucosal scrapings of bovine ileum tissue (36, 37). Similarly, a comparative analysis was conducted against the M. avium subsp. paratuberculosis...
K10 cell wall proteome. A total of 309 proteins were identified, with only 23 proteins found to overlap (25). These data suggest that methodologies employed in the cytoplasmic proteome, cell wall proteome, and secretome defined in this study are indeed isolating and enriching unique M. avium subsp. paratuberculosis subproteomes, as evidenced by the small overlap in protein identities in comparison to the total number of proteins identified in each proteomic data set.

One of the current challenges in Johne’s disease management is identifying M. avium subsp. paratuberculosis-specific components that can be exploited for diagnostic value. This challenge arises from T- and B-cell epitope cross-reactivity from environmental exposure to closely related mycobacteria, such as those belonging to the Mycobacterium avium complex. M. avium subsp. hominisuis shares 95% nucleotide sequence identity with M. avium subsp. paratuberculosis across 3,241 of 3,497 ORFs analyzed (39). For diagnostic purposes, comparison with M. avium subsp. hominisuis is of paramount importance in determining and eliminating cross-reactive epitopes. Using the rat model, we found that M. avium subsp. hominisuis CF-immunized serum cross-reacted with only a few antigens in the M. avium subsp. paratuberculosis SF2 preparation. In contrast, M. avium subsp. paratuberculosis CF-immunized serum reacted strongly and extensively with the same antigens. These data suggest that exposure to M. avium subsp. hominisuis, or other closely related mycobacteria, may not be problematic in generating cross-reactive antibodies against M. avium subsp. paratuberculosis.

### TABLE 1

| M. avium subsp. paratuberculosis gene | Pfam domain | Protein size (kDa) | Similar epitope (% similarity) | No. of common epitopes for M. avium subsp. paratuberculosis vs M. bovis/no. predicted*
|-------------------------------------|-------------|--------------------|------------------------------|-------------------------------------|
| 1569                                | FAP         | 36                 | Mav2859 (95) Mb1891 (74)     | M. avium 104 M. bovis AF2122/97 M. tuberculosis H37Rv M. leprae TN ML2055 (74) ML1918 (90) ML1638 (80) ML0081 (83) 2/12 2/7 1/7 1/15 |
| 0471                                | Shisa       | 28                 | Mav0566 (99) Mb3618c (94)    | 2/7 |
| 1981c                               | DUF164      | 27                 | Mav2210 (99) Mb2254c (90)    | 1/7 |
| 0196c                               | Septum_form| 46                 | Mav0192 (98) Mb3865 (81)     | 1/15 |

*a Standard protein BLAST was used to search for mycobacterial homologs, which were subsequently analyzed for percent similarity using FASTA sequence comparison (see http://fasta.bioch.virginia.edu). Bioinformatic prediction of common B-cell epitopes was performed for M. avium subsp. paratuberculosis homologs in M. bovis using Bepipred Linear Epitope Prediction (IEDB Analysis Resource) (see http://tools.immuneepitope.org). Data are presented as the total number of common epitopes over the total number of M. avium subsp. paratuberculosis epitopes predicted; only epitopes of ≥4 amino acids in length were considered.
these particular antigens. Moreover, antibodies against the 3 most frequently recognized F15 antigens were detected in serum from _M. avium_ subsp. _paratuberculosis_-infected cows, but only one of those antigens reacted with serum from _M. avium_ subsp. _paratuberculosis_-immunized rats. In light of this observation, our next initiative is to address whether antibodies against these antigens are generated in cows that have been immunized with _M. avium_ subsp. _paratuberculosis_ CF protein. Such analyses would allow us to distinguish antigens relevant to infection versus immunization.

As the SF2 preparation contained putative _M. avium_ subsp. _paratuberculosis_-specific antigens, this fraction was subjected to further immunogenic analyses. As _M. avium_ subsp. _paratuberculosis_ material often is obtained in small amounts, RPLC facilitated concentration with minimal loss and aided in high-throughput analysis by 1D SDS-PAGE by reducing sample complexity. This methodology yielded a different reactivity profile than 1D immunoblotting. The RPLC fractionation of _M. avium_ subsp. _paratuberculosis_ SF2 led to the identification of at least 10 seroreactive antigens not identified in the 1D immunoblot of the unfractionated SF2 preparation. These findings underline the importance of applying various methodologies beyond 1D and 2D immunoblotting in antigen-screening experiments.

A comparative study of serum ELISA and _M. avium_ subsp. _paratuberculosis_ fecal shedding results reported that the sensitivity of commercial ELISAs strongly correlated with the amounts of _M. avium_ subsp. _paratuberculosis_ shed in feces (10). The lowest sensitivity of 5% was evidenced in low _M. avium_ subsp. _paratuberculosis_ shedding (less than 10 CFU cultured) and increased to 88% in high _M. avium_ subsp. _paratuberculosis_ shedders (greater than 40 CFU cultured). The dependence of increased sensitivity on fecal shedding provides an inherent limitation in detecting the subset of early and subclinically infected animals that are most often categorized as low _M. avium_ subsp. _paratuberculosis_ shedders. Our study provides evidence suggesting that _M. avium_ subsp. _paratuberculosis_ F15 antigens are superior to commercial ELISA antigens as they are not limited in their ability to detect low- and high- _M. avium_ subsp. _paratuberculosis_ shedders. Serum from 3 high-shedding seronegative cows and 6 low-shedding (less than 7 CFU cultured) seropositive cows reacted with at least one of the three most frequently recognized antigen bands in _M. avium_ subsp. _paratuberculosis_ F15. In contrast to commercial ELISAs, our novel set of F15 antigens correctly identified all 9 samples as seropositive. Further validation of the sensitivity of these antigens with a larger sample population is required to confirm our observations. Lastly, preadsorption of serum (as required for commercial ELISAs) was dispensable in our studies, as reactivity to _M. avium_ subsp. _paratuberculosis_ F15 antigens was evident only in serum from _M. avium_ subsp. _paratuberculosis_-infected animals and not from negative-control animals. In contrast, all sera reacted extensively with _M. avium_ subsp. _paratuberculosis_ cell lysates, further supporting the notion that _M. avium_ subsp. _paratuberculosis_ F15 antigens could be used to differentiate infected from uninfected animals.

Bioinformatic analyses of the four fraction 15 antigens revealed proteins of high sequence similarity belonging to other mycobacterial species, including those found ubiquitously in the environment, such as _M. avium_ subsp. _hominisuis_ (Table 1). Although the negative-control sera were obtained from ELISA- and fecal-culture-negative animals, the sera reacted extensively with _M. avium_ subsp. _paratuberculosis_ cell lysates. These data suggest that these uninfected animals were exposed to shared epitopes belonging to environmental mycobacteria. However, these serum samples did not cross-react with _M. avium_ subsp. _paratuberculosis_ F15 proteins in immunoblotting, suggesting that epitope determinants belonging to these four antigens, although having highly homologous counterparts in other _Mycobacterium_, may elicit a unique immunological response in the context of _M. avium_ subsp. _paratuberculosis_ infections. The pathogen-mediated response to these proteins allows the use of these antigens to be exploited in discriminating infected from uninfected animals.

This study suggests that we have identified novel antigens that can be used to expand the diagnostic options for Johne’s disease. Based on our small test sample, four _M. avium_ subsp. _paratuberculosis_-specific proteins were identified that uniquely reacted with serum from _M. avium_ subsp. _paratuberculosis_-infected cows. In addition, sera from low _M. avium_ subsp. _paratuberculosis_ shedders (<10 CFU) also reacted with at least one of these antigens. The specificity of these proteins was demonstrated by calf and uninfected cow sera containing no detectable antibodies to these antigens and that being no need for serum preadsorption to remove antibodies to common antigens. We are currently validating these antigens, in addition to other targets, using a larger sample size from _M. avium_ subsp. _paratuberculosis_-infected animals and ELISA-negative cows from herds with positive animals. We believe that their use in an ELISA-based format may aid in the immediate management of Johne’s disease by detecting animals shedding low levels of _M. avium_ subsp. _paratuberculosis_, which current commercial serum ELISAs fail to do.

ACKNOWLEDGMENTS

We thank Janet I. MacInnes and Joseph S. Lam for their comments and suggestions in the preparation of the manuscript, Dyanne Brewer and Armen Charochgylan for their technical support in mass spectrometric analysis, and Niel Karrow for kindly providing bovine serum samples that assisted in our sample analysis. This work was supported by Alberta Beef Producers and NSERC-CRD grants to L.M.M. and D.F.K.

REFERENCES

1. Tiwari A, VanLeeuwen JA, Dohoo IR, Keefe GP, Weersink A. 2008. Estimate of the direct production losses in Canadian dairy herds with subclinical _Mycobacterium avium_ subspecies _paratuberculosis_ infection. Can. Vet. J. 49:569–576.
2. USDA. 2008. Johne’s disease on U.S. dairies. USDA, APHIS, Veterinary Services National Animal Health Monitoring System, Washington, DC. USDA publication 521.0408.
3. Harris NB, Barletta RG. 2001. _Mycobacterium avium_ subsp. _paratuberculosis_ in veterinary medicine. Clin. Microbiol. Rev. 14:489–512.
4. van Roermund HJW, Bakker D, Willemsen PTJ, de Jong MCM. 2007. Horizontal transmission of _Mycobacterium avium_ subsp. _paratuberculosis_ in cattle in an experimental setting: calves can transmit the infection to other calves. Vet. Microbiol. 122:270–279.
5. Collins M, Eggleston V, Manning E. 2010. Successful control of Johne’s disease in nine dairy herds: results of a six-year field trial. J. Dairy Sci. 93:1638–1643.
6. Wadhwa A, Hickling GJ, Eda S. 2012. Opportunities for improved serodiagnosis of human tuberculosis, bovine tuberculosis, and _paratuberculosis_. Vet. Med. Int. 2012:674238. doi:10.1155/2012/674238.
7. Collins MT, Gardner IA, Garry FB, Roussel AJ, Wells SJ. 2006. Consensus recommendations on diagnostic testing for the detection of _paratuberculosis_ in cattle in the United States. J. Am. Vet. Med. Assoc. 229:1912–1919.
8. Sweeney RW, Whitlock RH, McAdams S, Fyock T. 2006. Longitudinal study of ELISA seroreactivity to _Mycobacterium avium_ subsp. _paratuber-
culosis in infected cattle and culture-negative herd mates. J. Vet. Diagn. Invest. 18:2–6.
9. Singh S, Singh A, Singh P, Sohal J, Singh N. 2007. Evaluation of an indigenous ELISA for diagnosis of Johne’s disease and its comparison with commercial kits. Indian J. Microbiol. 47:251–258.
10. Clark D, Jr, Kozickiowski J, Radcliff R, Carlson R, Ellingson J. 2008. Detection of Mycobacterium avium subspecies paratuberculosis comparing fecal culture versus serum enzyme-linked immunosorbent assay and direct fecal polymerase chain reaction. J. Dairy Sci. 91:2620–2627.
11. Yokomizo Y, Yugi H, Merkal RS. 1985. A method for avoiding false-positive reactions in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine paratuberculosis. Jpn. J. Vet. Sci. 47:111–119.
12. Samanich KM, Keen MA, Vissa VD, Harder JD, Spencer JS, Belisle JT, Zolla-Pazner S, Laal S. 2000. Serodiagnostic potential of culture filtrate antigens of Mycobacterium tuberculosis. Clin. Diagn. Lab. Immunol. 7:662–668.
13. Waters WR, Palmer MV, Thacker TC, Bannantine JP, Vordermeier HM, Hewinson RG, Greenwald R, Esfandiari J, McNair J, Pollock JM, Andersen P, Lyashchenko KP. 2006. Early antibody response to experimental Mycobacterium bovis infection of cattle. Clin. Vaccine Immunol. 13:648–654.
14. Cho D, Collins MT. 2006. Comparison of the proteomes and antigenicities of secreted and cellular proteins produced by Mycobacterium paratuberculosis. J. Clin. Vaccin. Immunol. 13:1135–1161.
15. Shin SJ, Cho D, Collins MT. 2008. Diagnosis of bovine paratuberculosis by a novel enzyme-linked immunosorbent assay based on early secreted antigens of Mycobacterium avium subsp. paratuberculosis. Clin. Vaccine Immunol. 15:1277–1281.
16. Bannantine JP, Bayles DO, Waters WR, Palmer MV, Stabel Jr, Paustian ML. 2008. Early antibody response against Mycobacterium avium subspecies paratuberculosis antigens in subclinical cattle. Proteome Sci. 6:5; doi:10.1186/1477-5956-6-5.
17. Merkal R, Curran B. 1974. Growth and metabolic characteristics of Mycobacterium paratuberculosis. Appl. Microbiol. 28:276–279.
18. Blum H, Beier H, Gross HJ. 1974. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93–99.
19. Woodward M, Young W, Bloodgood R. 1985. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Methods 78:143–153.
20. Pukac LA, Carter JE, Morrison KS, Karnaovsky MJ. 1997. Enhancement of diaminobenzidine colorimetric signal in immunoblotting. Biotechniques 23:385–388.
21. Mattow J, Schaible UE, Schmidt F, Hagens K, Siejak F, Brestrich G, Haeselbarth G, Muller EC, Jungblut PR, Kaufmann SH. 2003. Comparative proteome analysis of culture supernatant proteins from virulent Mycobacterium tuberculosis H37Rv and attenuated M. bovis BCG Copenhagen. Electrophoresis 24:3405–3420.
22. Santema W, Overdijk M, Barends J, Krijgsveeld J, Rutten V, Koets A. 2009. Searching for proteins of Mycobacterium avium subspecies paratuberculosis with diagnostic potential by comparative qualitative proteomic analysis of mycobacterial tuberculins. Vet. Microbiol. 138:191–196.
23. Cho D, Sung N, Collins MT. 2006. Identification of proteins of potential diagnostic value for bovine paratuberculosis. Proteomics 6:5785–5794.
24. Leroy B, Roupie V, Noël-Georis I, Rosseels V, Walravens K, Govaerts M, Huyneg K, Wattiez R. 2007. Antigen discovery: a postgenomic approach to paratuberculosis diagnosis. Proteomics 7:1164–1176.
25. He Z, De Buck J. 2010. Localization of proteins in the cell wall of Myco- bacterium avium subsp. paratuberculosis K10 by proteomic analysis. Proteome Sci. 8:21; doi:10.1186/1477-5956-8-21.
26. Gouveia M, Johnson S, Genuaro ML. 2000. Identification of secreted proteins of Mycobacterium tuberculosis by a bioinformatic approach. In- fect. Immun. 68:2323–2327.
27. Målen H, Berven FS, Fladmark KE, Wiker HG. 2007. Comprehensive analysis of exported proteins from Mycobacterium tuberculosis H37Rv. Proteomics 7:1702–1718.
28. Champion PA, Cox JS. 2007. Protein secretion systems in mycobacteria. Cell Mol. Microbiol. 9:1376–1384.
29. Daleke DH, Ummels R, Bawono P, Heringa J, Van den Boucke-Grauls CM, Luirink J, Bitter W. 2012. General secretion signal for the mycobacterial type VII secretion pathway. Proc. Natl. Acad. Sci. U. S. A. 109:11342–11347.
30. Jeffry CJ, 1999. Moonlighting proteins. Trends Biochem. Sci. 24:8–11.
31. Henderson B, Martin A. 2011. Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. Infect. Immun. 79:3476–3491.
32. Tunio SA, Oldfield NJ, Berry A, Ala’Aldeen DAA, Wooldridge KG, Turner DPJ. 2010. The moonlighting protein fructose-1,6-bisphosphate aldolase of Neisseria meningitidis: surface localization and role in host cell adhesion. Mol. Microbiol. 76:605–615.
33. Cehovin A, Coates AR, Hu Y, Riffö-Vásquez Y, Tormay P, Botchan C, Altaraf F, Henderson B. 2010. Comparison of the moonlighting actions of the two highly homologous chaperonin 60 proteins of Mycobacterium tuberculosis. Infect. Immun. 78:3196–3206.
34. Raghavan S, Manzanillo P, Chan K, Dovey C, Cox JS. 2008. Secreted transcription factor controls Mycobacterium tuberculosis virulence. Nature 454:717–721.
35. Radosevich TJ, Reinhardt TA, Lippolis JD, Bannantine JP, Stabel JR. 2007. Proteomic and differential expression analysis of membrane and cytosolic proteins from Mycobacterium avium subsp. paratuberculosis strains K-10 and 187. J. Bacteriol. 189:1109–1117.
36. Weigoldt M, Meens J, Dolk K, Fritsch I, Molibus P, Goethe R, Geriach GF. 2011. Differential proteome analysis of Mycobacterium avium subsp. paratuberculosis grown in vitro and isolated from cases of clinical Johne’s disease. Microbiology 157:557–565.
37. Weigoldt M, Meens J, Bange FC, Pich A, Geriach GF, Goethe R. 2013. Metabolic adaptation of Mycobacterium avium subsp. paratuberculosis to the gut environment. Microbiology 159:380–391.
38. Xolalpa W, Vallecillo AJ, Lara M, Mendoza-Hernandez G, Comini M, Spallek R, Singh M, Espitia C. 2007. Identification of novel bacterial plasmogen-binding proteins in the human pathogen Mycobacterium tuberculosis. Proteomics 7:3332–3341.
39. Paustian ML, Kapur V, Bannantine JP. 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.