

Surface Proteome of “*Mycobacterium avium* subsp. *hominissuis*” during the Early Stages of Macrophage Infection

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“*Mycobacterium avium* subsp. *hominissuis*” is a robust and pervasive environmental bacterium that can cause opportunistic infections in humans. The bacterium overcomes the host immune response and is capable of surviving and replicating within host macrophages. Little is known about the bacterial mechanisms that facilitate these processes, but it can be expected that surface-exposed proteins play an important role. In this study, the selective biotinylation of surface-exposed proteins, streptavidin affinity purification, and shotgun mass spectrometry were used to characterize the surface-exposed proteome of *M. avium* subsp. *hominissuis*. This analysis detected more than 100 proteins exposed at the bacterial surface of *M. avium* subsp. *hominissuis*. Comparisons of surface-exposed proteins between conditions simulating early infection identified several groups of proteins whose presence on the bacterial surface was either constitutive or appeared to be unique to specific culture conditions. This proteomic profile facilitates an improved understanding of *M. avium* subsp. *hominissuis* and how it establishes infection. Additionally, surface-exposed proteins are excellent targets for the host adaptive immune system, and their identification can inform the development of novel treatments, diagnostic tools, and vaccines for mycobacterial disease.

The characterization of the surface-exposed proteome of a cell provides key insights into its nature. Surface-exposed proteins play a fundamental role in both how a cell interacts with its environment and how it is perceived by other cells. For infectious bacteria, surface proteins are essential components of many aspects of pathogenesis (47, 48). Host specificity, adhesion, invasion, molecular transport, and antimicrobial resistance are processes known to be directly mediated by proteins present at the surface interface (16, 17, 50, 81). These proteins are also the primary target of the host immune system. Effective cellular and humoral immune responses depend on targeting accessible molecules, which tend to be on the surface of a pathogen (87). With respect to mycobacteria, putative and known surface-exposed proteins comprise a substantial proportion of antigens observed in comprehensive screens using sera from mycobacterium-infected hosts (37, 42, 45, 85). The characterization of these proteins improves our understanding of bacterial pathogenesis and host immunity, providing insights that can lead to new diagnostic tools and vaccines (38, 87).

“*Mycobacterium avium* subsp. *hominissuis*” is a common environmental pathogen and a major source of disseminated mycobacterial disease in immune-compromised individuals (11). It is closely related to another pathogenic member of the *Mycobacterium avium* complex (MAC), *Mycobacterium avium* subsp. *paratuberculosis*. *M. avium* subsp. *hominissuis* also shares many genetic and structural features with its more virulent relatives, *Mycobacterium tuberculosis* and *Mycobacterium marinum*. Capable of surviving within a hijacked phagosome, *M. avium* subsp. *hominissuis* replicates within host macrophages (15). Like other pathogenic mycobacteria, *M. avium* subsp. *hominissuis* interferes with the typical process of vacuole maturation and establishes a stable niche. It is likely that, along with fully secreted molecules, proteins expressed on the bacterial surface play a role in the intracellular processes associated with survival and persistence. Despite residing within a vacuole for the majority of the intracellular phase of infection, very little is known about the interaction between *M.*

avium subsp. *hominissuis* and the host phagosome. Possible interference with the activation of toll-like receptors (TLRs), inactivation of host antimicrobial responses, and modulation of molecular transport into and out of the vacuole are all functions that may be mediated by surface proteins (19).

In this study, we employed an emerging technological approach to characterize the surface-exposed proteome of *M. avium* subsp. *hominissuis* that was cultured under conditions simulating early infection. The combination of the selective biotinylation of surface-exposed proteins, affinity purification, and so-called shotgun mass spectrometry is a powerful tool that can generate a snapshot of the surface proteome of a cell (20, 49, 70). While this general approach has been applied to a range of eukaryotic and prokaryotic cells, including several species of pathogenic bacteria (33, 73, 82), it has not been used to characterize the surface proteome in mycobacteria. In this report, we harness the technology to analyze the surface-exposed proteome of *M. avium* subsp. *hominissuis*, focusing on the earliest stages of macrophage infection. Combining all experimental conditions, our results identified more than 100 proteins from the surface of *M. avium* subsp. *hominissuis*. Within this total population, our analysis identified a set of core proteins that appeared to be constitutively abundant on the bacterial surface, as well as several sets of proteins whose surface exposure appears to be dependent on the experimental condition. The comparison of these results with previously reported

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surface-exposed proteome data from other mycobacterial species revealed that the majority of the proteins identified in this study have homologs across the genus that are known to be secreted, surface-exposed, or cell-wall-associated proteins. Furthermore, the proteins identified in this screen are disproportionately represented among the population of proteins that have previously been identified as dominant antigens in mycobacterial infection. This characterization of the *M. avium* subsp. *hominissuis* surface-exposed proteome builds upon previous reports and significantly expands our understanding of the proteins that mediate the earliest stages of *M. avium* subsp. *hominissuis* pathogenesis.

MATERIALS AND METHODS

Preparation of macrophage and *M. avium* subsp. *hominissuis* cultures.

RAW 264.7 cells (a mouse macrophage cell line) were purchased from ATCC (Manassas, VA). Adherent RAW 264.7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum, in 300-cm² glass trays, to a confluence of ~50%. Prior to the start of the experiment, *M. avium* subsp. *hominissuis* strain 109 (a clinical isolate from the blood of an HIV/AIDS patient) was cultured in 200 ml of Middlebrook 7H9 broth (Difco, Sparks, MD) supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) (Hardy Diagnostics, Santa Maria CA) broth at 37°C for 4 days with constant agitation, until the optical density at 600 nm (OD₆₀₀) was approximately 1. To initiate the experiment, bacteria were harvested by centrifugation at 4,000 × *g* and washed twice with Hank's buffered salt solution (HBSS) (Invitrogen, Carlsbad, CA). Bacteria were resuspended in 40 ml of HBSS and separated into eight equal (50-ml) aliquots.

Experimental culture conditions. For medium-only experimental conditions (Middlebrook 7H9 broth and DMEM), the 5-ml aliquot was added to 30 ml fresh medium in a 50-ml tube and placed in an incubator at 37°C with gentle shaking. For macrophage-exposed bacteria, each aliquot of washed bacteria was split among four trays of macrophages for a final multiplicity of infection (MOI) of ~10. Samples were harvested at 24 and 48 h postinfection. Extracellular bacteria (ECB) were isolated from infected macrophage cultures first. For the purposes of this study, ECB are defined as *M. avium* subsp. *hominissuis* incubated with macrophage cells but not phagocytosed. To isolate ECB, infected macrophage cultures were washed with HBSS three times to remove any bacteria that were not inside adherent cells. This wash solution was combined and centrifuged (1,500 × *g* for 10 min) to pellet the bacteria and any nonadherent macrophage cells. Nonadherent macrophages were then pelleted by low-speed centrifugation (100 × *g* for 2 min). Very few nonadherent macrophage cells were observed in intracellular *M. avium* subsp. *hominissuis* bacteria (ICB) samples from either time point. ICB samples then were isolated from infected macrophages. For the purpose of this study, ICB were defined as *M. avium* subsp. *hominissuis* cells that were incubated with and phagocytosed by the cultured macrophages. To isolate ICB, the infected macrophages were incubated for 5 min in cold differential lysis buffer (DLB; 90% H₂O, 9.8% [vol/vol] glycerol, 0.1% [vol/vol] Triton X-100, 0.1% [vol/vol] Tween 20). This buffer disrupts the membranes of RAW 264.7 cells but not those of the *M. avium* subsp. *hominissuis*, which are shielded by the robust mycobacterial cell envelope. The cells were scraped, completely removing all material, and combined into 50-ml centrifuge tubes. The samples were shaken and centrifuged at 1,500 × *g* for 15 min. This centrifugation collected both the bacteria and significant cell debris. The pellet was resuspended in 5 ml and transferred to clean tubes. Bacteria were isolated from cell debris by differential centrifugation in DLB. First, the majority of large cell debris was pelleted by low-speed centrifugation (100 × *g* for 2 min). Supernatant (containing bacteria in suspension) was transferred to clean tubes. This sequence of low- and high-speed centrifugation was repeated an additional two times. Bacteria were pelleted by centrifugation (4,000 × *g* for 5 min), and supernatant was discarded. Bacteria then were washed twice with BupH-PBS (150 mM NaCl, 100 mM

Na₂HPO₄, pH 7.3) prior to biotin labeling. Samples from medium-only culture conditions were collected by centrifugation and washed in the same fashion.

Biotin labeling and purification of *M. avium* subsp. *hominissuis* surface proteins. Bacterial pellets were resuspended in 1 ml of BupH-PBS. To biotinylated surface-exposed proteins, 500 μl of Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) was added at a concentration of 1 mg/ml in BupH-PBS to each sample. Bacteria were biotin labeled for 20 min at 23°C with gentle agitation. After labeling, bacteria were washed twice with BupH-PBS supplemented with glycine (10 mg/ml) and two times with plain BupH-PBS to inactivate and remove any unbound Sulfo-NHS-LC-Biotin. Labeled bacteria were resuspended in urea lysis buffer (ULB; 140 mM NaCl, 20 mM Na₂HPO₄, 7 M urea, 0.05% [vol/vol] Tween 20, 0.1% [wt/vol] deoxycholic acid, pH 7.2) and disrupted by bead milling with 100-μm glass beads (Sigma, St. Louis, MO). After disruption, samples were centrifuged (12,000 × *g* for 10 min) to remove particulates and other insoluble components, and the supernatant was transferred to a clean tube to be used for purification. Prior to affinity capture, samples were diluted 1:3 with WB-PBS (140 mM NaCl, 20 mM Na₂HPO₄, 0.05% [vol/vol] Tween 20, pH 7.2). Samples in this adjusted buffer, termed urea incubation buffer (UIB; 140 mM NaCl, 20 mM Na₂HPO₄, 1.75 M urea, 0.05% [vol/vol] Tween 20, 0.025% [wt/vol] deoxycholic acid, pH 7.2), were used for streptavidin affinity purification. Biotinylated protein was purified from total protein by affinity purification with magnetic streptavidin-coated C1 Dynabeads (Invitrogen). Briefly, aliquots of 1.5 ml of biotinylated protein solution was incubated for 30 min at 23°C with 80 μl of C1 Dynabeads. Samples were washed three times with 1 ml of UIB, twice with 1 ml WB-PBS, and once with 1 ml of ammonium bicarbonate buffer (ABB) (50 mM NH₄HCO₃, pH 7.8). Samples were resuspended in 50 μl ABB and digested with trypsin gold (1 μg; protease-to-substrate ratio of ~1:20) and ProteaseMAX according to the manufacturer's instructions (Promega, Madison, WI).

Preparation of negative controls. To eliminate nonspecific background and endogenously biotinylated proteins, samples of *M. avium* subsp. *hominissuis* were isolated from the conditions described above for use as negative controls. These samples were processed in the manner described above, except that Sulfo-NHS-LC-Biotin was not added. Data from negative controls were pooled to create a master list of false-positive identifications, and these proteins were then subtracted from the experimental data sets. The negative-control master list is included in Table S2 in the supplemental material.

Peptide purification, proteolysis, and LC-MS/MS analysis. Following enzymatic proteolysis, magnetic stands were used to remove the beads from the completed digests. Peptides from the resulting supernatant were purified and desalted on Vivapure C₁₈ microspin columns according to the manufacturer's instructions (Sartorius, Goettingen, Germany). After purification, peptides were dehydrated by speed vacuum and resuspended to a final concentration of approximately 500 ng/μl in mass spectrometry (MS) loading buffer (95% H₂O, 5% [vol/vol] acetonitrile [ACN], 0.1% [vol/vol] formic acid). Data-dependent liquid chromatography-tandem MS (LC-MS/MS) analyses were performed on an LTQ-FT Ultra mass spectrometer with an IonMax ion source (Thermo, West Palm Beach, FL) coupled to a nanoAcquity Ultra performance LC system (Waters, Milford, MA) equipped with a Michrom peptide CapTrap column and a C₁₈ column (Zorbax 300SB-C18; 250 by 0.3 mm; 5-μm volume; Agilent). A binary gradient system was used consisting of solvent A (0.1% aqueous formic acid) and solvent B (ACN containing 0.1% [vol/vol] formic acid). Two μl of C₁₈ column-purified peptides was trapped and washed with 3% solvent B at a flow rate of 5 μl/min for 3 min. Trapped peptides were then eluted into an analytical column using a linear gradient from 3% B to 30% B at a flow rate of 4 μl/min for 35 min. The column was maintained at 37°C during the run. The mass spectrometer was operated in a data-dependent acquisition mode. A full FT-MS scan (*m/z* 350 to 2,000) was alternated with collision-induced dissociation (CID) MS/MS scans of the 5 most abundant doubly or triply charged precursor ions. As the survey

scan was acquired in the ion cyclotron resonance (ICR) cell, the CID experiments were performed in the linear ion trap, where precursor ions were isolated and subjected to CID in parallel with the completion of the full FT-MS scan. CID was performed with helium gas at a normalized collision energy of 35% and an activation time of 30 ms. Automated gain control (AGC) was used to accumulate sufficient precursor ions (target value, 5×10^4 /microscan; maximum fill time, 0.2 s). Dynamic exclusion was used with a repeat count of 1 and exclusion duration of 60 s. Data acquisition was controlled by Xcalibur (version 2.0.5) software (Thermo Scientific).

Database search. Thermo Scientific data files were processed with Proteome Discoverer, version 1.2, using default parameters. A Mascot (version 2.2.04) search against the whole Swiss-Prot 2010 database (523,151 sequences; 184,678,199 residues) or an *M. avium* subsp. *hominissuis* (strain 104) database (obtained from UniProt; 5,040 sequences; 1,586,464 residues) was launched from Proteome Discoverer with the following parameters. The digestion enzyme was set to Trypsin/P, and two missed cleavage sites were allowed. The precursor ion mass tolerance was set to 5 ppm, while a fragment ion tolerance of 0.8 Da was used. Dynamic modifications included carbamidomethyl (+57.0214 Da) for Cys, oxidation (+15.9994 Da) for Met, and LC-Biotin (+339). Data from two experimental replicates were combined by MudPIT (multidimensional protein identification technology), and identified proteins from each sample were summarized with Scaffold 3 software (Proteome Software, Portland, OR). The inclusion of a protein in the final data set required that two or more unique peptides for that protein could be identified in at least one experimental condition. Only peptides that were identified with a confidence of greater than 50% were utilized to calculate the overall probability of protein identification.

Sequential fractionation and Western blotting. To assess the solubility of biotin-labeled proteins, three test samples (one negative-control and two experimental samples [DMEM and 7H9]) were subjected to sequential fractionation. Briefly, samples were prepared, washed, and disrupted as described above. Each extraction was repeated twice. First, BupH-PBS-soluble proteins were extracted, and the remaining material (insoluble in BupH-PBS) was pelleted by centrifugation at $12,000 \times g$ for 20 min. Pellets were then resuspended in ULB and incubated at 37°C for 10 min, with vigorous agitation. Soluble proteins were again separated by centrifugation ($12,000 \times g$ for 20 min), and the supernatant (containing ULB-soluble proteins) was transferred to a clean tube. The remaining pellets were resuspended in Laemmli SDS-PAGE buffer and heated to 95°C for 10 min. Protein concentrations were equilibrated between samples using the Bradford assay (Bio-Rad, Hercules, CA), and the proteins were separated by SDS-PAGE. Following SDS-PAGE separation, proteins were electrophoretically transferred to nitrocellulose membranes in preparation for Western blot analysis. IRDye-680 streptavidin (Licor, Lincoln, NE) was used to probe membranes by following the manufacturer's protocol. Biotinylation patterns were visualized on an Odyssey scanner (Licor).

Functional classification of identified proteins. Where a direct homolog between an identified *M. avium* subsp. *hominissuis* protein and its counterpart in *M. tuberculosis* existed, functional annotations were drawn from the Tuberculist database (44). For the purposes of this study, direct homologs were defined as those proteins having greater than 60% identity. Where a direct homolog in *M. tuberculosis* was absent, annotations were assigned based on BLAST and literature searches. The presence and identification of the direct homologs for the *M. avium* subsp. *hominissuis* proteins identified in this study are detailed in Table S1 in the supplemental material.

RESULTS

Selective biotinylation of surface-exposed proteins. The membrane impermeability and selective labeling of surface proteins with Sulfo-NHS-LC-Biotin has been demonstrated in a wide range of prokaryotic and eukaryotic cells (20, 24, 33). Because of the extensive washing required by the methodology used in this

study, the detected proteins were expected to be firmly associated with the bacterial cell envelope. To confirm the selective biotinylation of surface-exposed proteins, samples of Sulfo-NHS-LC-Biotin-labeled *M. avium* subsp. *hominissuis* were subjected to sequential fractionation by solubility, and the resulting fractions were analyzed by anti-biotin Western blotting (Fig. 1A). Most cytoplasmic proteins are expected to be soluble in BupH-PBS, while most membrane and cell wall-associated proteins are expected to be solubilized in ULB, which contains detergents and urea. The remaining, insoluble fraction was expected to be enriched in proteins that are highly hydrophobic, posttranslationally modified, tightly complexed with nonsoluble components, or otherwise resistant to solubilization. The results of this sequential fractionation demonstrate that most of the biotin-labeled proteins are indeed solubilized only with the addition of urea and detergent (Fig. 1). In contrast, relatively few proteins in the BupH-PBS and insoluble fractions were labeled with biotin. However, the presence of some biotinylated protein in the remaining insoluble fraction indicates that some surface-exposed proteins were not effectively extracted by the methods used in this study. Overall, the enrichment of biotinylated proteins in the ULB-soluble fraction suggests that the majority of biotin-labeled proteins are hydrophobic proteins that are membrane or cell wall associated, and that cytoplasmic *M. avium* subsp. *hominissuis* proteins were not significantly biotinylated.

Total protein identification. In total, our analysis identified in excess of 100 putative surface-exposed proteins (see Table S1 in the supplemental material). The identified proteins were distributed among a variety of functional classifications (Fig. 2A). Many previously described surface-exposed or secreted antigens of mycobacteria were identified in this study, including the antigen 85 complex (*ag85A*, locus tag MAV_0214; *ag85C*, MAV_0215; and *ag85B*, MAV_2816), heparin-binding hemagglutinin (*hbha*, MAV_4675), and superoxide dismutase (*sodA*, MAV_0182). The predominant functional group among the proteins detected in this study are categorized by Tuberculist as "Intermediary Metabolism and Respiration," which accounted for 34% of all identified proteins. As expected, proteins that are predicted to be involved in cell wall biogenesis and maintenance were well represented, comprising 14% of the total. An additional 14% of the total was represented by proteins with putative roles in lipid metabolism, many of which are also expected to have functions associated with the development of the mycobacterial cell envelope. Approximately 9% of the identified proteins are classified by Tuberculist as virulence related. These included multiple proteins that participate in the response to stress and host oxidative responses, such as catalase (*katG*, MAV_2753), alkylhydroperoxide reductase (*ahpC*, MAV_2839), and universal stress family proteins (MAV_2506 and MAV_3137). Several proteins with putative nucleotide binding functions, including multiple ribosomal proteins, were also identified. A large number of ribosomal proteins also were detected in negative controls, which indicates that at least some of the observed ribosomal proteins are persistent contaminants (see Table S2 in the supplemental material).

To assess the relevance of these results, the proteins identified in this screen were cross-referenced against the surface-exposed proteins identified in two published analyses of related mycobacterial species (32, 74). Unlike the selective labeling and affinity purification method employed here, the previous work employed either trypsin shaving or cell envelope solubilization to selectively

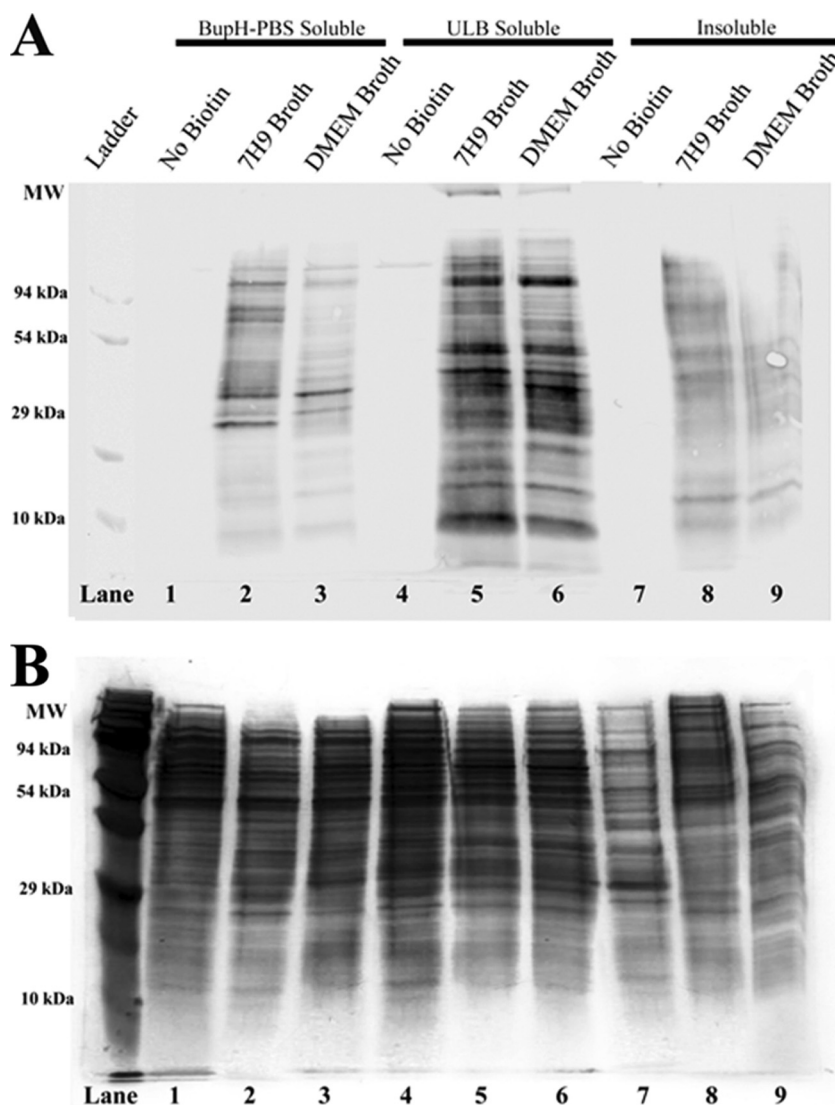


FIG 1 Biotin labeling of *M. avium* subsp. *hominissuis* proteins separated by solubility. Protein was analyzed from three *M. avium* subsp. *hominissuis* samples, one negative control (not biotinylated) and two experimental samples (biotinylated). Protein was isolated on the basis of solubility in three consecutive extractions. The first extraction was performed in BupH-PBS and isolated primarily PBS-soluble proteins that are expected to be soluble in native conditions (lanes 1 to 3). The pellet remaining from the first extraction was resuspended in ULB, which was expected to solubilize most of the membrane and cell envelope-associated proteins (lanes 4 to 6). The pellet remaining after the extraction of ULB-soluble proteins was resuspended in Laemmli buffer and heated to 95°C for 10 min to extract as much of the remaining protein as possible (lanes 7 to 9). (A) Anti-biotin Western blotting indicates that the majority of the biotin-labeled protein was present in the ULB fraction. Some signal was detected in the insoluble fraction, indicating that some surface-exposed proteins were unidentifiable in this study. The negative control shows minimal staining, suggesting low levels of endogenous biotinylation. (B) Silver-stained SDS-PAGE gel of the same protein samples.

isolate and identify proteins and peptides from the surface of several species of *Mycobacterium*, including *M. avium* subsp. *paratuberculosis*, *M. smegmatis*, and *M. tuberculosis*. The comparison between the findings of the three studies reveals considerable overlap between the previously identified mycobacterial proteins and the *M. avium* subsp. *hominissuis* surface-exposed proteins observed in this study (Fig. 2B; also see Table 4). Approximately 57% (21/37) of the surface-exposed proteins identified in *M. avium* subsp. *paratuberculosis* were also detected in this study (32). Similar results were observed for *M. tuberculosis* and *M. smegmatis*, 52% (13/25) and 56% (14/25), respectively (74). Interestingly, while there was a high degree of similarity between this study and the previous studies, there was substantially less overlap between

these studies themselves (Fig. 2B). This discrepancy, which is explored more thoroughly in the discussion, likely stems from critical differences between the methods used to isolate proteins for analysis. Beyond the comparisons visualized in Fig. 2, if the scope of reference is expanded to include proteins identified in published studies that comprehensively profile membrane, cell wall, and secreted proteins of *M. marinum*, *M. avium* subsp. *paratuberculosis*, and *M. tuberculosis*, the results indicate that ~90% of the proteins detected in this study are close homologues of proteins previously identified as plasma membrane, secreted, and/or cell wall-associated mycobacterial proteins (30, 32, 51, 54, 84, 87) (see Table S1 in the supplemental material).

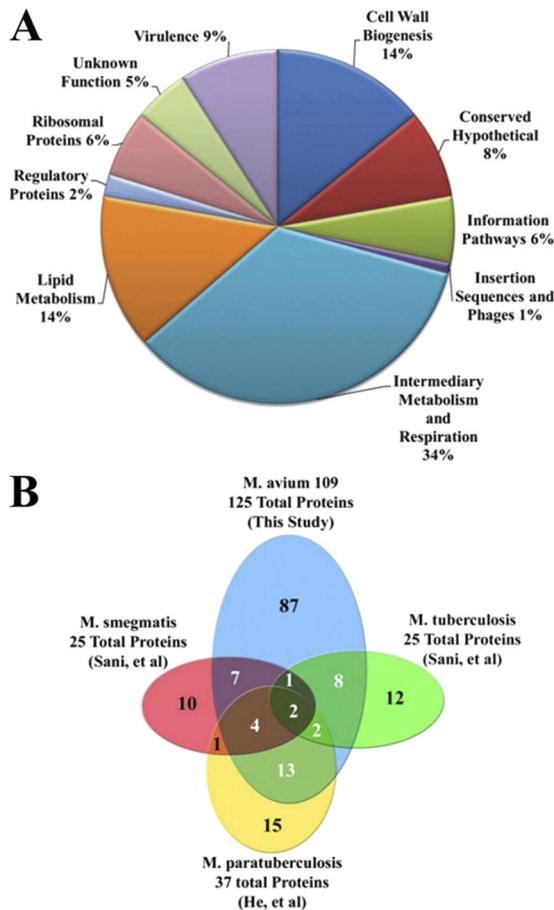


FIG 2 Functional grouping and interstudy cross-referencing of identified surface-exposed proteins. (A) Distribution of functional annotations of identified *M. avium* subsp. *hominissuis* surface-exposed proteins. Direct homologues of all *M. avium* subsp. *hominissuis* proteins identified in this study were identified for *M. tuberculosis*, *M. avium* subsp. *paratuberculosis*, *M. smegmatis*, and *M. marinum* (see Table S1 in the supplemental material). Functional classification for identified *M. avium* subsp. *hominissuis* proteins was assigned, where applicable, by using the annotated functional grouping of the *M. tuberculosis* homolog in the Tuberculist database. (B) Venn diagram illustrating overlap between surface proteins identified in published studies of the surface-exposed proteomes of *M. avium* subsp. *paratuberculosis*, *M. smegmatis*, and *M. tuberculosis* and the data presented here for *M. avium* subsp. *hominissuis*. The data sets for *M. smegmatis* and *M. tuberculosis* employed selective solubilization to isolate surface proteins and included only the 25 most abundant surface proteins. The data for *M. avium* subsp. *paratuberculosis* employed a trypsin-shaving approach and included all confidently identified surface proteins (30–32, 51, 54, 74, 84).

Core surface proteins of *M. avium* subsp. *hominissuis*. Expanding on the primary goal of characterizing the overall surface-exposed proteome of *M. avium* subsp. *hominissuis*, another objective of this study was to identify major changes within this subproteome that may occur during the initial stages of macrophage infection. While it is known that mycobacteria adapt to pathogenic growth conditions by modulating several aspects of their transcriptome and proteome (1, 40, 58, 84), the scope of this remodeling is poorly understood in *M. avium* subsp. *hominissuis*. The Western blot analysis of the biotinylation pattern of total protein from each experimental condition revealed similar overall patterns of labeled proteins between samples (Fig. 3). The lack of a

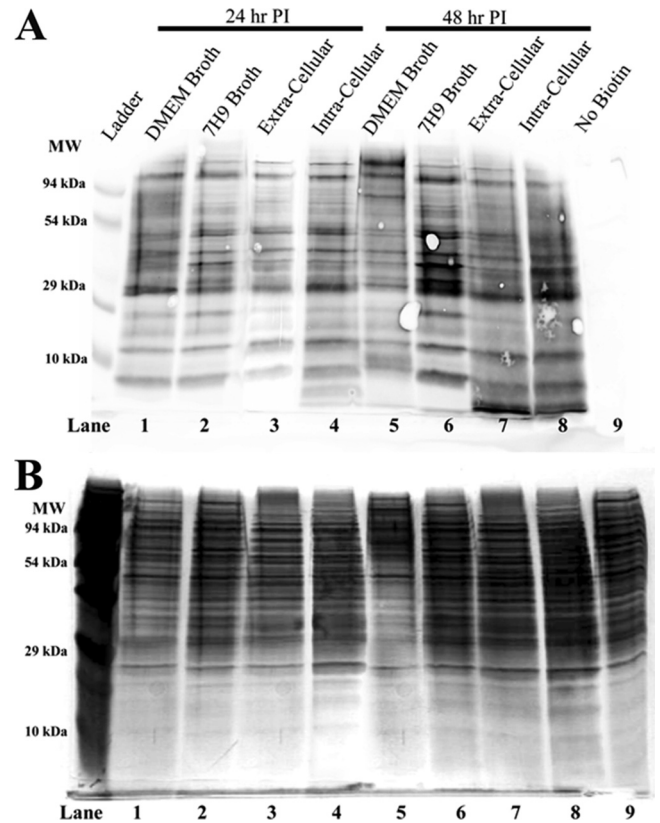


FIG 3 Comparison of biotinylation profiles from all experimental samples. (A) Biotinylation profiles of total protein from each experimental condition. To assess changes in global biotinylation patterns between conditions, aliquots of total protein were analyzed from each sample prior to affinity purification. Samples were from both time points, 24 h (lanes 1 to 4) and 48 h (lanes 5 to 9), and all culture conditions, DMEM (lanes 1 and 5), 7H9 medium (lanes 2 and 6), ECB (lanes 3 and 8), and ICB (lanes 4 and 9). Biotinylation profiles were analyzed by anti-biotin Western blotting. (B) Silver-stained SDS-PAGE gel of the same protein samples.

major remodeling of the surface proteome at the early time points assayed in this study is not particularly surprising due to the relatively low growth rate of *M. avium* subsp. *hominissuis*. The anti-biotin Western blotting results are also consistent with the mass spectrometry data, which indicate that many of the proteins identified in our analysis are present on the surface of *M. avium* subsp. *hominissuis* in most, if not all, of the experimental conditions. These consistently detected proteins may represent a set of core proteins which are likely to be highly abundant, easily detected, and constitutively expressed (Table 1). For the purposes of this study, core proteins were defined as proteins that were detected in all eight of the conditions tested. Approximately 19% (24/125) of the proteins detected in this study met this standard. As expected, a relatively high percentage of these universally detected proteins (42%; 10/24) were also observed on the surface of *M. avium* subsp. *paratuberculosis*, *M. tuberculosis*, and/or *M. smegmatis* (Table 1). In contrast to these core proteins, among the remaining *M. avium* subsp. *hominissuis* proteins, which may be less abundant or variably expressed, the percentage of overlapping identifications between studies was reduced (26%; 26/101).

The distribution of functional annotations among the core proteins was similar to the distribution observed for the complete

TABLE 1 *M. avium* subsp. *hominissuis* 109 surface-exposed proteins detected in all 8 experimental conditions

UniProt accession no.	EMBL annotation	MAV locus tag	No. of unique peptides found according to culture conditions and time point (h)							
			7H9 medium		DMEM		Extra-cellular		Intra-cellular	
			24	48	24	48	24	48	24	48
A0Q988_MYCA1	Superoxide dismutase	MAV_0182	4	4	5	4	3	2	4	3
CH602_MYCA1	60-kDa chaperonin 2	MAV_4707	18	11	19	17	7	10	10	12
A0QHU7_MYCA1	Aconitate hydratase 1	MAV_3303	9	5	7	5	9	8	6	2
A0QHY5_MYCA1	Glyceraldehyde-3-phosphate dehydrogenase	MAV_3341	7	7	8	8	6	9	3	6
A0QGG5_MYCA1	Antigen 85-B	MAV_2816	4	5	3	2	2	3	4	2
EFTU_MYCA1	Elongation factor Tu	MAV_4489	9	7	13	11	9	10	4	8
A0QI11_MYCA1	LprG protein	MAV_3367	2	1	2	1	2	3	2	1
A0QM99_MYCA1	Short-chain dehydrogenase/reductase	MAV_4916	6	8	3	3	2	1	4	5
A0QER4_MYCA1	Acyl carrier protein	MAV_2193	1	2	4	2	3	1	2	2
PGK_MYCA1	Phosphoglycerate kinase	MAV_3340	3	4	2	1	3	1	1	1
MDH_MYCA1	Malate dehydrogenase	MAV_1380	3	4	5	4	5	2	1	2
A0QME6_MYCA1	Phosphoenolpyruvate carboxykinase	MAV_4963	2	2	2	2	4	3	1	2
A0QLN4_MYCA1	Putative uncharacterized protein	MAV_4695	3	3	2	1	3	1	1	2
A0QEY3_MYCA1	Glutamine synthetase	MAV_2267	2	3	1	2	4	2	1	2
A0QMX5_MYCA1	Peroxisomal multifunctional enzyme type 2	MAV_5146	6	2	5	4	8	2	6	2
A0QMX6_MYCA1	Aldehyde dehydrogenase	MAV_5147	6	5	3	1	2	1	4	1
ATPA_MYCA1	ATP synthase subunit alpha	MAV_1525	7	9	6	5	1	7	5	14
ATPB_MYCA1	ATP synthase subunit beta	MAV_1527	10	11	10	10	4	8	1	10
A0QL09_MYCA1	30S ribosomal protein S17	MAV_4462	5	2	4	2	3	1	3	1
RS20_MYCA1	30S ribosomal protein S20	MAV_1770	4	3	3	1	3	3	2	4
A0QJ28_MYCA1	30S ribosomal protein S2	MAV_3744	2	1	1	1	1	1	1	2
RL19_MYCA1	50S ribosomal protein L19	MAV_3759	1	1	2	2	2	1	1	1
RL21_MYCA1	50S ribosomal protein L21	MAV_1729	2	1	1	2	2	1	1	1
RPOB_MYCA1	DNA-directed RNA polymerase subunit beta	MAV_4503	3	2	1	1	1	2	1	2

set of detected proteins. Proteins classified as intermediary metabolism and respiration continued to be the most populous, representing approximately 42% of the total (10/24). Multiple proteins with putative roles in lipid metabolism and cell wall biogenesis were detected under all conditions, such as antigen 85-B (*ag85B*, MAV_2816) and lipoprotein G (*lprG*, MAV_3367). Also represented among the identified core proteins are proteins that are implicated in defense, stress response, and virulence, such as superoxide dismutase (*sodA*) and the 60-kDa chaperonin 2 (*groEL2*, MAV_4707). Several unexpressed proteins were identified under all conditions, including elongation factor Tu (*tuf*, MAV_4489) and several ribosomal proteins. In most bacteria, elongation factor Tu serves a proof-reading function during ribosomal protein synthesis, a process which normally takes place in the cytoplasmic compartment (75). However, both elongation factor Tu and a range of ribosomal proteins have been consistently identified in studies of surface-exposed proteins in mycobacteria and related Gram-positive bacteria (1, 5, 32, 74, 80, 87).

Surface proteins uniquely detected after contact with macrophages. In addition to the core proteins, many additional surface-exposed proteins were identified that appeared to be differentially regulated in response to changes in culture conditions or following exposure to macrophage cells. One such subgroup is comprised of proteins whose detection appeared to be dependent on the exposure of the bacterium to macrophages (Table 2). Four proteins were detected in *M. avium* subsp. *hominissuis* that had

been exposed to macrophage cultures but were not detected in medium-only culture conditions. These proteins were alkylhydroperoxide reductase (*ahpC*, MAV_2839), isocitrate lyase (*aceA*, MAV_4682), a universal stress family protein (MAV_2506), and 5-methyl-tetrahydropteroyltrimethylglutamate-homocysteine methyltransferase (*metE*, MAV_1262) (Table 2). At least two of these genes, *ahpC* and *aceA*, are known to be important mycobacterial persistence factors that are upregulated during mycobacterial infection (22, 56, 59).

In contrast to the proteins detected only after exposure to macrophages, several additional proteins appeared to be uniquely absent from the surface of *M. avium* subsp. *hominissuis* following exposure to macrophages (Table 2). These proteins included a putative adhesion protein (*modD*, MAV_2859), a universal stress family protein (MAV_3137), a putative transposase (MAV_4302), and four uncharacterized proteins (MAV_4070, MAV_4583, MAV_4436, and MAV_3813). The best characterized of these proteins, *modD*, belongs to a family of alanine- and proline-rich proteins that have been described as surface exposed and highly immunogenic (26, 43). In *M. tuberculosis*, the *modD* gene is upregulated in response to nutrient deprivation, although the specific nutrients that influence expression remain undefined (8). In *M. smegmatis*, a close homologue of MAV_3137 has been identified as an abundant surface-exposed protein (74). Universal stress family proteins are a family of at least 10 proteins, many of which are differentially regulated during environmental stress and infection. The *M. avium*

TABLE 2 *M. avium* subsp. *hominissuis* 109 surface-exposed proteins differentially detected after contact with macrophages

Uniprot accession no. and protein detection status ^a	EMBL annotation	MAV locus tag	No. of unique peptides found according to medium and time point (h)							
			7H9 Medium		DMEM		Extra-cellular		Intra-cellular	
			24	48	24	48	24	48	24	48
Unique										
A0QGI8_MYCA1	Alkylhydroperoxide reductase	MAV_2839	0	0	0	0	0	2	4	3
A0QLM2_MYCA1	Isocitrate lyase	MAV_4682	0	0	0	0	1	2	1	4
A0QFL0_MYCA1	Universal stress protein family protein	MAV_2506	0	0	0	0	0	1	0	2
METE_MYCA1	5-Methyl-tetrahydropteroyltrimethylglutamate-homocysteine methyltransferase	MAV_1262	0	0	0	0	0	3	0	2
Not unique										
A0QGK7_MYCA1	ModD protein	MAV_2859	6	6	5	2	3	0	3	0
A0QJY3_MYCA1	Putative uncharacterized protein	MAV_4070	2	1	2	1	0	0	0	0
A0QLC4_MYCA1	Putative uncharacterized protein	MAV_4583	2	3	1	1	1	0	0	0
A0QHD3_MYCA1	Universal stress protein family protein	MAV_3137	2	1	3	2	1	0	3	0
A0QKY3_MYCA1	Putative uncharacterized protein	MAV_4436	1	1	2	1	0	1	0	0
A0QJ96_MYCA1	Putative uncharacterized protein	MAV_3813	1	2	1	1	0	1	0	0
A0QKK1_MYCA1	Putative transposase	MAV_4302	1	3	2	1	1	0	2	0

^a Unique refers to proteins that were uniquely detected after macrophage contact.

subsp. *hominissuis* genome encodes at least 4 members of this protein family (66).

Surface proteins differentially detected between phagocytosed and unphagocytosed populations of macrophage-exposed *M. avium* subsp. *hominissuis*. A focus of this study was the identification of proteins whose surface expression was uniquely different between *M. avium* subsp. *hominissuis* samples that had been exposed to macrophages and either phagocytosed (ICB) or not phagocytosed (ECB). The identification of differentially regulated, surface-exposed proteins can provide insight into the processes that influence the phagocytosis of *M. avium* subsp. *hominissuis* bacilli and their adaptation to the intracellular environment during early infection. Our results highlighted a few proteins that distinguished these two populations of *M. avium* subsp. *hominissuis* (Table 3). Four proteins were detected in ICB samples but not in ECB samples of *M. avium* subsp. *hominissuis*. These proteins included a 60-kDa chaperonin (*groEL1*, MAV_4365), a 10-kDa

chaperonin (*groES*, MAV_4366), a putative MarR family protein (MAV_4734), and an uncharacterized protein (MAV_4156). Mycobacterial *groEL1* and *groES* genes are expressed in an operon and are dominant antigens in many *Mycobacterium* infections (69, 72, 85). These proteins have been previously detected on the surface and in the culture filtrate of several *Mycobacterium* species, as well as on the more distantly related bacterium *Bacillus subtilis* (5, 32, 74, 80).

Several proteins were also detected in ECB *M. avium* subsp. *hominissuis* samples that were largely absent from ICB *M. avium* subsp. *hominissuis* samples, although the distinctions tended to be less well defined than those in the preceding comparisons (Table 3). The proteins in this group include a putative Rieske iron-sulfur protein (*qcrA*, MAV_2297), elongation factor G (*efg*, MAV_4490), and a putative uncharacterized protein (MAV_2964). Rieske iron-sulfur proteins and several associated dehydrogenases catalyze steps in the metabolism of various carbon sources (10). Homologs

TABLE 3 Proteins differentially detected after *M. avium* subsp. *hominissuis* 109 exposure to macrophages: phagocytosed versus unphagocytosed

Uniprot accession no. and detection status ^a	EMBL annotation	MAV locus tag	No. of unique peptides found according to medium and time point (h)							
			7H9 medium		DMEM		Extra-cellular		Intra-cellular	
			24	48	24	48	24	48	24	48
Phagocytosed										
CH601_MYCA1	60-kDa chaperonin 1	MAV_4365	4	4	5	3	0	0	1	3
CH10_MYCA1	10-kDa chaperonin	MAV_4366	4	4	6	6	0	0	4	4
A0QK66_MYCA1	Putative uncharacterized protein	MAV_4156	1	1	2	2	0	0	1	1
A0QLS3_MYCA1	MarR family protein	MAV_4734	4	1	3	2	0	0	2	1
Unphagocytosed										
A0QGW2_MYCA1	Putative uncharacterized protein	MAV_2964	0	1	1	0	1	2	0	0
EFG_MYCA1	Elongation factor G	MAV_4490	2	0	4	5	2	1	0	0
A0QF13_MYCA1	Putative ubiquinol-cytochrome <i>c</i> reductase, iron-sulfur subunit	MAV_2297	0	3	0	0	1	1	0	0

^a Phagocytosed means detected in phagocytosed *M. avium* subsp. *hominissuis* but not detected in unphagocytosed *M. avium* subsp. *hominissuis*. Unphagocytosed means detected in unphagocytosed *M. avium* subsp. *hominissuis* but not detected in phagocytosed *M. avium* subsp. *hominissuis*.

of each of these enzymes are known membrane or cell envelope-associated proteins in *M. tuberculosis* (30). The homolog of mycobacterial *qcrA* has also been identified as a surface-exposed protein in *B. subtilis* (80).

DISCUSSION

While previous studies have identified many of the cell membrane, cell wall, and surface-exposed proteins of related mycobacteria (30, 32, 51, 54, 74, 84, 87), little is known specifically about the surface-exposed proteome of *M. avium* subsp. *hominissuis*. In this work, we adapted an existing technology to selectively label and capture surface-exposed proteins. We utilized this approach to profile the surface-exposed proteome of *M. avium* subsp. *hominissuis* and to highlight modulations that may occur in this subproteome during the initial stages of infection. In total, we were able to identify more than 100 putative surface proteins in *M. avium* subsp. *hominissuis*. These data shed new light on the surface proteome of *M. avium* subsp. *hominissuis* and highlight some of the proteomic features that may be central to pathogenesis. The overall findings of this study are broadly consistent with the surface proteomes observed in studies of other mycobacteria, which suggests that *M. avium* subsp. *hominissuis* shares much of its surface-exposed proteome with related species (Fig. 2B and Table 4). These proteomic similarities lend support to the argument that many of the functions that are essential to mycobacterial pathogenesis (e.g., attachment, the invasion of host cells, and the manipulation of the host immune response) are shared between the various species of *Mycobacterium* (55).

Surface-exposed proteins that are both constitutively expressed and highly abundant are excellent targets for diagnostic tests and rational vaccine design (38). In this study, we were able to identify a group of 24 core proteins that were detected in all of the tested conditions, both in medium-cultured *M. avium* subsp. *hominissuis* and *M. avium* subsp. *hominissuis* that had been in contact with macrophages (Table 1). Not unexpectedly, this group includes many proteins that have also been reported to be abundant and surface exposed in other mycobacteria. Many of the core proteins observed in this study are dominant antigens and/or play a central role in mycobacterial pathogenesis, most notably antigen 85-B (*ag85b*, MAV_2816) and lipoprotein G (*lprG*, MAV_3367) (18, 83). Antigen 85-B is a mycolyl transferase that is involved in the biosynthesis of the cell envelope (39). It is a well-known surface protein that has been identified as an adhesion protein with a strong affinity for fibronectin (67). The *ag85B* protein is also a dominant antigen in mycobacterial infection and is released into the phagosome after ingestion by host macrophages (4, 85). Like *ag85B*, *lprG* has been previously observed on the mycobacterial surface and has been implicated in mycobacterial virulence (9, 21). Also a dominant antigen of mycobacterial infection, *lprG* is a posttranslationally glycosylated, surface-exposed lipoprotein (27). It is a toll-like receptor 2 (TLR2) agonist and is involved in the modulation of the innate immune response to mycobacterial infection (18, 25).

Several proteins that are normally expected to localize to the cytoplasm also appeared to be abundantly and constitutively represented at the bacterial surface, most notably multiple ribosomal proteins (MAV_4462, MAV_1770, MAV_3744, MAV_3759, and MAV_1729) and elongation factor Tu (*tuf*, MAV_4489). The question of whether these proteins are truly components of the surface-exposed proteome or merely persistent contaminants re-

mains unresolved. On one hand, ribosomal proteins are abundant and are excellent substrates for trypsin proteolysis because of their abundant lysine and arginine residues. These features may cause samples to become contaminated with easily detected ribosomal peptides. This possibility is supported by the detection of numerous ribosomal proteins in negative-control samples (see Table S2 in the supplemental material). On the other hand, ribosomal proteins have been detected in numerous studies that have profiled the surface-exposed, secreted, and cell wall-associated proteins of mycobacteria and many other species of Gram-positive bacteria (1, 5, 32, 74, 80, 87). In bacteria, some ribosomal proteins may have modified functions beyond the translation of mRNA into protein (79). Similarly, elongation factor Tu has been identified both as a surface-exposed protein and an essential virulence factor for several bacteria (14, 28, 35, 52). Ribosomes may also be complexed with protein-translocating channels, making them partially exposed to the extracellular environment, which may also cause them to be detected in screens of surface-exposed proteins (57).

In addition to the ubiquitously observed core proteins, several sets of proteins appeared to be differentially expressed at the bacterial surface in response to specific culture conditions. *M. avium* subsp. *hominissuis* actively endeavors to adhere to host cells, invade them, and establish persistent intracellular infections, a feat which is achieved, in part, by modulating aspects of its surface-exposed proteome (6, 7, 88). This study identified multiple *M. avium* subsp. *hominissuis* proteins that appeared to be either uniquely expressed or uniquely absent following exposure to macrophages. Of particular interest are two proteins, alkylhydroperoxide reductase (*ahpC*, MAV_2839) and isocitrate lyase (*aceA*, MAV_4682), that were detected only in samples of macrophage-exposed *M. avium* subsp. *hominissuis*. The detection of proteins that are differentially regulated following contact with macrophages may help elucidate some of the specific mechanisms that are utilized by *M. avium* subsp. *hominissuis* to overcome the host defense responses and nutrient limitation encountered during pathogenic growth. In the case of *ahpC* and *aceA*, the genes for both proteins are known to be transcriptionally upregulated in *M. tuberculosis* during infection, and both have been implicated in mycobacterial pathogenesis (36, 53). Alkylhydroperoxide reductase is a protein that catalyzes peroxide reduction and has been observed on the surface of several bacteria, including *M. avium* subsp. *paratuberculosis*, *M. smegmatis*, and *B. subtilis* (32, 74, 80). It is also known to play a role in isoniazid resistance by compensating for deletions in *katG* (77), and it is essential for intracellular survival and virulence (53, 86). Experiments using sera harvested from ruminants infected with *M. avium* subsp. *paratuberculosis* and human patients suffering from Crohn's disease indicate that *ahpC* is a dominant antigen in both cases (64, 65). Consistently with the findings presented here, the *ahpC* gene in *M. tuberculosis* has been shown to be highly upregulated after phagocytosis by THP-1 macrophage cells (22). Isocitrate lyase is a key enzyme in the glyoxylate shunt, a metabolic process utilized by mycobacteria to maintain metabolism in carbon-limited conditions (76). The glyoxylate shunt allows the bacteria to directly use fatty acids and acetate as basic carbon sources, a process that is important during nutrient-limited stages of infection. In *M. avium* subsp. *hominissuis*, isocitrate lyase has been shown to be uniquely expressed in response to phagocytosis by host macrophages (36). Isocitrate lyase has also been demonstrated to be a necessary persistence

TABLE 4 Cross-referenced list of proteins identified in studies profiling the surface-exposed proteome of *M. avium* subsp. *hominissuis* 109, *M. avium* subsp. *paratuberculosis*, *M. tuberculosis*, and *M. smegmatis*^a

Uniprot annotation	Protein identification			
	<i>M. avium</i> subsp. <i>hominissuis</i> 109 (this study)	<i>M. avium</i> subsp. <i>paratuberculosis</i> (32)	<i>M. tuberculosis</i> (74)	<i>M. smegmatis</i> (74)
60-kDa chaperonin 2	MAV_4707	MAP3936	Rv3417c	MSMEG_0880
Chaperone protein dnaK	MAV_4808	MAP3840	Rv0350	MSMEG_0709
Elongation factor Tu	MAV_4489	MAP4143	NA	MSMEG_1401
Acyl carrier protein	MAV_2913	MAP1997	Rv2244	NA
Heparin-binding hemagglutinin	MAV_4675	MAP3968	NA	NA
Alkylhydroperoxide reductase C	MAV_2839	MAP1589c	NA	MSMEG_4891
Adenosylhomocysteinase	MAV_4211	MAP3362c	NA	NA
3-Oxoacyl-[acyl-carrier-protein] synthase 1	MAV_2192	MAP1998	NA	NA
10-kDa chaperonin	MAV_4366	MAP4264	NA	NA
FadA2	MAV_4915	MAP3693	NA	NA
Transcriptional regulator, Crp/Fnr family protein	MAV_0453	MAP0398c	NA	NA
Succinyl-coenzyme A ligase	MAV_1074	MAP0896	NA	NA
Uncharacterized oxidoreductase	MAV_3816	MAP3007	NA	NA
Glyceraldehyde-3-phosphate dehydrogenase	MAV_3341	MAP1164	NA	MSMEG_3084
Wag31	MAV_2345	MAP1889c	NA	NA
DNA-directed RNA polymerase subunit alpha	MAV_4398	MAP4233	NA	NA
Electron transfer protein, beta subunit	MAV_3876	MAP3061c	NA	MSMEG_2351
Unknown protein	MAV_3813	MAP3005c	NA	NA
Peroxisomal multifunctional enzyme	MAV_5146	MAP3567	NA	NA
ATP synthase alpha chain	MAV_1525	MAP2453c	Rv1308	NA
ATP synthase beta chain	MAV_1527	NA	Rv1310	NA
ATP synthase gamma chain	MAV_1526	NA	Rv1309	NA
ATP synthase delta chain	MAV_1524	NA	Rv1307	NA
Aconitate hydratase 1	MAV_3303	NA	Rv1475c	MSMEG_3143
Unknown protein	MAV_3615	NA	Rv2721c	NA
MetE	MAV_1262	NA	Rv1133c	NA
Oxidoreductase	MAV_4916	NA	Rv0242c	NA
Catalase-peroxidase	MAV_2753	NA	Rv1908c	NA
Glutamine synthase	MAV_2267	NA	NA	MSMEG_4290
Succinyl-CoA ligase	MAV_1075	NA	NA	MSMEG_5524
Universal stress protein	MAV_3137	NA	NA	MSMEG_3811
Ketol-acid reductoisomerase	MAV_3850	NA	NA	MSMEG_2374
60 kDa chaperonin 1	MAV_4365	NA	NA	MSMEG_1583
Ribosomal protein S8	MAV_4451	NA	NA	MSMEG_1469
50S ribosomal protein L7/L12	MAV_4507	NA	NA	MSMEG_1365
Putative uncharacterized protein	MAV_2412	NA	Rv2091c	NA
MihF protein	Negative control	MAP1122	NA	MSMEG_3050
DNA binding protein HU	Negative control	MAP3024c	NA	NA
30S Ribosomal protein 3	Negative control	MAP4167	NA	NA
Malate synthase G	Negative control	NA	Rv1837c	NA
Ribosomal protein L18	Negative control	NA	NA	MSMEG_1471
Enolase	NA	MAP0990	NA	NA
FadE3	NA	MAP3651c	NA	NA
PPE protein PPE26	NA	MAP1506	NA	NA
PPE protein PPE30	NA	MAP1519	NA	NA
DesA2	NA	MAP2698c	NA	NA
Unknown protein	NA	MAP1563c	NA	NA
PPE protein PPE61	NA	MAP3532	NA	NA
SerA	NA	MAP3033c	NA	NA
FadE24	NA	MAP3188	NA	NA
Alkylhydroperoxide reductase D	NA	MAP1588c	NA	NA
FadE18	NA	MAP2228	NA	NA
ATP-dependent Clp protease proteolytic subunit	NA	MAP2280c	NA	NA
WXG100 protein EsxP	NA	MAP1508	NA	NA
Proline-rich protein	NA	NA	Rv1078	NA
Conserved membrane protein	NA	NA	Rv0227c	NA
Serine protease HtrA	NA	NA	Rv1223	NA
Unknown protein	NA	NA	Rv1006	NA

(Continued on following page)

TABLE 4 (Continued)

Uniprot annotation	Protein identification			
	<i>M. avium</i> subsp. <i>hominissuis</i> 109 (this study)	<i>M. avium</i> subsp. <i>paratuberculosis</i> (32)	<i>M. tuberculosis</i> (74)	<i>M. smegmatis</i> (74)
Band protein	NA	NA	Rv1488	NA
FabE10	NA	NA	Rv0873	NA
Unknown protein	NA	NA	Rv1836c	NA
Conserved 35-kDa alanine-rich protein	NA	NA	Rv2744c	NA
<i>Trans</i> -acting enoyl reductase	NA	NA	Rv2953	NA
Unknown protein	NA	NA	Rv0831c	NA
Hypothetical protease	NA	NA	Rv2224c	NA
Glycerol kinase	NA	NA	NA	MSMEG_6759
Fatty acid synthase	NA	NA	NA	MSMEG_4757
Methoxy mycolic acid synthase	NA	NA	NA	MSMEG_0913
Isocitrate dehydrogenase, NADP dependent	NA	NA	NA	MMEG_1654
Unknown protein	NA	NA	NA	MSMEG_6431
Ribosomal protein S1	NA	NA	NA	MSMEG_3833
Ribonucleoside-diphosphate reductase, alpha subunit	NA	NA	NA	MSMEG_1019
Pyruvate dehydrogenase E1	NA	NA	NA	MSMEG_4323
Citrate synthase I	NA	NA	NA	MSMEG_5672

^a NA, not applicable.

factor of infectious mycobacteria in both macrophage and animal models (56, 59), and it has been identified as an ideal drug target for the treatment of tuberculosis infection (60).

Several additional proteins with known roles in mycobacterial pathogenesis were observed to be uniquely present or absent, depending on their interaction with macrophages, including an alanine- and proline-rich protein (*modD*, MAV_2859) and two chaperonin proteins, *groEL1* (MAV_4365) and *groES* (MAV_4366). The *modD* protein, which appeared to be uniquely absent following exposure to macrophages, is thought to play a role in bacterial attachment to the extracellular matrix (71). It has been described as a surface-exposed protein that is posttranslationally glycosylated and is a dominant antigen in several infection models (12, 26, 41). The interspecies variation of *modD*, along with its antigenicity, has been used as the basis for the development of species-specific diagnostic screens (78).

An unexpected observation among the differentially detected proteins was the apparent absence of the *groEL1* and *groES* proteins (MAV_4365 and MAV_4366) on the surface of ECB samples of *M. avium* subsp. *hominissuis* despite being detected in all of the other conditions tested in this study. These proteins, which are traditionally identified as chaperone proteins, are also important for virulence in mycobacteria (23). While chaperone proteins are commonly involved in protein folding and stress responses, *groEL1* and *groES* have been implicated in several additional processes in mycobacteria, including cell envelope biosynthesis, persistence, and the invasion of host cells and virulence (34, 46, 63, 69). These proteins appear to be absent from the surface of *M. avium* subsp. *hominissuis* samples that had been exposed to macrophages but not phagocytosed, but they are present in all other conditions. This observation provides additional evidence that these proteins have functions related to attachment and invasion. At the same time, important questions remain, such as whether these proteins are removed by the bacteria in the ECB population or whether there is a mixed population (between those with low

levels and high levels of these proteins at their surfaces) and the invasion process is sufficient to segregate the two populations.

The treatment of mycobacterial infections is notoriously challenging, but the identification of potential drug targets that are unique for specific phases of infection should be a positive step toward the development of new and improved treatment strategies. This analysis revealed some variations between culture conditions that provide support for the hypothesis that modulations in the expression of dominant antigens during the course of mycobacterial infection undermines the ability of the host to develop an effective adaptive immune response (19, 40). For example, these data suggest that two well-known dominant antigens, *ag85A* and *ag85C* (MAV_0214 and MAV_0215), were well represented in *M. avium* subsp. *hominissuis* cultured in standard broth medium (7H9) but largely absent from other conditions (see Table S1 in the supplemental material). A potential consequence of this sort of modulation is that a robust overall immune response to mycobacterial infection may be insufficient to clear infection, because the immune response may focus on antigenic targets whose expression is downregulated during the course infection. As a result, the immune system may end up failing to effectively target bacteria that have established a stable infection (87). This type of result also suggests that screens for antigens and immunogens are limited by the culture conditions used to generate the reference protein samples.

Although putative surface and/or secreted proteins are a small fraction of the mycobacterial proteome, they represent a large portion of known bacterial antigens (3, 37, 38, 45, 87). This is largely because the surface-exposed molecules of the bacterium are primarily what the immune system sees when it targets a pathogen. Antibodies are most likely to be effective if their target antigens are easily accessible, surface-exposed molecules. The comprehensive profiling of the surface proteome of a pathogen like *M. avium* subsp. *hominissuis* can generate a list of viable targets for rational vaccine design. A central challenge in designing

both vaccines and diagnostic tests is identifying targets which are both abundant and specific. As a result of the similarities between the surface-exposed proteomes of different mycobacteria, cross-reactivity among mycobacterial antigens is a common problem (68). The comprehensive profiling of *M. avium* and other mycobacteria can help resolve this issue by identifying subsets of potential targets that may be unique to a particular species of mycobacteria. Additionally, the identification of targets that may also be unique to a particular phase of pathogenesis (e.g., intracellular growth) is of significant importance. In the case of pathogenic *Mycobacterium*, many of which establish long-term latent infections, this methodology could assist the development of therapeutic vaccines that can aid in the treatment of chronic infection.

While our analysis of the surface-exposed proteome of *M. avium* subsp. *hominissuis* was highly consistent with previous proteomic profiles of *M. avium* subsp. *paratuberculosis* (32), *M. smegmatis*, and *M. tuberculosis* (74), the methods used in each study are significantly different, which inevitably affects the respective results (Fig. 2B and Table 4). The referenced studies of mycobacterial surface proteins employed trypsin shaving (*M. avium* subsp. *paratuberculosis*) or the selective solubilization of the cell envelope (*M. tuberculosis* and *M. smegmatis*) to isolate surface-exposed peptides and proteins for analysis. In contrast, this study used the covalent labeling of surface-exposed proteins with biotin, followed by affinity purification. The primary challenge of each approach is to efficiently purify the target molecules while minimizing background contamination. The various capacities of each experimental approach to overcome this challenge may explain some of the observed similarities and differences between the respective data sets.

In addition to the proteins highlighted in Tables 1 to 3, numerous other putative surface-exposed proteins were identified that did not fit into well-defined groups based on the conditions in which they were detected (see Table S1 in the supplemental material). Most of these proteins were detected in only a few conditions with apparently random distribution. The hit-or-miss nature of these protein identifications highlights the fundamental limitation of the conclusions that can be drawn from these data. The peptide mixtures analyzed in each sample are both complex and variable. Most of the protein identifications are dependent on the identification of a couple of peptides, which is a fingerprint and not a comprehensive profile. In addition, many of these peptides are not abundant and near the detection threshold. Sample handling during protein extraction, affinity purification, enzymatic digestion, and peptide cleanup introduces variability that affects which peptides are ultimately detected with confidence and which are not. Therefore, while positive identifications have a good probability of being accurate, a failure to detect a protein does not necessarily indicate that it is truly absent. Because of this limitation, the data presented here cannot be considered a comprehensive or exhaustive profile.

There are two additional limitations of the data presented in this study that should be taken into account. First, the fractionation of total protein, on the basis of solubility, from biotinylated *M. avium* subsp. *hominissuis* indicated that some proteins were not effectively solubilized by the buffers used in this study (Fig. 1). This result suggests that some surface proteins are poorly represented or are not identified by this analysis. For example, no members of the PPE and PE families of mycobacterial proteins were observed in this study, although many among them are known to

be surface-exposed proteins in many species of *Mycobacterium*, including the closely related *M. avium* subsp. *paratuberculosis* (29, 32, 61). Overall, many proteins that are expected to be surface exposed were not identified in this analysis (62). Subsequent work in our laboratory has shown that several of these proteins are indeed present on the surface of *M. avium* subsp. *hominissuis*, but the efficient solubilization and purification of these proteins require the presence of additional reagents, such as 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) or SDS (data not shown). A similar effect of solubility may explain the minimal overlap of identified proteins between the two previous studies used in this study as comparative references for mycobacterial surface-exposed proteins (Fig. 2B and Table 4). For example, whereas the trypsin shaving of *M. avium* subsp. *paratuberculosis* identified 3 PPE family proteins on the surface of *M. avium* subsp. *paratuberculosis*, the solubilization of the *M. tuberculosis* outer membrane did not identify any members of this family, although they are known to be prominent members of the surface proteome of *M. tuberculosis* (2, 13, 29, 32). The likely cause of this discrepancy is the bias of the solubilization method toward proteins that are more easily solubilized. While the results generated here correlate well with the results of both previous methods, it is nonetheless apparent that a population of proteins remains refractory to each method of analysis.

The final limitation of note applies to all of the methods that use trypsin to generate peptides for proteomic analysis. Trypsin cleaves after lysine and arginine, a fact which can cause some proteins to be overrepresented and others underrepresented. Complicating matters, the biotinylation of lysine side chains inhibits cleavage at that residue, which may further contribute to the underrepresentation of lysine-poor proteins in the data set. Many DNA-binding and ribosomal proteins are rich in both lysine and arginine, which make them excellent substrates for trypsin and may cause them to appear to be more abundant than they truly are. Along with their general abundance, this feature may explain why ribosomal proteins are commonly identified in profiles of surface-exposed proteins, including in this study. While beyond the scope of this study, the limitations discussed here may be overcome in future analyses through improvements in protein extraction and purification protocols, the use of alternative protease enzymes to create complementary sets of peptides, improved peptide cleanup, and optimized LC-MS/MS technologies.

The selective labeling of surface proteins to facilitate their purification and identification has been employed in studies analyzing both prokaryotic and eukaryotic cells. In this report, we streamlined the technology and adapted it for use in the study of the opportunistic pathogen *M. avium* subsp. *hominissuis*. In total, this study identified more than 100 putative surface-exposed proteins. The comparison of data derived from *M. avium* subsp. *hominissuis* cultured in both standard media and conditions simulating the initial stages of infection highlighted several groups of proteins that may be uniquely modulated during early pathogenesis. These analyses provide a unique insight into the molecular mechanisms of *M. avium* subsp. *hominissuis* pathogenesis that are mediated by surface-exposed proteins. In turn, the data and methods presented here can contribute to the development of better tools for the diagnosis and treatment of mycobacterial disease.

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