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Proteome and Differential Expression Analysis of Membrane and Cytosolic Proteins from *Mycobacterium avium* subsp. *paratuberculosis* Strains K-10 and 187▽†

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Little is known of protein expression in *Mycobacterium avium* subsp. *paratuberculosis* and how this contributes to pathogenesis. In the present study, proteins from both membranes and cytosol were prepared from two strains of *M. avium* subsp. *paratuberculosis*, i.e., laboratory-adapted strain K-10 and a recent isolate, strain 187, obtained from a cow exhibiting clinical signs of Johne's disease. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cytosol and membrane proteins from K-10 and 187 showed marked differences in protein expression. Relative levels of protein expression from both *M. avium* subsp. *paratuberculosis* strains were measured by using amine-reactive isobaric tagging reagents (iTRAQ) and tandem mass spectroscopy. Protein identification and relative expression data were obtained for 874 membrane and cytosolic proteins from the *M. avium* subsp. *paratuberculosis* proteome. These data showed a number of significant differences in protein expression between strain K-10 and clinical isolate 187. Examples of proteins expressed at higher levels in clinical isolate 187 compared to strain K-10 are AtpC, RpoA, and several proteins involved in fatty acid biosynthesis. In contrast, proteins such as AhpC and several proteins involved in nitrogen metabolism were expressed at higher levels in strain K-10 compared to strain 187. These data may provide insights into the proteins whose expression is important in natural infection but are modified once *M. avium* subsp. *paratuberculosis* is adapted to laboratory cultivation. Results from these studies will provide tools for developing a better understanding of *M. avium* subsp. *paratuberculosis* infection in the host and offer potential as diagnostic reagents and vaccine candidates.

Paratuberculosis (Johne's disease) is a significant economic problem in cattle and sheep worldwide (33). The causative agent of this chronic enteric disease is *Mycobacterium avium* subsp. *paratuberculosis*. *M. avium* subsp. *paratuberculosis* is a gram-positive intracellular pathogen that can persist and replicate within macrophages of the infected host (41). It is thought that neonatal cattle become infected by ingesting *M. avium* subsp. *paratuberculosis* shed in the feces of cattle that are in the clinical phase of the disease. Infections can persist for several years in a subclinical phase that is difficult to identify because of the absence of clinical signs. Once the disease enters the clinical phase, thickening of the intestinal wall leads to weight loss, decreased milk production, diarrhea, shedding of *M. avium* subsp. *paratuberculosis* in the feces, and eventual death (11, 16, 33). Diagnosis of Johne's disease is often complicated because of the slow progression of the disease and the prevalence of genetically similar mycobacterial species in the environment.

The recent sequencing and annotation of the K-10 strain of *M. avium* subsp. *paratuberculosis* has led to significant progress in the analysis of the genetic regulation of *M. avium* subsp. *paratuberculosis* (22). Microarray analysis has provided extensive data on gene expression in several species of mycobacteria, including *M. avium* subsp. *paratuberculosis*. Although single proteins have been identified as potential diagnostic tools for *M. avium* subsp. *paratuberculosis*, comprehensive surveys of the *M. avium* subsp. *paratuberculosis* proteome have been lacking until now. Recent studies have reported success in the use of methods such as two-dimensional electrophoresis and surface-enhanced laser desorption ionization-time of flight mass spectrometry (MS) to profile protein expression in *M. avium* subsp. *paratuberculosis* (10, 15). Direct comparison of gene expression between strains or within a single strain grown under differing conditions has proven useful in identifying mycobacterial growth and pathogenicity characteristics (14, 17). Analysis of the *M. avium* subsp. *paratuberculosis* proteome offers another level of regulation for study.

In postgenomic studies, high-throughput high-performance liquid chromatography coupled with MS has been used to identify greater than 25% of the predicted *M. tuberculosis* coding sequences (25). The identification of large sets of proteins like this is possible with extensive fractionation of the protein sample. Large-scale proteome analysis from multiple samples has thus far been limited by an inability to quantify MS results. Use of recently developed amine-labeled isobaric tags now allows the analysis of relative protein expression levels between samples (1). By using this method, we analyzed the levels of protein expression from two *M. avium* subsp. *paratuberculosis* strains.

Studies on *M. avium* subsp. *paratuberculosis* have shown that

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adaptation to laboratory growth conditions often is accompanied by changes in phenotype (5, 38). It is unknown how changes in phenotype correspond to protein expression profiles. The two strains used in this study were chosen to evaluate effects of in vitro propagation of *M. avium* subsp. *paratuberculosis* on protein expression. Comparing the proteome expression profiles of two *M. avium* subsp. *paratuberculosis* strains of different passage numbers may help us elucidate the roles of key components involved in *M. avium* subsp. *paratuberculosis* growth and pathogenesis. In the present study, our goals were to obtain a comprehensive survey of the *M. avium* subsp. *paratuberculosis* proteome and to demonstrate the differences in protein expression in two *M. avium* subsp. *paratuberculosis* strains, multiple-passage laboratory-adapted strain K-10 and low-passage strain 187 obtained from a cow with clinical Johne's disease.

MATERIALS AND METHODS

Culture strains and protein samples. *M. avium* subsp. *paratuberculosis* strain K-10 was chosen as the reference strain because of its use in sequencing of the *M. avium* subsp. *paratuberculosis* genome and extensive study. Strain K-10 was passed 16 times in our laboratory, a value that does not account for the multiple passages performed in the laboratory of origin. *M. avium* subsp. *paratuberculosis* strain 187 was isolated from the ileum of a clinical cow. It was confirmed to be *M. avium* subsp. *paratuberculosis* by PCR IS900 (37; data not shown) and grown to passage 5. Twenty-milliliter volumes of actively growing cultures of both strains were individually transferred to 500 ml of Middlebrook 7H9 broth (pH 5.9) containing 0.05% Tween 80 and ferric mycobactin J (2 mg/liter; Allied Monitor, Fayette, MO). Culture growth was monitored, and cultures were harvested in log phase (A_{540} = 0.23 to 0.24). Expansion of each strain to log phase after passage took between 2 and 3 weeks because of the slow replication rate that is characteristic of *M. avium* subsp. *paratuberculosis*. Cultures were collected and washed three times in cold phosphate-buffered saline (PBS, pH 7.4). Cell sonicates were prepared as previously reported (4). Briefly, cells were resuspended in 5 ml of buffer (250 mM sucrose, 10 mM HEPES [pH 7.5], 1 mM EGTA) and sonicated three times for 10 min in an ice-water slurry. After each sonication step, the samples were cooled for 10 min prior to the start of the next sonication interval. The sonicates were centrifuged at 10,000 × *g* for 15 min to remove unbroken cells, and cellular debris and the supernatants were decanted. The supernatants were centrifuged at 100,000 × *g* for 1 h to pellet membranes. The supernatant was collected and served as the *M. avium* subsp. *paratuberculosis* cytosolic protein fraction. The pellet was resuspended in an equal volume of sonication buffer and served as the *M. avium* subsp. *paratuberculosis* membrane protein fraction. All samples were assayed for protein content by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and stored at −20°C.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with precast 4 to 12% Bis-Tris buffered minigels in morpholinepropanesulfonic acid (MOPS) buffer (Invitrogen Corporation, Carlsbad, CA). Samples were diluted in 4× NuPAGE loading buffer and denatured at 95°C for 5 min. Silver staining was done with the SilverQuest silver staining kit (Invitrogen).

Immunoblot assays. Electrophoretic transfer of proteins to nitrocellulose (Invitrogen) was done with a Criterion blotting cell (Bio-Rad) in NuPAGE (Invitrogen) blotting buffer with 10% methanol for 30 min at 100 V. Membranes were washed for 5 min in PBS and blocked overnight at 4°C on a rotating platform with SuperBlock (Pierce Chemical Company, Rockford, IL) plus 0.05% Tween 20. Three *M. avium* subsp. *paratuberculosis* monoclonal antibodies were used in this study. MAP1643 codes for the AceAb protein, MAP3840 codes for the DnaK protein, and MAP2121c codes for the 35-kDa major membrane protein that is involved in *M. avium* subsp. *paratuberculosis* invasion of intestinal epithelial cells (3). Monoclonal antibodies were diluted in 15 ml of SuperBlock plus 0.05% Tween 20 at the following rates: anti-MAP1643, 1:300; anti-MAP3840, 1:200; anti-MAP2121c, 1:500. Blots were incubated with each antibody at room temperature for 1 h on a rocking platform. Following primary-antibody incubation, the blots received six 5-min washes in PBS containing 0.05% Tween 20. Next, the blots were incubated at room temperature on a rocking platform for 45 min with horseradish peroxidase-labeled anti-mouse secondary antibody (1:60,000 in 20 ml of SuperBlock plus 0.05% Tween 20). The blots were washed six times for 5 min in PBS plus 0.05% Tween 20. Specific banding was detected with the WestDura detection system (Pierce Immunochemicals, Rockford, IL).

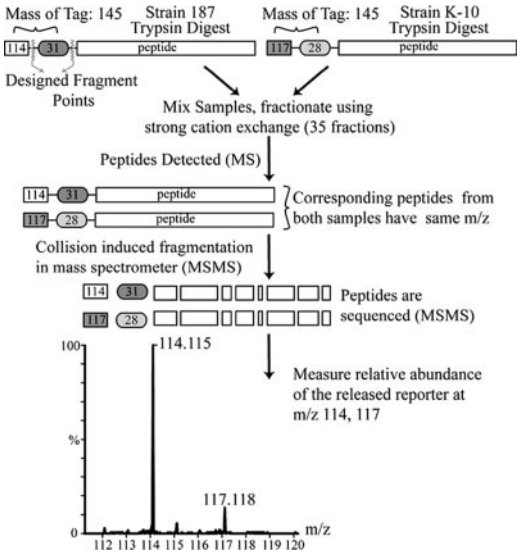

FIG. 1. Schematic representation of iTRAQ protein expression analysis. The cytosolic protein fractions of strains 187 and K-10 were digested with trypsin, and the resulting peptides were labeled with appropriate iTRAQ tags. Samples were combined, and peptides were separated by strong cation exchange and analyzed with a Q-TOF Ultima API mass spectrometer. The ratio of peak areas between 114 and 117 was used to determine the relative abundance of proteins in the original sample. The procedure was repeated with the membrane-enriched fractions from both strains.

iTRAQ labeling. One hundred micrograms of protein from each sample was dried and processed with the iTRAQ kit (Applied Biosystems, Foster City, CA). *M. avium* subsp. *paratuberculosis* strain 187 was labeled with the 114 iTRAQ tag, and *M. avium* subsp. *paratuberculosis* strain K-10 was labeled with the 117 iTRAQ tag (Fig. 1). Peptides were digested with trypsin followed by labeling with the iTRAQ reagent. Subsequently, the peptides from the two samples were combined, dried, resuspended in 300 μl of 20 mM formic acid–20% acetonitrile (ACN), and further purified.

Strong cation exchange. The iTRAQ samples were chromatographed on a cation-exchange column (Mono S PC 1.6/5; Amersham, Piscataway, NJ). The gradient solutions are mobile phase A (20 mM formic acid, 20% ACN, pH 2.7) and mobile phase B (20 mM formic acid, 20% ACN, 350 mM ammonium bicarbonate, pH 4.7). Sample fractions (0.5 ml) were collected over a 150-min gradient of 0 to 35% mobile phase B, followed by 5 min in 90% mobile phase B (the flow rate was 300 μl/min). Samples were dried and resuspended in 30 μl of 0.1% formic acid in 5% ACN and subjected to mass spectroscopy as follows.

High-performance chromatography and tandem mass spectroscopy of the samples. Each strong-cation-exchange fraction was analyzed by capillary high-pressure liquid chromatography (CapLC; Waters, Milford, MA) in line with a Q-TOF Ultima API mass spectrometer (Waters, Milford, MA) (24). An Altantis C_{18} NanoEase column (75 μm by 100 mm) was used for peptide separation. The system was configured to concentrate and wash the injected sample on a Symmetry 300 C_{18} precolumn. Seven minutes after the start of sample loading, the precolumn was switched in line with the analytical column to allow the trapped peptides to be eluted onto the analytical column. Mobile phase A was 0.1% formic acid in 5% ACN. Mobile phase B was 0.1% formic acid in 95% ACN. The gradient was 95% A for 5 min and then ramped linearly to 60% A over 85 min. Over the next 2.5 min, it was ramped to 10% A and held for an additional 10 min before equilibration of the column. The flow rate was approximately 300 nl/min. The analytical column was connected to a Waters Lockspray-nanospray interface on the front of the mass spectrometer. The Lockspray used the peptides [Glu^1]fibrinopeptide B and leucine enkephalin as standards (Sigma, St. Louis, MO). The capillary voltage was 3,500 V and was tuned for signal intensity. The five most intense ions with charge states between 2 and 4 were selected in each survey scan if they met the switching criteria. Three collision energies were used

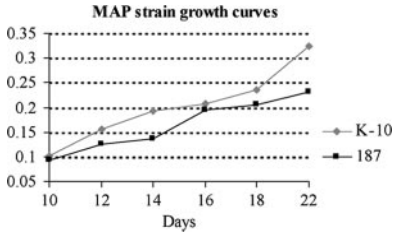

FIG. 2. Growth curves generated for *M. avium* subsp. *paratuberculosis* strains 187 and K-10 in Middlebrook 7H9 broth supplemented with Tween 80 and ferric mycobactin J. On the basis of their rates of growth, strain K-10 was harvested on day 18 (A_{540}, 0.235) and strain 187 was harvested on day 22 (A_{540}, 0.232).

to fragment each peptide ion on the basis of its mass-to-charge (*m*/*z*) values. Each fraction was run four times, once collecting MS-MS data on the full range of parent masses, followed by runs collecting MS-MS data on parent masses in three mass ranges (400 to 635, 635 to 750, and 750 to 1,500) (32).

Bacterial processing. All MS data files were processed into pkl files with Protein Lynx Global Server 2.0 (PLGS 2.0; Waters, Milford, MA) and analyzed with Mascot (Matrix Science, London, United Kingdom) with the NCBI nonredundant database with bacterial taxonomy. Peptide expression was determined by using the peak area of each of the iTRAQ labels, after the peaks were summed, smoothed, and centered. Data were organized to remove duplication of query assignments and duplicate proteins. All proteins without at least two MS-MS spectra and a probability of greater than 95% were removed. Only MS-MS spectra with abundance information for both iTRAQ labels were retained (23). Changes in protein expression were calculated as described by Ross and coworkers [187 label/(187 label + K-10 label)] (28). The range of this calculation is between 0 and 1, with a result of 0.5 meaning no change in protein expression. The average iTRAQ calculation for all MS-MS spectra in the membrane fraction was 0.569, with a standard deviation of 0.086. For the cytosol fraction, the average was 0.428, with a standard deviation of 0.057. A change of less than 2 standard deviations was deemed no change in protein expression.

RESULTS

Strains 187 and K-10 show different proteome expression profiles. Differences in protein expression between *M. avium* subsp. *paratuberculosis* strains likely correlate with phenotypic traits such as differences in growth rates and virulence (2). To investigate to what extent this occurs in *M. avium* subsp. *paratuberculosis*, we analyzed two strains, low-passage *M. avium* subsp. *paratuberculosis* clinical isolate 187 and laboratory-adapted *M. avium* subsp. *paratuberculosis* strain K-10. Strain 187 grew at a slower rate than strain K-10 (Fig. 2). Protein samples from *M. avium* subsp. *paratuberculosis* strains 187 and K-10 showed different protein banding patterns on silver-stained SDS-PAGE gels (Fig. 3A). The regions with brackets show differences in banding patterns between the strains for both the cytosol and membrane-enriched fractions. Together, these data illustrate both phenotypic and proteomic profile differences between the two strains.

iTRAQ analysis identifies differentially expressed proteins. Protein expression data were obtained for 550 proteins from the membrane fractions, of which 385 proteins were present only in the membranes and not the cytosol of the *M. avium* subsp. *paratuberculosis* strains (see the membrane protein data in the supplemental material). Of the 550 proteins identified in the *M. avium* subsp. *paratuberculosis* membranes, 266 of the proteins identified were hypothetical proteins in the SwissProt database. Within the membrane fractions of each strain, 37 proteins were expressed at higher abundance in strain K-10

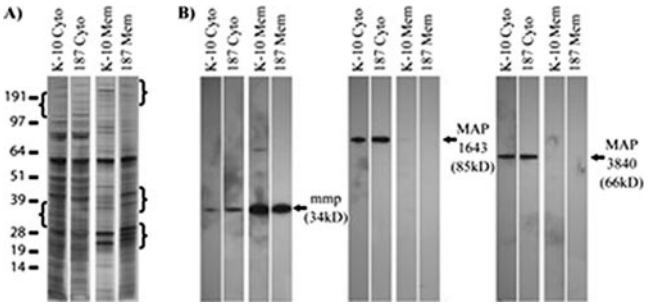

FIG. 3. Protein expression differences observed on both one- and two-dimensional silver-stained PAGE gels. (A) SDS-PAGE of cytosolic and membrane protein samples (0.5 μg/lane) from strains 187 and K-10. Differences in banding patterns between the strains are noted in the bracketed regions. The values on the left are molecular sizes in kilodaltons. (B) Immunoblot analysis of *M. avium* subsp. *paratuberculosis* strains 187 and K-10 with three available anti-*M. avium* subsp. *paratuberculosis* monoclonal antibodies. MAP1643 and MAP3840 are found predominantly in the cytosolic fractions, while MAP2121c is found mainly in the membrane fractions.

than strain 187 (Table 1). In contrast, 35 *M. avium* subsp. *paratuberculosis* membrane proteins were expressed at higher abundance in strain 187 than in strain K-10 (Table 2). The incomplete and/or electronic-only annotation of the *M. avium* subsp. *paratuberculosis* genome limits the conclusions that can be made about the differentially regulated proteins observed in this study. It is of interest that membrane-associated proteins in clinical isolate 187 are generally upregulated to a greater extent (2.9- to 6.9-fold) than those in laboratory-adapted strain K-10 (Table 2). In contrast, upregulation of membrane-associated proteins in strain K-10 over those seen in strain 187 was more moderate (1.5- to 3.5-fold; Table 1). The greatest increase in protein expression was demonstrated for AtpC, a component of the ATP synthase complex, at 6.91-fold higher expression noted for strain 187 compared to strain K-10. The MVIN-like protein (MAP4336), a putative virulence factor in other organisms, was expressed 4.75-fold more in strain 187 than in strain K-10. Stress-associated proteins MAP4265 (GroEL1) and Map2281c (ClpP) were both expressed about threefold more in strain 187 than in strain K-10. Slower-growing clinical isolate 187 expressed 3.4-fold more of cell division protein MAP1894c (FtsZ) in the membrane fractions than was observed in laboratory-adapted strain K-10 (Table 2). Finally, a number of fatty acid metabolism proteins, FadE_2, InhA, FadE23, FadA1, FadE5, and FadB1 (2.9- to 4-fold), as well as RNA polymerase proteins RpoA and RpoB (3.6- to 3.9-fold; Table 2), were also upregulated in the recent clinical isolate (strain 187).

From *M. avium* subsp. *paratuberculosis* cytosolic fractions, protein expression data were obtained for 489 proteins, of which 324 proteins were identified only in the cytosolic fraction (see the cytosolic protein data in the supplemental material). Out of 489 proteins identified in *M. avium* subsp. *paratuberculosis* cytosol, 185 of the proteins identified were hypothetical proteins in the SwissProt database. Within the cytosolic fractions of each strain, 22 proteins were expressed at higher abundance in strain K-10 than in strain 187 (Table 3). In contrast, 18 cytosolic proteins were expressed at higher abundance in strain 187 than in strain K-10 (Table 4). The greatest increase

TABLE 1. Proteins expressed at higher relative abundance in strain K-10 membrane fractions compared to strain 187 membrane fractions

NCBI no.	Protein	Score[a]	Description[b]	Mean (SD) of 114/114 +117[c]	Fold increase[d]	No. of unique peptides[e]	MS-MS[f]
gi 41407687	MAP1589c	171.26	AhpC	0.224 (0.012)	3.46	2	2
gi 41395410	MAP0961c	209.71	Hypothetical protein	0.251 (0.037)	2.98	4	5
gi 41399117	MAP4185	254.35	RpmD	0.273 (0.092)	2.66	2	8
gi 41396278	MAP1826c	137.78	Hypothetical protein	0.291 (0.182)	2.44	2	7
gi 41408218	MAP2120c	599.40	nifS-like protein	0.301 (0.105)	2.32	7	20
gi 41397444	MAP2987c	345.10	GlnB	0.322 (0.170)	2.11	7	9
gi 41397250	MAP2793	120.59	Hypothetical protein	0.325 (0.113)	2.08	3	4
gi 41398325	MAP3396c	110.75	Hypothetical protein	0.329 (0.136)	2.04	2	3
gi 41395411	MAP0962c	139.86	Hypothetical protein	0.342 (0.139)	1.92	3	3
gi 41395766	MAP1316c	223.80	AppC	0.346 (0.063)	1.89	4	15
gi 41399115	MAP4183	70.03	RplR	0.349 (0.031)	1.86	2	3
gi 41398647	MAP3717c	181.06	Hypothetical protein	0.350 (0.161)	1.86	3	4
gi 41395491	MAP1042	195.72	Hypothetical protein	0.350 (0.041)	1.86	2	2
gi 41395079	MAP0631c	174.39	Hypothetical protein	0.351 (0.041)	1.85	4	5
gi 41395078	MAP0630c	850.22	Hypothetical protein	0.356 (0.097)	1.81	13	58
gi 41394589	MAP0143	257.33	Hypothetical protein	0.357 (0.026)	1.80	4	6
gi 41398430	MAP3501	86.81	Hypothetical protein	0.360 (0.109)	1.78	2	4
gi 41395429	MAP0980c	395.83	Hypothetical protein	0.364 (0.097)	1.75	6	8
gi 41398294	MAP3365c	144.71	Hypothetical protein	0.368 (0.158)	1.72	3	4
gi 41394511	MAP0233c	163.76	Hypothetical protein	0.373 (0.050)	1.68	2	3
gi 41394679	MAP1179	63.14	CtaB	0.374 (0.049)	1.67	2	4
gi 41395629	MAP3171c	116.67	FtsX	0.378 (0.041)	1.64	2	3
gi 41398089	MAP1292c	124.01	Hypothetical protein	0.379 (0.096)	1.64	2	3
gi 41395742	MAP2939c	160.68	Hypothetical protein	0.379 (0.158)	1.64	4	4
gi 41397396	MAP0940	82.59	Hypothetical protein	0.381 (0.038)	1.62	2	2
gi 41395389	MAP1940c	292.78	CtaC	0.382 (0.037)	1.62	4	4
gi 41396393	MAP2433	688.67	Hypothetical protein	0.382 (0.086)	1.62	12	25
gi 41396889	MAP2988c	112.85	Amt_2	0.383 (0.052)	1.61	2	3
gi 41397445	MAP3287	258.26	Hypothetical protein	0.384 (0.099)	1.61	4	5
gi 41398216	MAP2057	656.43	Hypothetical protein	0.386 (0.071)	1.59	14	26
gi 41396510	MAP3698c	1656.99	Hypothetical protein	0.388 (0.064)	1.58	27	72
gi 41398628	MAP3092	243.76	FecB	0.389 (0.130)	1.56	3	4
gi 41398010	MAP1510	479.82	Hypothetical protein	0.393 (0.061)	1.55	6	10
gi 41395961	MAP1738	223.21	MmpL5	0.394 (0.075)	1.54	4	5
gi 41396190	MAP3291c	336.52	Hypothetical protein	0.400 (0.068)	1.50	3	3
gi 41398220	MAP4169	242.16	RpmC	0.400 (0.087)	1.50	5	5

[a] Protein score assigned by Mascot.
[b] Descriptions are from the SwissProt database.
[c] Mean and standard deviation for all of the MS spectra for a given protein.
[d] Fold increase compared to the other strain. A ratio of 0.5 is no change in protein expression.
[e] Number of unique peptides identified for the protein.
[f] MS-MS refers to the total number of MS spectra found for the given protein.

in expression was demonstrated for Ffh, a signal recognition particle that participates in the secretory pathway for bacteria. Other major cytosolic proteins that were upregulated were GlnD, Icd2, and AtpC, all of which are involved in energy and nitrogen metabolism.

A total of 874 unique proteins were identified from *M. avium* subsp. *paratuberculosis* in this study, and 165 of these proteins were found in both the *M. avium* subsp. *paratuberculosis* membrane and cytosol preparations (see the supplemental material). Much of the protein function data available for *M. avium* subsp. *paratuberculosis* is inferred from electronic annotation and therefore must be used with that caveat in mind. Of the 58 proteins expressed at higher relative abundance in the strain K-10 membrane and cytosolic fractions compared to strain 187, 34 (57%) were hypothetical proteins (Fig. 4A). Of the 53 proteins expressed at higher relative abundance in the membrane and cytosol of strain 187 compared to strain K-10, 18 (34%) were hypothetical proteins (Fig. 4B). For the total *M. avium* subsp. *paratuberculosis* proteome, 763 proteins showed no expression changes and 374 (42%) were hypothetical proteins (Fig. 4C). Approximately 71% of the 4,350 annotated proteins from the sequencing of *M. avium* subsp. *paratuberculosis* are currently listed as hypothetical proteins (22). In each proteome grouping, hypothetical proteins were the largest category, followed by metabolism and ribosome-protein synthesis (Fig. 4A, B, and C). Clearly, as functions are attributed to the many hypothetical proteins identified, more important details will emerge from these data.

Immunoblot analysis confirms iTRAQ results. Monoclonal antibodies to MAP1643, MAP2121c, and MAP3840 were used to confirm *M. avium* subsp. *paratuberculosis* iTRAQ expression data. None of these MAP proteins showed any change in expression in comparisons of *M. avium* subsp. *paratuberculosis* strains 187 and K-10 by either the iTRAQ method or immunoblotting (Fig. 3B; see the supplemental material). MAP1643 was only found in *M. avium* subsp. *paratuberculosis* cytosol, regardless of the method used. MAP3840 was identified in both the *M. avium* subsp. *paratuberculosis* membrane and cytosolic fractions by mass spectroscopy, but immunoblot data showed it to be present predominately within *M. avium* subsp.

TABLE 2. Proteins expressed at higher relative abundance in strain 187 membrane fractions compared to strain K-10 membrane fractions

NCBI no.	Protein	Score[a]	Description[b]	Mean (SD) of 114/114 +117[c]	Fold increase[d]	No. of unique peptides[e]	MS-MS[f]
gi 41408548	MAP2450c	95.36	AtpC	0.874 (0.046)	6.91	2	3
gi 41394941	MAP0494	160.55	Hypothetical protein	0.852 (0.059)	5.77	3	6
gi 41397135	MAP2679c	145.98	Hypothetical protein	0.828 (0.112)	4.82	2	5
gi 41399268	MAP4336	243.44	Hypothetical protein	0.826 (0.095)	4.75	4	7
gi 41395817	MAP1367	530.25	ArgG	0.820 (0.093)	4.55	8	16
gi 41399164	MAP4232	361.15	RpsD	0.805 (0.058)	4.13	4	8
gi 41395238	MAP0790	1251.23	FadB1	0.801 (0.099)	4.02	17	50
gi 41398624	MAP3694c	209.19	FadE5	0.799 (0.117)	3.99	3	4
gi 41399062	MAP4130	573.43	RpoB	0.797 (0.111)	3.93	8	15
gi 41407408	MAP1310	87.39	PykA	0.793 (0.057)	3.83	2	2
gi 41395106	MAP0658c	647.22	DesA1	0.791 (0.082)	3.77	10	14
gi 41399165	MAP4233	429.61	RpoA	0.784 (0.150)	3.63	7	13
gi 41397348	MAP2891c	325.60	GpsI	0.784 (0.085)	3.62	4	6
gi 41395237	MAP0789	282.86	FadA1	0.779 (0.094)	3.53	4	5
gi 41396346	MAP1894c	545.69	FtsZ	0.774 (0.088)	3.43	7	13
gi 41396700	MAP2246c	130.37	Hypothetical protein	0.773 (0.115)	3.41	2	3
gi 41395960	MAP1509	104.02	Hypothetical protein	0.773 (0.118)	3.41	2	2
gi 41397138	MAP2682c	81.60	Hypothetical protein	0.768 (0.041)	3.32	2	5
gi 41396537	MAP2084	113.61	Hypothetical protein	0.768 (0.056)	3.32	2	4
gi 41399075	MAP4143	884.65	Tuf	0.764 (0.120)	3.23	8	37
gi 41396008	MAP1557c	275.96	Gnd	0.762 (0.085)	3.20	6	11
gi 41396732	MAP2787	161.39	ClpX	0.762 (0.078)	3.20	3	3
gi 41398576	MAP3646	353.58	PckA	0.758 (0.115)	3.14	9	11
gi 41398107	MAP3189	304.13	FadE23	0.756 (0.085)	3.09	4	5
gi 41396735	MAP2281c	168.83	ClpP	0.755 (0.139)	3.09	3	6
gi 41395318	MAP0870c	89.73	Hypothetical protein	0.755 (0.062)	3.08	2	2
gi 41397978	MAP3060c	643.12	FixB	0.755 (0.074)	3.08	8	17
gi 41399185	MAP4253	164.27	GlmS	0.754 (0.075)	3.07	3	5
gi 41399197	MAP4265	601.67	GroEL1	0.752 (0.075)	3.04	7	16
gi 41398243	MAP3314c	169.50	Hypothetical protein	0.752 (0.074)	3.04	2	3
gi 41407308	MAP1210	168.78	InhA	0.751 (0.032)	3.02	2	2
gi 41397954	MAP3036c	343.67	IlvC	0.750 (0.075)	3.00	6	9
gi 41398500	MAP3750c	119.53	FadE2	0.744 (0.094)	2.91	2	2
gi 41398385	MAP3456c	442.78	Icd2	0.743 (0.069)	2.89	8	17
gi 41409122	MAP3024c	374.91	hupB	0.743 (0.091)	2.89	6	32

[a] Protein score assigned by Mascot.
[b] Descriptions are from the SwissProt database.
[c] Mean and standard deviation for all of the MS spectra for a given protein.
[d] Fold increase compared to the other strain. A ratio of 0.5 is no change in protein expression.
[e] Number of unique peptides identified for the protein.
[f] MS-MS refers to the total number of MS spectra found for the given protein.

paratuberculosis cytosol preparations. In contrast, MAP2121c was identified only within the *M. avium* subsp. *paratuberculosis* membranes by mass spectroscopy but faint banding was observed in cytosolic fractions by immunoblotting (Fig. 3B; see the supplemental material). The correlation of immunoblot analysis and iTRAQ data with regard to both protein expression and subcellular localization supports the iTRAQ experimental results.

DISCUSSION

The aims of this study were to obtain a proteomic profile of *M. avium* subsp. *paratuberculosis* and to obtain information on the effects of in vitro growth on the relative protein expression data for two *M. avium* subsp. *paratuberculosis* strains. With just over 20% of potential *M. avium* subsp. *paratuberculosis* proteins identified by this shotgun MS-MS approach, we demonstrated that with the proper prefractionation, data sets large enough to look at global protein expression are obtainable for *M. avium* subsp. *paratuberculosis*. Moreover, this study is the most comprehensive proteomic survey of *M. avium* subsp. *paratuberculosis* performed to date. Of the 874 proteins found in both strains, 763 (87%) proteins showed no significant change in relative expression, indicating that both *M. avium* subsp. *paratuberculosis* strains expressed the majority of proteins at similar levels. As a screening protocol, the iTRAQ method proved capable of identifying 111 proteins with significantly different relative protein expression levels between the strains. The differences in protein expression ranged from no change to a sixfold increase in the expression of AtpC in strain 187 compared to strain K-10 membranes.

Since the membrane proteins are the first proteins presented to the host in an infection, it was of particular interest to us that MAP4336 was upregulated 4.7-fold in the membrane fraction of clinical isolate 187 over that seen in laboratory-adapted strain K-10. Since strain 187 was a recent isolate of *M. avium* subsp. *paratuberculosis*, it was anticipated that potential virulence proteins would be more highly expressed in strain 187 membranes. MAP4336 is an integral membrane protein from the MVIN family of proteins which, although biochemically uncharacterized, have been found to be virulence factors in

TABLE 3. Proteins expressed at higher relative abundance in strain K-10 cytosolic fractions compared to strain 187 cytosolic fractions

NCBI no.	Protein	Score[a]	Description[b]	Mean (SD) of 114/114 +117[c]	Fold increase[d]	No. of unique peptides[e]	MS-MS[f]
gi 41397440	MAP2983c	87.30	Ffh	0.165 (0.176)	5.06	2	3
gi 41397443	MAP2986c	167.87	GlnD	0.180 (0.030)	4.55	2	3
gi 41394518	MAP0072c	266.55	Hypothetical protein	0.225 (0.076)	3.44	5	8
gi 41395953	MAP1502	179.81	Hypothetical protein	0.234 (0.092)	3.28	3	4
gi 41398385	MAP3456c	367.30	Icd2	0.238 (0.131)	3.21	5	13
gi 41398458	MAP3529	72.89	Hypothetical protein	0.245 (0.110)	3.08	2	4
gi 41406377	MAP0279	84.58	Hypothetical protein	0.246 (0.042)	3.07	2	5
gi 41399048	MAP4116c	182.64	MmaA4	0.246 (0.091)	3.07	3	7
gi 41399128	MAP4196	239.31	Hypothetical protein	0.262 (0.134)	2.82	3	6
gi 41399156	MAP4224c	126.33	RmlC	0.265 (0.070)	2.78	2	3
gi 41398646	MAP3716c	211.65	FadE6	0.266 (0.098)	2.75	4	13
gi 41394854	MAP0407c	93.72	Acs	0.271 (0.031)	2.70	2	2
gi 41409122	MAP3024c	260.00	hupB	0.276 (0.038)	2.63	4	20
gi 41398803	MAP3872	89.36	Hypothetical protein	0.276 (0.002)	2.62	2	2
gi 41398763	MAP3832c	83.46	Hypothetical protein	0.276 (0.091)	2.62	2	6
gi 41394457	MAP0011	167.86	PpiA	0.280 (0.068)	2.57	3	5
gi 41396466	MAP2013c	87.44	Hypothetical protein	0.285 (0.089)	2.51	2	3
gi 41409145	MAP3047	86.80	Hypothetical protein	0.286 (0.069)	2.50	2	3
gi 41396510	MAP2057	150.58	Hypothetical protein	0.290 (0.034)	2.44	3	6
gi 41394882	MAP0435c	134.69	Ppa	0.295 (0.083)	2.39	2	6
gi 41395817	MAP1367	516.06	ArgG	0.299 (0.074)	2.35	6	9
gi 41395429	MAP0980c	115.41	Hypothetical protein	0.310 (0.022)	2.23	2	3

[a] Protein score assigned by Mascot.
[b] Descriptions are from the SwissProt database.
[c] Mean and standard deviation for all of the MS spectra for a given protein.
[d] Fold increase compared to the other strain. A ratio of 0.5 is no change in protein expression.
[e] Number of unique peptides identified for the protein.
[f] MS-MS refers to the total number of MS spectra found for the given protein.

other bacteria and therefore are important in bacterial pathogenesis (8, 29).

The higher expression of both GroEL1 and ClpP in clinical isolate 187 compared to strain K-10 may reflect the stress associated with the adaptation of strain 187 to laboratory culture medium. The function of GroEL1 is to promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions (6). In association with this, the function of ClpP is in the degradation of misfolded proteins (21). Both of these proteins are upregulated in strain 187 to the same degree, and their complementary functions to prevent accumulation of incorrectly folded proteins make sense phys-

TABLE 4. Proteins expressed at higher relative abundance in strain 187 cytosolic fractions compared to strain K-10 cytosolic fractions

NCBI no.	Protein	Score[a]	Description[b]	Mean of 114/114 +117[c]	SD of 114/114 +117[c]	Fold increase[d]	No. of unique peptides[e]	MS-MS[f]
gi 41397329	MAP2872c	184.54	FabG5_2	0.703	0.179	2.36	2	5
gi 41397167	MAP2710c	357.16	Hypothetical protein	0.692	0.125	2.24	6	14
gi 41395789	MAP1339	82.60	Hypothetical protein	0.665	0.001	1.98	2	2
gi 41394941	MAP0494	383.10	Hypothetical protein	0.616	0.036	1.60	4	8
gi 41397454	MAP2997c	351.82	Hypothetical protein	0.613	0.116	1.58	6	16
gi 41408548	MAP2450c	113.18	AtpC	0.608	0.051	1.55	2	10
gi 41395775	MAP1325	563.23	RpsA	0.601	0.159	1.50	9	33
gi 41396962	MAP2506c	117.11	Hypothetical protein	0.587	0.059	1.42	2	4
gi 41396907	MAP2451c	657.81	AtpD	0.586	0.178	1.42	11	31
gi 41396721	MAP2267c	194.66	Rne	0.578	0.128	1.37	3	3
gi 41394596	MAP0150c	620.65	FadE25_2	0.578	0.078	1.37	7	32
gi 41398994	MAP4063c	153.40	Hypothetical protein	0.576	0.032	1.36	2	4
gi 41394917	MAP0470	98.56	Hypothetical protein	0.571	0.069	1.33	2	2
gi 41395572	MAP1123	117.28	Gmk	0.559	0.061	1.27	2	4
gi 41396735	MAP2281c	209.32	ClpP	0.554	0.090	1.24	4	6
gi 41394796	MAP0349	180.79	Hypothetical protein	0.552	0.025	1.23	2	5
gi 41398481	MAP3551c	178.96	Hypothetical protein	0.550	0.077	1.22	3	3
gi 41399272	MAP34340	150.17	TrxC	0.549	0.004	1.22	2	2

[a] Protein score assigned by Mascot.
[b] Descriptions are from the SwissProt database.
[c] Mean and standard deviation for all of the MS spectra for a given protein.
[d] Fold increase compared to the other strain. A ratio of 0.5 is no change in protein expression.
[e] Number of unique peptides identified for the protein.
[f] MS-MS refers to the total number of MS spectra found for the given protein.

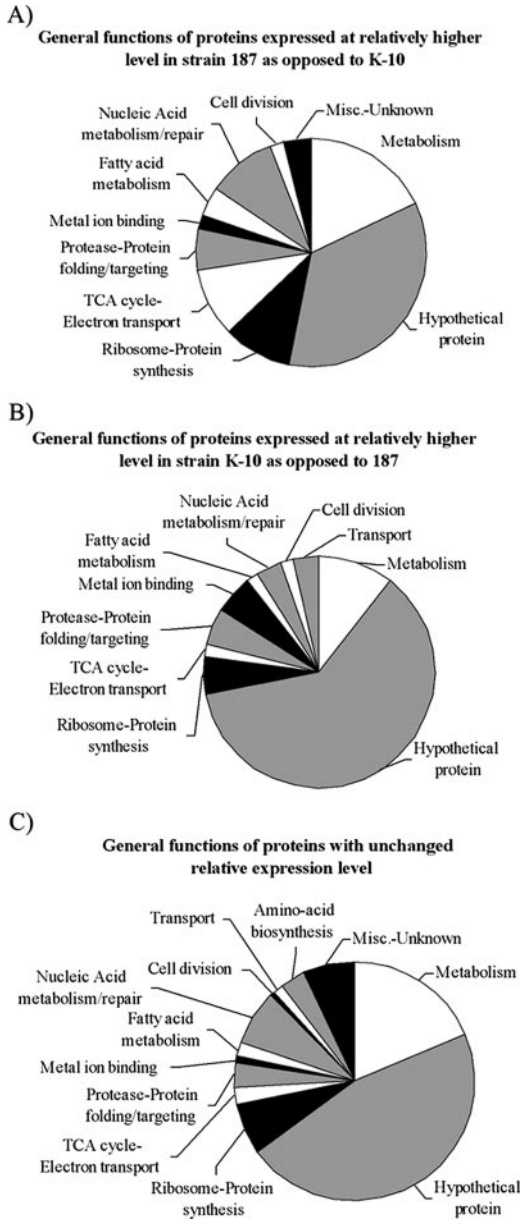

FIG. 4. General functions of the proteins identified. (A) Fifty-three proteins were identified in strain 187 in both the membrane and cytosolic fractions at a significantly higher level compared to strain K-10 when grouped by function. The protein functions listed are from the SwissProt database and were inferred from electronic annotation. (B) Fifty-eight proteins were identified in strain K-10 in both the membrane and cytosolic fractions at a significantly higher level compared to strain 187 when grouped by function. (C) Seven hundred sixty-three proteins were identified in both strains whose relative expression level was found to be the same when grouped by function. Proteins identified in both the membrane and cytosolic fractions were counted one time. TCA, trichloroacetic acid; Misc., miscellaneous.

iologically as this would likely occur during the transitional stress from the host environment to laboratory culture.

The slower growth of strain 187 in laboratory medium was not surprising since it was a recent isolate. But the high expression of a protein essential to cell division (FtsZ) is puzzling. Perhaps the higher expression of FtsZ in strain 187 can be explained by a slower completion of the cell division process due to the stress of movement to a laboratory environment. While in the preceding discussion the high expression of GroEL1 was attributed to stress, recent work has shown that GroEL1 associates with FtsZ in the cell division process (26). Therefore, this could explain part of the upregulation of GroEL1 seen in strain 187 compared to strain K-10.

Laboratory-adapted and low-passage strains of bacteria have been shown to possess differing characteristics (35). Of particular interest are membrane proteins which serve as the host interaction sites for these intracellular pathogens (12, 39). Low passage number strains of *M. tuberculosis* have been shown to retain protein expression profiles that include important antigenic markers that are not expressed in higher passage number laboratory attenuated strains (2). This is critical information suggesting that a loss of virulence genes may occur during in vitro propagation of the bacteria, which could potentially result in misdirected searches for immunogenic proteins. Fifty years ago, Segal and Bloch noted that *M. tuberculosis* grows in vivo by metabolizing fatty acids as opposed to carbohydrates in vitro (7, 30). The *M. avium* subsp. *paratuberculosis* membrane proteins FadE_2, InhA, FadE23, FadA1, FadE5, and FadB1 and cytosolic proteins FadE25_2 and FabG5_2 are all involved in fatty acid metabolism as determined by homology to proteins in the KEGG database (19, 20). All eight of these proteins were expressed at significantly higher levels in *M. avium* subsp. *paratuberculosis* clinical isolate 187 compared to in vitro-adapted *M. avium* subsp. *paratuberculosis* strain K-10. Laboratory medium for cultivation of *M. avium* subsp. *paratuberculosis* is supplemented with fatty acids for optimal growth. It has been shown that *M. avium* subsp. *paratuberculosis* cultured in medium lacking fatty acids shows distinct morphological changes, reduced resistance to acid conditions, and altered protein expression (35). These data are also indicative that these proteins are important for the survival of *M. avium* subsp. *paratuberculosis* within the host, enabling optimal assimilation of fatty acids for generation of the cell wall and cellular membrane. Consequently, the proteins involved may be missed or underrepresented during screening of in vitro-adapted *M. avium* subsp. *paratuberculosis* to obtain potential targets for identification of *M. avium* subsp. *paratuberculosis*-infected animals.

The RNA polymerase proteins RpoA and RpoB were both expressed at higher levels in strain K-10 compared to strain 187. RpoB, in particular, has been intensely scrutinized in *M. tuberculosis* as mutations in RpoB confer resistance to the antibiotic rifampin (13, 36). With a relative expression of RpoB greater than sixfold higher in strain 187 than in the laboratory-adapted strain, RpoB may prove an interesting candidate for study in *M. avium* subsp. *paratuberculosis*. Whether this represents an adaptation toward optimized growth in the laboratory strain remains to be investigated. GlnB, GlnD, and the nifs-like protein are all involved in nitrogen metabolism and were found at higher relative abundance in strain K-10 compared to strain 187, perhaps a reflection of a readily assessable nitrogen source for growth (18, 34). However, this indicates once again that strain-specific protein expression differences may also lead to an overemphasis on proteins that could be laboratory growth artifacts.

Although a necessary cofactor, excess iron can lead to oxi-

dative stress in bacteria (9). It is thought that in response to excess iron, bacteria may induce the synthesis of proteins involved in iron binding in order to reduce iron-mediated oxidative damage (31). *M. tuberculosis* has more than 40 enzymes for which iron is a necessary cofactor (40). Although several iron-binding proteins were identified in the present survey, only FecB was expressed at a higher level in the strain K-10 membrane fraction. Alkyl hydroperoxide reductase C (AhpC) reduces organic hyperoxides and was present at threefold higher levels in the K-10 membrane fraction. Together, these two proteins may represent part of the adaptation response of *M. avium* subsp. *paratuberculosis* strain K-10 to oxidative stress (12). Additionally, AhpC has proven useful as an immunological identifier to distinguish *M. avium* from *M. avium* subsp. *paratuberculosis*, as antibodies against AhpC were found in the sera of *M. avium* subsp. *paratuberculosis*-infected goats (27). However, if field strains of *M. avium* subsp. *paratuberculosis* show lower expression of the protein in cattle, this may limit the usefulness of AhpC to screen for infection.

The results from this study demonstrate the potential for analyzing relative protein levels from large data sets. In addition to being the most comprehensive proteome to date for *M. avium* subsp. *paratuberculosis*, 451 hypothetical proteins inferred from the nucleotide sequence have been identified. Proteins expressed at relatively higher levels in the low-passage strain may prove useful in the development of diagnostic techniques, while proteins expressed at relatively higher levels in the high-passage strain need to be analyzed with that in mind. Although the present study is based upon a limited number of high- and low-passage *M. avium* subsp. *paratuberculosis* strains, we have identified differences in relative protein levels that both confirm expectations and challenge previous findings. It is clear that laboratory adaptation of *M. avium* subsp. *paratuberculosis* through continuous passage in complete medium will alter the growth pattern and protein expression profile, but how this directly affects the virulence of the bacterium remains to be tested. The broad scope of proteomic analysis coupled with the added power of direct comparison of individual proteins holds the potential for applications similar to that found in nucleic acid chip technology.

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