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Francisella tularensis LVS Surface and Membrane Proteins as Targets of Effective Post-Exposure Immunization for Tularemia

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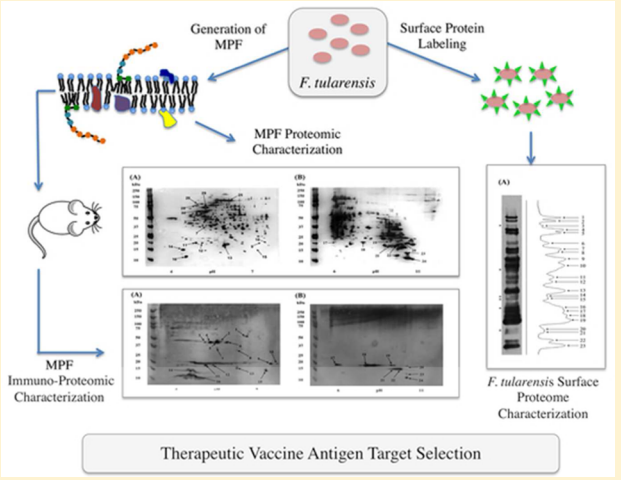
Supporting Information

ABSTRACT: *Francisella tularensis* causes disease (tularemia) in a large number of mammals, including man. We previously demonstrated enhanced efficacy of conventional antibiotic therapy for tularemia by postexposure passive transfer of immune sera developed against a *F. tularensis* LVS membrane protein fraction (MPF). However, the protein composition of this immunogenic fraction was not defined. Proteomic approaches were applied to define the protein composition and identify the immunogens of MPF. MPF consisted of at least 299 proteins and 2-D Western blot analyses using sera from MPF-immunized and *F. tularensis* LVS-vaccinated mice coupled to liquid chromatography−tandem mass spectrometry identified 24 immunoreactive protein spots containing 45 proteins. A reverse vaccinology approach that applied labeling of *F. tularensis* LVS surface proteins and bioinformatics was used to reduce the complexity of potential target immunogens. Bioinformatics analyses of the immunoreactive proteins reduced the number of immunogen targets to 32. Direct surface labeling of *F. tularensis* LVS resulted in the identification of 31 surface proteins. However, only 13 of these were reactive with MPF and/or *F. tularensis* LVS immune sera. Collectively, this use of orthogonal proteomic approaches reduced the complexity of potential immunogens in MPF by 96% and allowed for prioritization of target immunogens for antibody-based immunotherapies against tularemia.



KEYWORDS: *Francisella tularensis, surface proteome, membrane proteome, adaptive immunity, antibodies*

1. INTRODUCTION

Francisella tularensis, the etiological agent of tularemia, causes an acute infection with several clinical manifestations, including a pneumonic presentation that is the most severe and ulceroglandular disease that is the most common.[1,2] *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B) both cause disease in humans, but type-B infections are rarely fatal. In contrast, pneumonic disease caused by *F. tularensis* subsp. *tularensis* results in mortalities ranging between 30 and 60% if left untreated.[3] *F. tularensis* infections are treatable by a wide array of antibiotics including gentamicin, but these need to be administered in a timely manner to avoid increased chance of relapse.[3] The importance of the humoral response against *F. tularensis* to control and clear infection is also recognized. Foshay et al. showed that passive transfer of *F. tularensis* immune sera provided prophylactic protection in humans.[4] Similarly, Drabick et al. demonstrated that passive transfer of immune sera protected mice against a lethal high dose challenge with *F. tularensis* subsp. *holarctica* live vaccine strain (LVS), and this protection was abrogated by preabsorption of the serum with a *F. tularensis* LVS lysate, thus implicating antibodies as the protective component.[5] Passively transferred *F. tularensis* LVS immune serum also decreased the duration and severity of a type A infection in rats as well as reduced systemic bacterial burden to the liver and spleen.[6]

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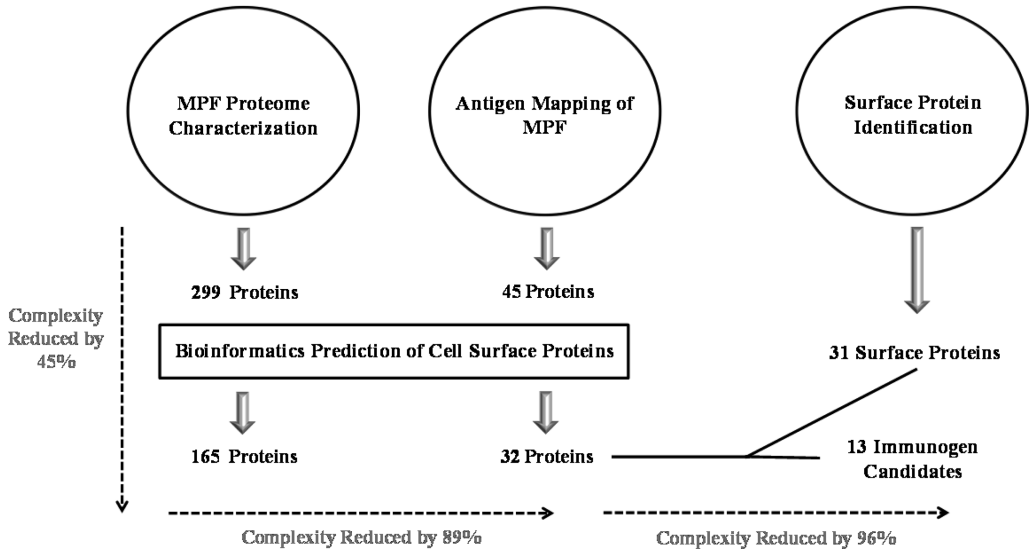

Figure 1. Schematic of the experimental workflow used to identify *F. tularensis* LVS MPF immunogens.

Membrane components of *F. tularensis* have shown protective efficacy in prophylactic and postexposure therapeutic models of tularemia.[7−9] Ireland et al. demonstrated the protective effects of adjuvant complexed with a membrane protein fraction (MPF) when administered prophylactically 3 days prior to a virulent *F. tularensis* SCHU S4 challenge in mice.[8] Huntley et al. isolated outer membrane proteins and lipopolysaccharide (LPS) from *F. tularensis* LVS and found that vaccination with these provided 50 and 15% increase in survival, respectively, in mice challenged with *F. tularensis* SCHU S4.[9] While LPS provided a degree of protection in immunized mice, passive transfer of *F. tularensis* LVS LPS immune sera provided little to no protection against a *F. tularensis* SCHU S4 challenge.[10,11]

To evaluate membrane-based immunotherapeutic methods that enhance chemotherapy, we created a murine model of tularemia treated with a subtherapeutic regimen of gentamicin. Using this model, it was demonstrated that postexposure vaccination with the MPF of *F. tularensis* LVS provided full protection in the presence of a subtherapeutic dose of gentamicin against a type A *F. tularensis* strain SCHU S4 infection (100% survival at day 40 of infection).[7] Moreover, the passive transfer of the MPF immune sera restored complete efficacy to the suboptimal gentamicin regime, indicating antibodies as the protective component in this model. The protective immune sera from our postexposure subtherapeutic gentamicin and MPF vaccination murine model showed high IgM, IgG3, and IgG2a titers with the IgM response directed at LPS and the IgG response directed toward membrane proteins.[7] Additionally, these mice showed a reduced severity of disease once the adaptive immune response initiated the production of high IgG titers, indicating that MPF proteins were important immunogenic components of MPF. However, the protein targets of these protective antibodies were not defined.

In the present study, we characterized the MPF proteome and applied the principles of reverse vaccinology to identify the likely immunogens of MPF (Figure 1). The concept behind reverse vaccinology is that successful protein-based bacterial immunotherapies are formulated with surface-exposed or -secreted bacterial proteins. Reverse vaccinology utilizes orthogonal high-throughput bioinformatics and proteomic pipelines to identify surface proteins, dramatically reducing the number of candidate immunogens to test in animal models.[12,13] The immunogen signatures profiled in this study included bioinformatic predictions of membrane and surface localization and secretion, immunoreactivity to corresponding murine immune sera (MPF immunized and *F. tularensis* LVS vaccinated), and experimental validation of cell surface localization. The MPF consisted of at least 299 proteins, of which 45 immunoreactive proteins were identified. Of the immunoreactive proteins, 13 localized to the bacterial cell surface, suggesting they are the immunogenic protein components of the *F. tularensis* LVS MPF.

2. MATERIALS AND METHODS

2.1. Bacteria, Culture Conditions, and MPF Isolation

F. tularensis LVS was provided by Dr. Jeannine Petersen (Centers for Disease Control and Prevention, Fort Collins, CO). For identification of surface proteins, *F. tularensis* LVS was grown on CHAB medium (cysteine heart agar supplemented with 9% chocolatized sheep blood) for 48 h at 37 °C. Cells were collected by scraping and suspended in phosphate-buffered saline (PBS) (pH 7.4) for surface labeling. *F. tularensis* LVS, used for the generation of MPF, was grown on CHAB plates for 48 h at 37 °C and subcultured in 50 mL of modified Mueller Hinton (MMH) broth supplemented with 2% Isovitalex (BD, Sparks, MD), 0.1% glucose, and 0.025% ferric pyrophosphate with shaking (150 rpm) at 37 °C for 12 h, followed by inoculation of 1 L of MMH broth. After 12 h, cells were collected by centrifugation at 3000*g* and washed in PBS, pelleted by centrifugation, and stored at −80 °C. The MPF of *F. tularensis* strain LVS was prepared, quantified, and tested for foreign endotoxin contamination as described previously.[7]

2.2. Mice

Specific pathogen-free, 6−8 week old BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were provided with sterile water and food ad libitum, and all research involving animals was conducted in accordance with Animal Care and Use guidelines and approved by the

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Animal Care and Use Committees at Colorado State University and Rocky Mountain Laboratories.

2.3. Immunization of Mice

For generation of MPF immune sera, mice were given 10 μg of MPF diluted in 5% dextrose water (D5W) and intraperitoneal (i.p.). Mice were immunized twice, 4 days apart, and serum was drawn via cardiac puncture 7 days after the first injection. Anti-MPF antibody titers were confirmed via ELISA with naïve serum used as a control.[7]

Mice were immunized twice (2 weeks apart) subcutaneously (s.c.) with *F. tularensis* LVS diluted in PBS (200 CFU in 200 μL). The CFU concentration of the inoculum was confirmed by plating on CHAB medium and enumerating colonies after 48 h. Unvaccinated mice served as negative sera controls. All mice were bled via the tail vein for serum collection 12 days after immunization.

2.4. Surface Labeling of Proteins and Label Localization

Suspensions of *F. tularensis* LVS were pelleted by centrifugation at 4500*g*, washed three times in PBS, and adjusted to an A_{600} OD of 0.15 to 0.19 in PBS. An aliquot (80 μL) of EZ-Link Sulfo-NHS-LC-Biotin (LC-Biotin) (Thermo/Pierce, Rockford, IL) at 6.6 mg/mL was added per 1 mL of the cell suspension and incubated at room temperature for 1 h with gentle rocking. The cells were collected by centrifugation and washed once in Tris-buffered saline (TBS) (pH 7.4), and twice in PBS. The final biotin-labeled cell pellet was suspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.3 M Tris, 50% glycerol, 10% sodium dodecyl sulfate, 25% β-mercaptoethanol, and trace bromophenol blue) for analysis by SDS-PAGE or in 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS) buffer (1% CHAPS in PBS) for affinity purification of labeled proteins.

F. tularensis LVS whole cell lysates (WCLs) from cells grown on CHAB were prepared by suspending cells in breaking buffer [PBS, pH 7.4, 60 μg of DNase (Sigma, St. Louis, MO), 60 μg of RNase (Sigma), 50 μg of lysozyme (Sigma), and one Complete Protease Inhibitor tablet (Roche Applied Sciences, Indianapolis, IN) per 50 mL of buffer]. Cells were placed on ice and lysed by nine repetitions of pulsed sonication using a 4710 series ultrasonic homogenizer (Cole and Palmer Instrument Company, Vernon Hills, IL) employing the following instrument parameters: 50% duty, output-5, 60 s pulses, and 60 s pauses. Unbroken cells were removed from the lysate by centrifugation at 4500*g*, 4 °C, for 20 min. Lysates were labeled with LC-Biotin as described for whole cells.

2.5. Biotinylated Protein Purification

Surface-labeled proteins were extracted for affinity purification by suspending cells in 1% CHAPS buffer for 15 min at 120 °C with intermittent vortexing. Extracts were added to immobilized streptavidin resin (Thermo/Pierce) pre-equilibrated in PBS and incubated overnight at 4 °C with gentle shaking. The resin was washed with 10 vol of 1% CHAPS buffer, followed by 10 vol of PBS. Biotinylated proteins were eluted from the resin in SDS-PAGE sample buffer at 120 °C for 10 min.

2.6. Polyacrylamide Gel Electrophoresis

2D-PAGE was performed with 100 μg aliquots of MPF. Sample preparation was performed with the ReadyPrep 2-D Cleanup Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The resulting protein pellet was air-dried and suspended in 22.5 μL of 50 mM Tris (pH 7.1), 8 M urea, 2 M thiourea, 100 mM dithiothreitol (DTT), 4% (w/v) CHAPS, 1% (w/v) ASB-14, 0.7% pH 4−7 ampholytes, and 0.3% pH 3−10 ampholytes. The proteins were sonicated and incubated for 4 h at room temperature. Samples were diluted with a 130 μL of solution of 8 M urea, 2 M thiourea, 20 mM DTT, 4% (w/v) CHAPS, 1% (w/v) ASB-14, 0.7% pH 4−7 ampholytes, and 0.3% pH 3−10 ampholytes and centrifuged for 30 min at 13 000*g* to remove any insoluble material. Supernatants (130 μL) were applied to an Immobiline dry strip (GE Healthcare Life Sciences, Piscataway, NJ) with either pH gradients of 4−7 or 6−11 following the manufacturer's instructions.

Isoelectric focusing (IEF) was performed using the Multiphore II unit (GE Healthcare Life Sciences) at 50, 100, 150, 200, 250, and 300 V sequentially for 6 min, followed by 500 V for 12 min and 3000 V for 5 h. The focused Immobiline strips were rinsed in ultrapure H_2O and incubated in 0.375 M Tris (pH 7.1), 6 M urea, 2% SDS, 30% glycerol, 1% DTT, and trace bromophenol blue for 15 min at room temperature. Immobiline strips were again rinsed in ultrapure H_2O and incubated in 0.375 M Tris (pH 7.1), 6 M urea, 2% SDS, 30% glycerol, 2.5% iodoacetamide, and trace bromophenol blue before a final rinsing in ultrapure H_2O. Proteins were resolved in the second dimension by SDS-PAGE.

Aliquots of labeled proteins and MPF were applied to SDS-PAGE using a 4−12% Bis-Tris SDS-polyacrylamide gels (Life Technologies, Carlsbad, CA) under denaturing conditions.[14]

Detection and staining of protein in polyacrylamide gels was accomplished with the Pierce Silver Stain Kit (Thermo/Pierce) or SimplyBlue SafeStain (Life Technologies) according to the respective manufacturer's protocols. Gel images were converted to digital data using a HP Scanjet 4850 photo scanner (Hewlett-Packard Company, Palo Alto, CA).

2.7. Western Blotting

Biotinylated surface proteins and MPF resolved by SDS-PAGE were transferred to nitrocellulose membranes,[15] incubated in TBS containing 0.1% Tween 20 and 5% nonfat milk and washed in TBS containing 0.1% Tween 20 (TBST). The nitrocellulose membranes were probed with pooled sera (1:200) from vaccinated or control mice or with antibiotin antibody conjugated to horseradish peroxidase (HRP) (Cell Signaling Technology, Danvers, MA) and subsequently washed in TBST. Immune sera Western blots were incubated with alkaline-phosphatase-conjugated goat-antimouse IgG (1:5000) to detect primary antibodies and washed with TBST, and antibody-reactive proteins were detected by BCIP/NBT SigmaFAST tablets (Sigma). Western blot images were digitized using a HP Scanjet 4850 photo scanner (Hewlett-Packard Company, Palo Alto, CA). Membranes receiving the antibiotin-HRP as the primary probe were developed with LumiGLO (Cell Signaling Technology) using CL-Exposure film (Thermo/Pierce). Digitized images of antibiotin Western blots were analyzed with Imagequant TL software (GE Health Care Life Sciences), and the number of reactive protein bands in each sample was determined based on densitometry.

2.8. Fractionation of MPF Tryptic Peptides

An aliquot (400 μg) of MPF suspended in 50 μL of 0.2 M ammonium bicarbonate was digested with 10 μg modified trypsin (Roche Applied Science) at 37 °C for 4 h, followed by overnight digestion with an additional 10 μg of trypsin. Samples were dried under vacuum, reconstituted in H_2O, and dried three times before suspension in 120 μL of 3% acetonitrile (ACN) containing 0.1% acetic acid. Insoluble material was

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removed from the sample by centrifugation at 16 000*g* for 10 min.

Peptides were separated by strong cation exchange (SCX) chromatography using an Alliance 2695 HPLC (Waters, Milford, MA) coupled to a PolyLC polysulfethyl A column (4.6 mm × 100 mm) (The Nest Group, Southboro, MA) with an increasing linear gradient of KCl (0 to 500 mM) in 10 mM KH_2PO_4, 25% ACN and a flow rate of 1 mL/min. A total of 12 fractions were collected and dried under vacuum. The fractions were reconstituted in 100 μL of 0.1% trifluoroacetic acid and desalted using OMIX C18 tips according to the manufacturer's instructions (Varian, Palo Alto, CA). Desalted samples were dried under vacuum and suspended in 11 μL of 3% ACN, 0.1% formic acid. Samples were sonicated for 5 min, followed by centrifugation for 10 min at 16 000*g* and transferred to an autosampler vial for LC−MS/MS analyses as described below.

2.9. Protein Identification by Liquid Chromatography−Mass Spectrometry

Affinity purified surface proteins and MPF proteins resolved by SDS-PAGE were subjected to in-gel proteolytic digestion with trypsin or chymotrypsin as described previously.[16]

Peptides from affinity purified surface proteins were applied to capillary C18 reverse-phase columns (Zorbax 300SB C18, 3.5 μm particle size, 0.3 × 150 mm, Agilent Technologies, Santa Clara, CA) and eluted with an increasing linear gradient of ACN in 0.1% formic acid at a flow rate of 5 μL/min using an Agilent 1100 capillary HPLC solvent delivery system. Effluent was introduced directly into a ThermoFinnigan (San Jose, CA) LTQ mass spectrometer (LTQ) operated with Xcalibur software ver. 2.0 SR2. For ionization and fragmentation, the mass spectrometer was configured with an electrospray voltage of 4 kV, a N_2 sheath gas flow of 15, a capillary temperature of 200 °C, and a normalized collision energy of 35%. The top five most intense ions from the full MS scan (m/z range of 400 to 2000 Da) were selected for MS/MS (a maximum of two times per precursor). Selected precursor ions were then placed on the dynamic exclusion list for one min.

LC−MS/MS of tryptic digests of 2D-PAGE protein spots and MPF peptides separated by SCX was achieved using an Agilent 1200 nano flow LC system coupled via a Chip Cube interface to an Agilent 6520 quadrapole time-of-flight mass spectrometer (Q-TOF) operated with MassHunter Workstation Software ver. B.06.00 (Agilent). Peptides were resolved with an increasing linear gradient (36 min, 10 to 90%) of ACN applied to 43 mm 300 Å C18 chip in-line with a 40 nL trap column (ProtID-Chip-43) (Agilent). Peptides were eluted directly into the mass spectrometer at a rate of 0.5 μL/min and MS spectra were collected in positive ion mode over a m/z range of 250 to 2400 Da. MS/MS data were collected using a ramped collision energy with a slope of 3.7 and an offset of 2.5. Three precursor ions were selected for each MS/MS cycle. These analyses were performed in the BioMolecular Analysis Core at Colorado State University (http://www.rmrce.colostate.edu/pages/scientific-cores/BioMolecular-Analysis).

Tryptic digests of selected low-abundance 2D-PAGE protein spots were analyzed using a Thermo Scientific Orbitrap Velos (Orbitrap) operated with Xcalibur ver.2.2 SP1 software (Thermo Scientific). Peptides were applied to an in-line enrichment column (Thermo scientific EASY-Column, 2 cm, ID 100 μm, 5 μm, C18-A1), and subsequent chromatographic separation was performed on a reversed-phase nanospray column (Thermo Scientific EASY-Column 10 cm, ID 75 μm, 3 μm, C18-A2) using an increasing linear gradient (10 to 30%) of ACN at a flow rate of 0.4 μL/min. Peptides were eluted directly into the mass spectrometer, and data-dependent spectral acquisition was performed over a m/z range of 400 to 2000 Da at a normalized collision energy of 35%. The instrument was operated in Orbitrap-LTQ mode, where precursor measurements were acquired at 60 000 resolution, and MS/MS spectra were acquired in the LTQ ion trap using dynamic exclusion of two MS/MS spectra per precursor ion over 30 s and an exclusion duration of 90 s. These analyses were performed in the Proteomics and Metabolomics Facility at Colorado State University (www.pmf.colostate.edu).

2.10. Database Searching

Q-TOF derived MS/MS data were extracted using MassHunter Workstation software. LTQ and Orbitrap data were extracted using MSConvert (Proteowizard, http://proteowizard.sourceforge.net). All MS/MS data were searched using Mascot (Matrix Science, London, U.K.; ver. 2.3.02) and X! Tandem (The Global Proteome Machine Organization, www.thegpm.org; ver. CYCLONE (2010.12.01.1)). Mascot and X! Tandem were set to search the NCBInr_011014 database (*F. tularensis*, 7532 entries). MS/MS data acquired from the Q-TOF were searched with a fragment ion mass tolerance of 0.01 Da and a parent ion tolerance of 20 ppm. LTQ data were searched with a fragment ion mass tolerance of 1.0 Da and a parent ion tolerance of 2.5 Da. Orbitrap-derived MS/MS data were searched with a fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 20 ppm. For all data, variable modifications of glutamic acid to pyroglutamic acid of the N-terminus, ammonia-loss of the N-terminus, glutamine to pyroglutamic acid of the N-terminus, and oxidation of methionine were considered. For affinity-purified surface proteins and for protein spots, biotinylation of lysine (339.16 Da) and carbamidomethylation of cysteine, respectively, were also considered as variable modifications.

Scaffold ver. 4.3.2 (Proteome Software, Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >90% probability by the Peptide Prophet algorithm with Scaffold delta-mass correction.[17] Protein identifications were accepted if they could be established at >99% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[18] Proteins that contained similar peptides and could not be differentiated based on MS/MS analyses alone were grouped to satisfy the principles of parsimony. For protein spots or fractions where biological replicates were obtained, data files were combined using the "mudpit" function to generate a composite list of the proteins identified in the specific sample. Protein and peptide false discovery rates (FDRs) were calculated using the assigned probabilities estimated from the results of Peptide and Protein Prophet. For all samples, peptide and protein FDR were <1.1 and 0%, respectively.

2.11. Bioinformatic Analyses of Protein Subcellular Localization

Bioinformatic approaches were applied to proteins identified from the *F. tularensis* LVS genome sequence (accession number: NC_007880). Subcellular protein localizations were predicted with PSORTb (ver. 3.0.2) (http://www.psort.org/psortb). Classical signal peptides were detected with SignalP (ver. 4.1) (http://www.cbs.dtu.dk/services/SignalP), and signal

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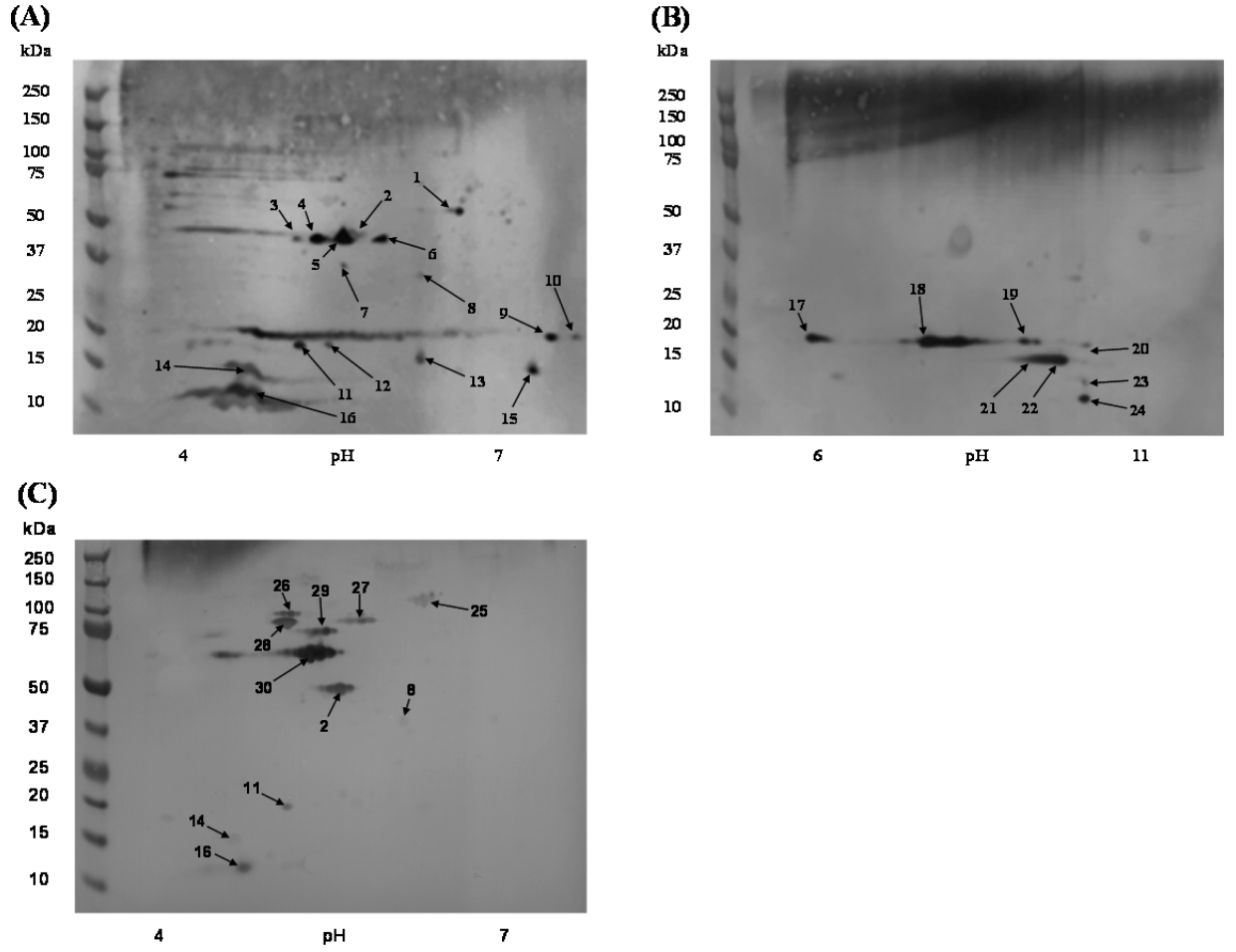

Figure 2. 2D Western blot of MPF and *F. tularensis* LVS immune sera to MPF proteins. (A) MPF resolved in a pH range of 4−7 and probed with MPF immune sera. (B) MPF resolved in a pH range of 6−11 and probed with MPF immune sera. (C) MPF resolved in a pH range of 4−7 and probed with *F. tularensis* LVS immune sera. The numbered arrows correspond to the spots labeled in Figure 1 and the protein identifications presented in Table 1 and Table S2 in the Supporting Information.

peptides of lipoproteins were predicted with LipoP (ver. 1.0) (http://www.cbs.dtu.dk/services/LipoP). Nonclassically secreted proteins were predicted with SecretomeP (ver. 2.0) (http://www.cbs.dtu.dk/services/SecretomeP).

3. RESULTS AND DISCUSSION

3.1. Characterization of the MPF Proteome

To first determine the complement of proteins in the protective MPF component of *F. tularensis*, we performed proteome characterization via multidimensional LC−MS/MS on a tryptic digest of MPF. This resulted in the identification of 284 proteins with a high degree of confidence (Table S1 in the Supporting Information). Bioinformatic analyses indicated that 40% (114 out of 284) of these proteins contained at least one signature or motif consistent with membrane or surface localization (translocated, 67 proteins; cell envelope localization, 58 proteins; or β-barrel motif, seven proteins). The remaining proteins were predicted to have a cytosolic or an unknown subcellular localization. The identification of a large number of predicted nonmembrane or nontranslocated proteins was not unexpected because experimental detection of presumably cytosolic proteins in bacterial membrane preparations is commonly reported.[19,20] Of the 170 proteins not predicted to be membrane associated, 41 were described in previous studies as localized to the cell envelope (membrane, secreted, or surface protein). (See Table S1 in the Supporting Information.) In addition to adding validity to the identification and localization of the proteins comprising the MPF, the bioinformatics analyses served as a filter to reduce the complexity of the potential immunogens in MPF by 55% (155 of 284 proteins with predicted or experimentally determined membrane localization).

3.2. Sera from MPF Immunization and *F. tularensis* LVS Vaccination Recognize a Small Subset of MPF Proteins

We previously established that passive transfer of sera from mice immunized with MPF protects against a *F. tularensis* SCHU S4 challenge in a murine model and shows reactivity to specific components of MPF by 1D-Western blot analyses.[7] To fully identify the immunoreactive proteins of MPF and further narrow the number of potential immunogen candidates, 2D-Western blots were probed with immune sera. 2D-PAGE analyses of MPF resulted in the resolution of 295 and 271 individual protein spots using pH 4−7 and pH 6−11 IEF gradients, respectively (Figure S1A and S1B in the Supporting Information). This was in concordance with a previous report, indicating a similar number of spots in *F. tularensis* membrane preparations.[21] Proteins with close-to-neutral isoelectric points resolved effectively with the pH 4−7 IEF gradient (Figure S1A in the Supporting Information); however, only proteins with

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Table 1. Proteins Identified in MPF Spots

spot no.	protein name	locus[a]	signal peptide[b]	PSORTb localization (score)[c]	sera reactivity[d]	previously identified as immunoreactive and reference	previously identified as membrane (M), secreted (T), or surface (S) protein and reference
1	dihydrolipoamide dehydrogenase (Lpd)[e]	FTL_0311		Cyto (9.97)	X	21	M[21,37]
	ATP-dependent protease, ATP-binding subunit (HslU)	FTL_0964		Cyto (9.97)			
	glyceraldehyde-3-phosphate dehydrogenase (GapA)[f,e]	FTL_1146		Cyto (9.97)		38−40	T[35,41]
	glutathione reductase (Gor)[e]	FTL_1248	SpI	unknown			
2	elongation factor Tu (TufA)[f]	FTL_1751		Cyto (9.97)	X, Y	21,38,40,42−46	M[21]
3−4	could not be identified with confidence				X		
5	outer membrane associated protein FopA1[f]	FTL_1328	SpI	OM (9.93)	X	21,38,40,42,45,46	M,[21,23,47,48] T,[35] S[25,28]
6	universal stress protein (Usp)	FTL_0166		Cyto (9.97)	X	39	M[37]
	outer membrane associated protein FopA1[f]	FTL_1328	SpI	OM (9.93)		21,38,40,42,45,46	M,[21,23,47,48] T,[35] S[25,28]
7	could not be identified with confidence				X		
8	outer membrane associated protein FopA1[f]	FTL_1328	SpI	OM (9.93)	X, Y	21,38,40,42,45,46	M,[21,23,47,48] T,[35] S[25,28]
9−10	could not be identified with confidence				X		
11	acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit (AccB)[f]	FTL_1592		unknown	X, Y	21,39,40,43−46	M[21,47]
12	acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit (AccB)[f]	FTL_1592		unknown	X	39,40,43−46	M[21,47]
13	hypothetical protein[f]	FTL_0617		Cyto (8.96)	X	21,38,39,45	M,[21,37,49] T,[35,41] S[25]
	50S ribosomal protein L9 (RplI)[f]	FTL_1026		Cyto (9.97)		38,40,45,46	T[35]
	F0F1 ATP synthase subunit delta (AtpH)	FTL_1798		Cyto (9.26)			M[49]
14	50S ribosomal protein L7/L12 (RplL)[f]	FTL_1745		unknown	X, Y	21,38−40,42,46	M[21]
15	F0F1 ATP synthase subunit epsilon (AtpC)	FTL_1794	NC (SP)	unknown	X		M[49]
16	hypothetical protein	FTL_0105	SpI	unknown	X, Y	40,45,46	M[48]
17	30S ribosomal protein S7 (RpsG)	FTL_0233		Cyto (9.97)	X		
	50S ribosomal protein L5 (RplE)	FTL_0248		Cyto (9.97)			
	30S ribosomal protein S5 (RpsE)[e]	FTL_0253	NC (SP)	Cyto (9.26)		46	
	50S ribosomal protein L15 (RplO)	FTL_0255	NC (SP)	Cyto (9.26)			
	outer membrane protein OmpH[f,e]	FTL_0536	SpI	unknown		38,40	M[37,47]
	(3*R*)-hydroxymyristoyl-ACP dehydratase (FabZ)[e]	FTL_0538		Cyto (9.97)			
	50S ribosomal protein L13 (RplM)	FTL_1187	NC (SP)	Cyto (9.26)			
18	hypothetical protein	FTL_0571	SpII	unknown	X	45	M[47]
19	could not be identified with confidence				X		
20	30S ribosomal protein S7 (RpsG)	FTL_0233		Cyto (9.97)	X		
	50S ribosomal protein L16 (RplP)	FTL_0243		Cyto (9.97)			
	50S ribosomal protein L5 (RplE)	FTL_0248		Cyto (9.26)			
	30S ribosomal protein S8 (RpsH)	FTL_0250		Cyto (9.26)			
	30S ribosomal protein S5 (RpsE)[e]	FTL_0253	NC (SP)	Cyto (9.26)		46	
	50S ribosomal protein L15 (RplO)	FTL_0255	NC (SP)	Cyto (9.26)			
	50S ribosomal protein L17 (RplQ)	FTL_0262		Cyto (9.97)			
	hypothetical protein (annotated as a pseudogene)[e]	FTL_0349		unknown			
	peptide methionine sulfoxide reductase (MsrB)[e]	FTL_0379	SpI	Cyto (9.26)			
	lipoprotein (LpnA)[f]	FTL_0421	SpII	OM (10.00)		21,40,43,44,46	M,[21,23,47,49] S[26]
	30S ribosomal protein S9 (RpsI)	FTL_1186	NC (SP)	Cyto (9.97)			
	50S ribosomal protein L10 (RplJ)	FTL_1746		Cyto (9.26)			M[37]

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Table 1. continued

spot no.	protein name	locus[a]	signal peptide[b]	PSORTb localization (score)[c]	sera reactivity[d]	previously identified as immunoreactive and reference	previously identified as membrane (M), secreted (T), or surface (S) protein and reference
	50S ribosomal protein L11 (RplK)[e]	FTL_1748	NC (SP)	Cyto (9.26)			T[35]
	Sau5/YciO/YrdC family protein[e]	FTL_1913		unknown			
21	lipoprotein (LpnA)[f]	FTL_0421	SpII	OM (10.00)	X	21,40,43,44,46	M,[21,23,47,49] S[26]
22	lipoprotein (LpnA)[f]	FTL_0421	SpII	OM (10.00)	X	21,40,43,44,46	M,[21,23,47,49] S[26]
	50S ribosomal protein L10 (RplJ)	FTL_1746		Cyto (9.26)			M[37]
23	50S ribosomal protein L10 (RplJ)	FTL_0235		Cyto (9.26)	X		
	50S ribosomal protein L22 (RplV)[e]	FTL_0241		Cyto (9.26)			
	50S ribosomal protein L24 (RplX)	FTL_0247		Cyto (9.97)			
	hypothetical protein[f]	FTL_0617		Cyto (8.96)		21,38,39,45	M,[21,37,49] T,[35,41] S[25]
	histone-like protein HU form B (HupB)[e]	FTL_0895	NC (SP)	Cyto (9.26)		21	M,[21] T[35]
24	histone-like protein HU form B (HupB)[e]	FTL_0895	NC (SP)	Cyto (9.26)	X	21	M,[21] T[35]
	50S ribosomal protein L31 (RpmE)[e]	FTL_1303	NC (SP)	Cyto (9.26)			
	30S ribosomal protein S16 (RpsP)[e]	FTL_1738		Cyto (9.26)			
25	pyruvate dehydrogenase, E1 component (AceE)	FTL_0309		Cyto (9.97)	Y	40,50	M[23,50]
26	chitinase family 18 protein (ChiA)[f,e]	FTL_1521	SpI	unknown	Y	38,45,46	M[23]
27	peroxidase/catalase (KatG)[f]	FTL_1504	NC (SP)	Cyto (9.26)	Y	21,38−40,42,45,46,50	M,[21,23,47,50] T[35,41]
28	chaperone protein DnaK[f]	FTL_1191	NC (SP)	Cyto (9.97)	Y	21,38−40,42−46,50	M,[21,23,50] T,[35,41] S[25]
29	dihydrolipoamide acetyltransferase (AceF)	FTL_0310	NC (SP)	Cyto (9.97)	Y	21,40,45,50	M[21,37,47,50]
	outer membrane associated protein FopA1[f]	FTL_1328	SpI	OM (9.93)		21,38,40,42,45,46	M,[21,23,47,48] T,[35] S[25,28]
	chaperonin GroEL[f]	FTL_1714		Cyto (9.97)		21,38−40,42−46	M,[21,23,43] T,[35,41] S[25]
30	chaperonin GroEL[f]	FTL_1714		Cyto (9.97)	Y	21,38−40,42−46	M,[21,23,43] T,[35,41] S[25]
	dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (SucB)	FTL_1783		Cyto (9.97)		21,40,45,46,50	M[21,37,50]

[a]NCBI reference sequence identification codes matching each loci are listed in Table S2 in the Supporting Information. [b]Signal peptide prediction; SpI, signal peptide cleaved by signal peptidase I; SpII, signal peptide cleaved by signal peptidase II; NC (SP), nonclassical signal peptide. [c]Subcellular localization predicted with PSORTb. Peri, periplasmic; Cyto, cytosol; OM, outer membrane; CM, cytoplasmic membrane. [d]X and Y designate proteins were reactive to sera from mice immunized with MPF and *F. tularensis* LVS vaccination, respectively. [e]Protein was not identified in the multidimensional LC−MS/MS analysis of MPF. [f]Protein was identified on the surface of *F. tularensis* LVS in this study.

higher isoelectric points were well-resolved with a basic pH gradient (Figure S1B in the Supporting Information).

Western blot analyses with MPF immune serum revealed 16 and 8 immunoreactive protein spots in MPF preparations resolved with the pH 4−7 and pH 6−11 IEF gradients, respectively (Figure 2A,B). Additionally, immune serum from mice vaccinated with *F. tularensis* LVS was evaluated to compare effective humoral-based immune responses targeting MPF components.[22] Immune serum from mice vaccinated with *F. tularensis* LVS was reactive to 11 protein spots (Figure 2C), of which five were also recognized by the anti-MPF immune sera.

Thirty immuno-reactive protein spots (those numbered in Figure S1 in the Supporting Information and Figure 2) were subject to LC−MS/MS analyses for protein identification (Table 1 and Table S2 in the Supporting Information). Q-TOF-based identifications were successful for 18 of these protein spots, and six additional spots (spots 1, 13, 17, 20, 23 and 24) of low protein abundance were identified using an Orbitrap platform. In total, 45 proteins were identified from the immunoproteome analyses, thus significantly reducing the number of potential target immunogens. Multiple proteins were identified in 10 of the protein spots (spots 1, 6, 13, 17, 20, 22−24, 29, and 30). In particular, protein spots 17, 20, and 23 were composed of a large number of proteins (7, 14, and 5 proteins, respectively), but this was not unexpected given the poor resolution in these areas of the 2D-gels (Figure S1 in the Supporting Information). A majority of the proteins identified from 2D-gels were also identified in the multidimensional LC−MS/MS analyses of MPF (30 proteins). Fifteen proteins detected from 2D-gels but not identified in the multidimensional LC−MS/MS approach were present in protein spots 1, 17, 20, 23, 24, and 26. Thus, the total number of MPF proteins was increased to 299 (Figure 1).

The dominant antigens reactive to the MPF immune sera displayed a mass of <50 kDa. Single proteins were identified in protein spots 2, 5, 8, 11, 12, 14−16, 18, 21, and 25−28. Protein spot 5 was identified as outer-membrane-associated protein (FopA1, FTL_1328). Spot 8 (~36 kDa) was also identified as FopA1, despite its lower observed molecular mass as compared with spot 5 (~43 kDa). This finding was in agreement with a previous report where immunoreactive FopA1 resolved at multiple molecular masses and similar isoelectric points.[23] When multiple proteins were identified in a single spot (spots

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1, 6, 13, 17, 20, 22−24, 29, and 30), the identification of the specific anti-MPF reactive antigen(s) was difficult. Nonetheless, previously identified immunoreactive proteins were present in each of these spots. (See Table 1.) The proteins of six immunoreactive spots (spots 3, 4, 7, 9, 10, and 19) could not be identified with confidence, presumably due to low abundance (Figure S1A in the Supporting Information and Figure 1B). Several areas (~20 kDa and 75 to 100 kDa) of the anti-MPF pH 4−7 MPF Western blot displayed immunoreactivities defined by a smear (Figure 2A). These smears occurred in areas with minimal protein, as detected by silver staining (Figure S1A in the Supporting Information), and thus were not pursued for protein identification.

When *F. tularensis* LVS immune sera was used to probe 2D-Western blots of MPF, there were fewer immunoreactive protein spots as compared with the MPF immune sera (compare Figure 2A,B to 2C). These 11 protein spots (spots 2, 8, 11, 14, 16, and 25−30) resolved only in the pH range of 4−7 and were predominantly of a mass greater than or equal to 50 kDa (spots 2 and 25−30) (Figure 2C). From these spots, 15 proteins were identified by MS (Table 1). The proteins in spots 25−30 were uniquely recognized by the *F. tularensis* LVS immune sera. Five protein spots were reactive with both MPF and *F. tularensis* LVS immune sera (spots 2, 8, 11, 14, and 16).

3.3. Surface-Exposed Proteome of *F. tularensis* LVS

Immunogenic proteins are often exposed on the surface of bacteria, and we hypothesized that this is true for the proteins targeted by the protective IgG response against *F. tularensis*.[12,13] Bioinformatics analyses of the 45 MPF proteins identified in immunoproteome analyses predicted that 71.1% (32 of 45) could be surface-localized. This was an overall 89% reduction in the potential MPF target immunogens (Figure 1). However, the bioinformatics applied were not specifically designed to predict surface localization. As such, a direct analysis of surface protein localization on *F. tularensis* LVS was performed using a membrane-impermeable biotin label, LC-Biotin. Initial experiments to optimize surface labeling were performed with LC-Biotin concentrations in a range between 6.6 and 26.4 mg/mL, with incubation times of 10, 30, 60, and 120 min (data not shown). This established that treatment of freshly harvested *F. tularensis* with 6.6 mg/mL LC-Biotin for 60 min provided optimal labeling. After surface labeling of *F. tularensis* LVS, the proteins were extracted from the cells and detected by Western blot using an HRP-conjugated antibiotin antibody as the probe (Figure 3, lane 3). This resulted in the detection of 17 distinct bands. In comparison, labeling of *F. tularensis* LVS cytosolic proteins as a control resulted in a different and more complex pattern of proteins (Figure 3, lane 2), thus providing further evidence that labeling of intact cells was selective for surface-exposed products.

Identification of the surface proteins was facilitated by purification of the biotin-tagged products with immobilized streptavidin affinity chromatography. Antibiotin Western blot analysis of the purified material demonstrated the enrichment of 23 antibiotin reactive bands (Figure 4A). An aliquot of affinity-purified material was resolved by SDS-PAGE, the gel was cut into nine slices based on molecular mass (Figure 4B), and LC−MS/MS-based protein identification resulted in 31 proteins (Table 2 and Table S3 in the Supporting Information). A positive correlation was observed between the calculated molecular mass of each protein identified and the observed molecular mass based on SDS-PAGE migration. Of the 31

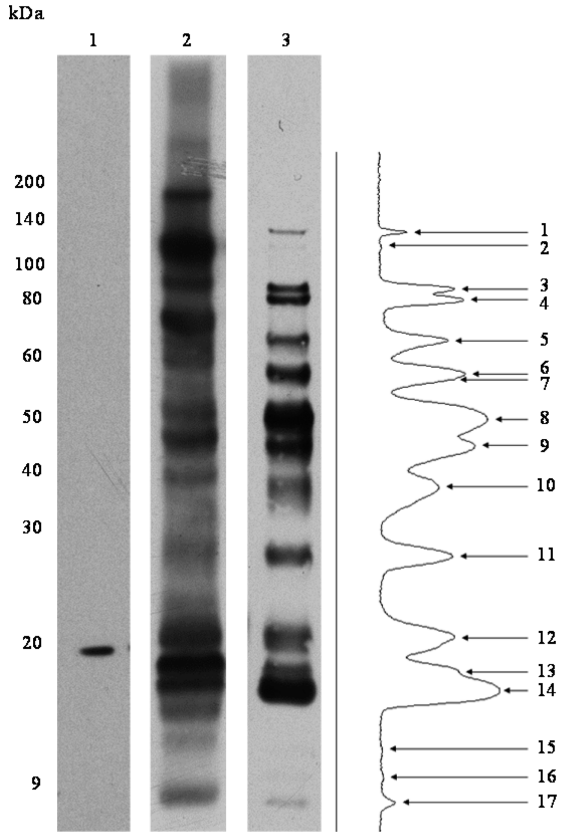

Figure 3. Biotinylation of *F. tularensis* LVS surface-exposed proteins. Antibiotin Western blots to detect biotinylated proteins after labeling *F. tularensis* LVS surface proteins with LC-Biotin. Lane 1, WCL of *F. tularensis* LVS unlabeled control; Lane 2, labeled *F. tularensis* LVS WCL (15 s exposure); Lane 3, labeled *F. tularensis* LVS intact cells (2 min exposure) accompanied by densitometry analysis of reactive protein bands.

identified surface-exposed proteins, 27 were detected in the MPF by multidimensional LC−MS/MS and 2D-PAGE. A bioinformatic analysis including previous literature and predictive algorithms revealed only 4 of the 31 identified surface proteins lacked supporting evidence of membrane/surface localization (Table 2). Prior studies directly identified 12 *F. tularensis* or *F. novicida* proteins as surface-localized.[24−29] However, only five of these were identified by our surface-labeling analyses (Table 2) and also were the only previously defined surface proteins identified in the immunoproteome analyses (Table 1).

The results of the predictive bioinformatic algorithms as well as predicted function indicate that nearly half of the 31 identified surface proteins could be classified as cytosolic products. Thus, the application of a reverse vaccinology immunogen selection approach based solely on bioinformatics algorithms for subcellular localization would have resulted in greater ambiguity in target immunogen selection. As with membrane proteins, experimental identification of known or predicted cytosolic proteins in subcellular fractions of surface proteins is not uncommon. There is a growing list of presumed intracellular proteins identified on the surface of pathogenic bacteria that appear to be multifunctional based on their subcellular location.[30] For example, we identified glycolytic enzyme 2-phosphoglycerate dehydratase GapA (FTL_1146) as being surface-exposed. This is consistent with findings in both

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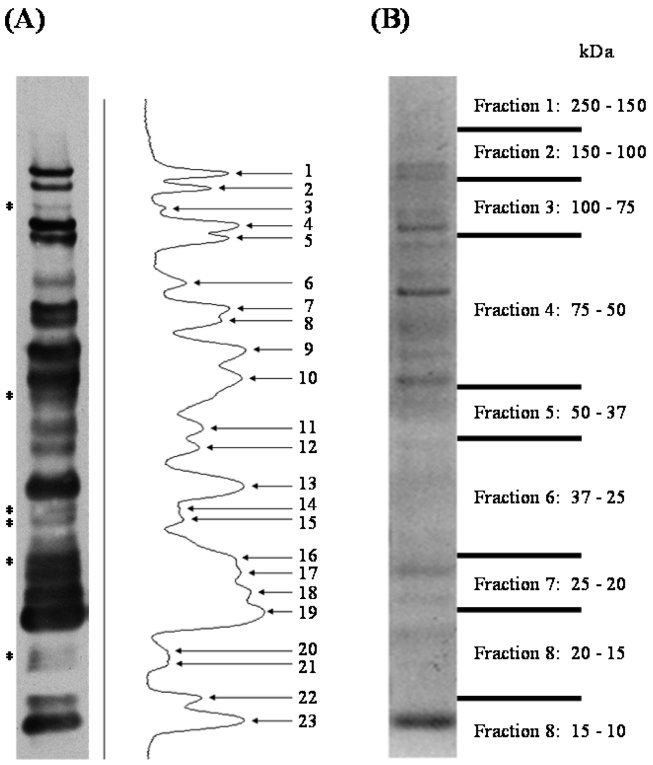

Figure 4. Purified *F. tularensis* LVS LC-Biotin labeled surface proteins. Biotinylated surface proteins purified using immobilized streptavidin were separated by SDS-PAGE and analyzed by antibiotin Western blot. (A) Antibiotin Western blot of purified biotinylated surface proteins accompanied by densitometry analysis of reactive protein bands. (B) Simply Blue stained gel of affinity-purified material. Gel fractions excised for LC−MS/MS analysis are denoted on the right by the molecular mass range. *indicates reactive protein bands only visualized after affinity purification.

Gram-negative and Gram-positive pathogens where this protein was demonstrated to participate in bacterial adhesion and was identified on cell surfaces despite lacking predicted signal peptides.[19,31] Likewise, TufA (FTL_1751) was observed as surface-associated, and homologues of this gene product in *Lactobacillus*, *Mycoplasma*, and *Pseudomonas* species are described as acting as adhesions and plasminogen ligands on the bacterial surface.[20,32] The bacterial GroEL and DnaK chaperones or stress response proteins are also known to interact with the innate immune response and enhance antigen presentation.[33] Although they are cytosolic functioning chaperones, they have been noted as surface-exposed in other bacteria.[34] Thus, the surface presentation of GroEL (FTL_1714) and chaperone protein DnaK (FTL_1191) in *F. tularensis* was not unexpected. Recently, Konecna et al. identified 22 predicted cytosolic proteins that were expelled into the culture supernatant by *F. tularensis* LVS and SCHU S4;[35] four of these were found in our analyses as surface products of *F. tularensis* LVS including GroEL (FTL_1714), glutamate dehydrogenase (Gdh, FTL_0269), GapA (FTL_1146), and hypothetical protein FTL_0617. An alternative explanation for identification of predicted cytosolic proteins as surface structures is unexpected membrane permeability of the LC-Biotin reagent and labeling of the most abundant cytosolic proteins. However, this seems unlikely as the protein profile obtained from cell surface labeling was markedly different from that obtained by LC-Biotin labeling of WCL. It is also possible that some cytosolic contaminants may have been copurified with biotinylated surface proteins.

3.4. Grouping and Prioritization of MPF Immunogens

When *F. tularensis* LVS surface protein characterization was combined with the bioinformatics and the immunoproteome analyses, the potential number of immunotherapeutic targets in MPF is reduced from a total of 299 to 13 (Figure 1 and Table 2), a 96% reduction in target complexity. However, the further grouping of MPF surface immunogens based on reactivity to MPF and *F. tularensis* LVS immune sera provides an additional means to prioritize the testing of multiple purified proteins in a postexposure vaccine model. Such immunogen grouping also enables the ability to test whether postexposure vaccine candidates can be identified and selected based on differential reactivity to various immunization approaches.

The immunoreactive proteins of the MPF could be placed into three seroreactive groups: group-1, proteins recognized by both MPF and *F. tularensis* LVS immune sera; group-2, proteins recognized specifically by *F. tularensis* LVS immune sera; and group-3, proteins recognized specifically by MPF immune sera.

Five proteins of MPF fell into group-1: TufA (FTL_1751); FopA1 (FTL_1328); acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit (AccB, FTL_1592); 50S ribosomal protein L7/L12 RplL (FTL_1745); and hypothetical protein FTL_0105 (Table 1). All but one of these proteins (FTL_0105) were also identified as surface antigens of *F. tularensis* LVS (Table 2). Of the group-1 MPF products, FopA1 has been the most extensively evaluated as a surface-exposed immunogen of *F. tularensis*.[9,23,25] FopA1 and anti-FopA1 antibodies provided prophylactic protection against a lethal *F. tularensis* LVS challenge in mice but not against type A *F. tularensis*.[10,36] Huntley et al. demonstrated that immunization with an outer membrane protein adjuvant complex, including FopA1, produced high titers of IgM and IgG (IgG2a and IgG3) and provided a significant level of protection against type A *F. tularensis*.[9] This suggested that a multiple immunogen complex including FopA1 is required to elicit prophylactic protection. We hypothesize the same would be required for a immunotherapeutic vaccine, and the group-1 proteins represent those selected by the reverse vaccinology approach as being the highest priority to test.

Eight proteins were identified in the six immunoreactive spots that were exclusively reactive to sera from vaccination with *F. tularensis* LVS (Table 1). Of these eight group-2 proteins, four were also identified as surface-localized (Table 2): DnaK (FTL_1191), KatG (FTL_1504), ChiA (FTL_1521), and GroEL (FTL_1714). Given the demonstrated effectiveness of *F. tularensis* LVS vaccination, it could be hypothesized that the *F. tularensis* LVS immunoreactive proteins might also be effective as immunotherapeutic targets. Additionally, it is noted that all group-2 proteins were previously recognized as immunogens (Table 1).

A total of 35 proteins were identified in the 13 protein spots immunoreactive only to the anti-MPF sera (group-3), with 12 of these previously identified as antigens (Table 1). The inclusion of surface-labeling data reduced the number of potential group-3 immunotherapeutic targets to five: LpnA (FTL_0421), hypothetical protein FTL_0617, RplI (FTL_1026), OmpH (FTL_0536), and GapA (FTL_1146). The most notable of these is LpnA (FTL_0421), which has previously been studied as a subunit vaccine immunogen.[10]

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Table 2. Surface-Associated Proteins of *F. tularensis* LVS

protein name	locus[a]	signal peptide[b]	PSORTb localization (score)[c]	sera reactivity[d]	previously identified as membrane (M), secreted (T), or surface (S) protein and reference
			Group 1 Surface Immunogens[e]		
outer membrane associated protein FopA1[f]	FTL_1328	SpI	OM (9.93)	X,Y	M,[21,23,47,48] T,[35] S[25,28]
acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit (AccB)[f]	FTL_1592		unknown	X,Y	M[21,47]
50S ribosomal protein L7/L12 (RplL)[f]	FTL_1745		unknown	X,Y	M[21]
elongation factor Tu (TufA)[f]	FTL_1751		Cyto (9.97)	X,Y	M[21]
			Group 2 and 3 Surface Immuogens[e]		
lipoprotein (LpnA)[f]	FTL_0421	SpII	OM (10.00)	X	M,[21,23,47,49] S[26]
outer membrane protein OmpH[f]	FTL_0536	SpI	unknown	X	M[37,47]
hypothetical protein[f]	FTL_0617		Cyto (8.96)	X	M,[21,37,49] T,[35,41] S[25]
50S ribosomal protein L9 (RplI)[f]	FTL_1026		Cyto (9.97)	X	T[35]
glyceraldehyde-3-phosphate dehydrogenase (GapA)[f]	FTL_1146		Cyto (9.97)	X	T[35,41]
chaperone protein DnaK[f]	FTL_1191	NC (SP)	Cyto (9.97)	Y	M,[21,23,50] T,[35,41] S[25]
peroxidase/catalase (KatG)[f]	FTL_1504	NC (SP)	Cyto (9.26)	Y	M,[21,23,47,50] T[35,41]
chitinase family 18 protein (ChiA)[f]	FTL_1521	SpI	unknown	Y	M[23]
chaperonin GroEL[f]	FTL_1714		Cyto (9.97)	Y	M,[21,23,43] T,[35,41] S[25]
			Nonimmunoreactive Surface Proteins		
outer membrane protein	FTL_0009	SpI	Peri (9.84)		M,[21,37,47,49] T[35]
intracellular growth locus, subunit B (IglB)[f]	FTL_0112		Cyto (9.97)		M[50]
intracellular growth locus, subunit C (IglC)[f]	FTL_0113		unknown		M,[21,37] T[51]
elongation factor G (FusA)[f]	FTL_0234		Cyto (10.00)		
heat shock protein 90 (HtpG)[f]	FTL_0267		Cyto (9.97)		
glutamate dehydrogenase (Gdh)[f]	FTL_0269		unknown		M,[37] T[35]
OmpA family protein[f]	FTL_0325	SpII	unknown		M[21,23,47]
peptidoglycan-associated lipoprotein[f]	FTL_0336	SpII	OM (10.00)		M[23,47−49]
hypothetical protein[f]	FTL_0569	SpI	unknown		
AhpC/TSA family protein[f]	FTL_1015	NC (SP)	unknown		M,[21] T[35,41]
lipoprotein (DsbG, FipB)[f]	FTL_1096	SpII	unknown		M[23,37,47−49,52]
hypothetical protein	FTL_1225	SpII	unknown		
hypothetical protein	FTL_1494	SpII	unknown		
succinyl-CoA synthetase subunit beta (SucC)[f]	FTL_1553		Cyto (8.96)		M,[50] T[41]
aconitate hydratase (AcnA)[f]	FTL_1772		Cyto (9.97)		M[21,37,50]
citrate synthase (GltA)	FTL_1789		Cyto (9.97)		
cell division protein FtsZ[f]	FTL_1907		Cyto (9.12)		M[21,49]
30S ribosomal protein S1 (RpsA)[f]	FTL_1912		Cyto (9.97)		

[a]NCBI reference sequence identification codes matching each loci are listed in Table S3 in the Supporting Information. [b]Signal peptide prediction; SpI, signal peptide cleaved by signal peptidase I; SpII, signal peptide cleaved by signal peptidase II; NC (SP), nonclassical signal peptide. [c]Subcellular localization predicted with PSORTb; Peri, periplasmic; Cyto, cytosol; OM, outer membrane; CM, cytoplasmic membrane. [d]X and Y designate proteins were reactive to sera from mice immunized with MPF and *F. tularensis* LVS vaccination, respectively. [e]Group 1 immunogens are jointly recognized by *F. tularensis* LVS immune sera, group 2 immunogens by *F. tularensis* LVS immune sera, and group 3 immunogens by MPF immune sera. [f]Indicates the protein was identified in the MPF by multidimensional LC−MS/MS or from MPF 2D-PAGE protein spots.

4. CONCLUSIONS

Our previous studies demonstrated that passive transfer of sera from MPF immunized mice to naïve *F. tularensis* SCHU S4 infected animals enhanced gentamicin therapy and the sera possessed high titers of IgM and IgG (IgG2a and IgG3), targeting LPS and MPF proteins, respectively.[7] Moreover, it was observed that with the MPF postexposure vaccination the presence of an IgG response at day-7 corresponded to initial recovery from infection.[7] This work confirmed that the humoral immune response significantly contributes to the control and clearance of *F. tularensis* infections.[5,9,10] However, it did not define the molecular identity of proteins that were immunogenic with MPF postexposure vaccination. In this present study, we characterized the MPF proteome as being composed of at least 299 proteins, identified the repertoire of MPF proteins recognized by MPF immunization, and compared these with the MPF proteins recognized by *F. tularensis* LVS vaccination. This reduced the number of target immunogens to 45, and bioinformatics indicated that 32 of these had the potential to be surface-localized. To further reduce the complexity of immunogens prioritized as immunotherapeutic targets, a direct analysis of *F. tularensis* LVS surface proteins was performed. This reduced the complexity of target immunogens to 13. Thus, comparative proteomics evaluations of MPF based on serological reactivity and surface localization provided a means to select and prioritize potential immunogens for further evaluation. From

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the collective data, we hypothesize that 13 proteins on the surface of *F. tularensis* and found to be immunoreactive compose the primary candidates for a defined postexposure vaccine. These 13 candidates are FopA1 (FTL_1328), AccB (FTL_1592), RplL (FTL_1745), TufA (FTL_1751), OmpH (FTL_0536), LpnA (FTL_0421), FTL_0617, RplI (FTL_1026), GapA (FTL_1146), DnaK (FTL_1191), KatG (FTL_1504), ChiA (FTL_1521), and GroEL (FTL_1714).

■ ASSOCIATED CONTENT

Supporting Information

Table S1: Complete list of the 284 proteins identified in MPF by multidimensional LC−MS/MS, accompanied by corresponding MS/MS data and bioinformatic predictions. Table S2: LC−MS/MS analyses of MPF 2D-PAGE resolved protein spots. Table S3: LC−MS/MS analyses of the identified surface proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MPF, membrane protein fraction; type A, *F. tularensis* subsp. *tularensis*; type B, *F. tularensis* subsp. *holarctica* (type B); LVS, live vaccine strain; CLDC, cationic liposomal DNA complex; LC-biotin, EZ-Link sulfo-NHS-LC-biotin; WCL, whole cell lysate

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