

Identification, Recombinant Expression, Immunolocalization in Macrophages, and T-Cell Responsiveness of the Major Extracellular Proteins of *Francisella tularensis*

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A safer and more effective vaccine than the previously developed live attenuated vaccine is needed for combating *Francisella tularensis*, a highly infectious bacterial pathogen. To search for potential candidates for inclusion in a new vaccine, we characterized the proteins present in the culture filtrates of a virulent recent clinical isolate and the attenuated live vaccine strain of *F. tularensis* using a proteomic approach. We identified a total of 12 proteins; among these, catalase-peroxidase was much more abundant in the culture filtrate of the virulent clinical isolate, whereas bacterioferritin was more abundant in the culture filtrate of the live vaccine strain. Streptolysin O treatment of infected human macrophages indicated that catalase-peroxidase and the heat shock protein GroEL are released intracellularly by actively growing *F. tularensis*. Mice immunized with *F. tularensis* developed significant cell-mediated immune responses to catalase-peroxidase, the heat shock protein GroEL, and bacterioferritin as measured by splenic lymphocyte proliferation and gamma interferon production. Finally, we expressed the major culture filtrate proteins that are promising vaccine candidates in *Escherichia coli* at high levels in soluble form to facilitate study of their immunobiology and potential role in vaccines.

The gram-negative bacterium *Francisella tularensis* is the causative agent of the zoonotic disease tularemia. Humans acquire tularemia from contact with infected tissues or materials, insect bites, consumption of contaminated food or water, or inhalation of aerosols (35). *F. tularensis* consists of three subspecies—*tularensis*, *holarctica*, and *mediasiatica*—which differ in their geographic distributions and in their virulence in humans (9). *F. tularensis* subsp. *tularensis* (found almost exclusively in North America) is highly virulent for humans. As few as 10 organisms subcutaneously or 25 organisms by inhalation can lead to a severe infection (31, 32). *F. tularensis* subsp. *holarctica* (found in North America and in Europe) and subsp. *mediasiatica* (found in Asia) are of lower virulence. Because of its high infectivity and capacity to cause severe morbidity and mortality, *F. tularensis* subsp. *tularensis* is considered a potential agent of bioterrorism.

An attenuated live vaccine strain (LVS) derived from a colony variant of *F. tularensis* subsp. *holarctica*, developed in the former Soviet Union in the 1930s, confers substantial protective immunity against virulent *F. tularensis* in humans. However, this vaccine suffers major drawbacks. First, the vaccine does not provide full protection against aerosol challenge with relatively low doses (10 to 50 organisms) of virulent *F. tularensis* (32). Second, the vaccine retains significant virulence raising concerns regarding its safety, especially in children and immunocompromised individuals. In addition, the immunoprotective moieties in the vaccine have not been determined. In view

of these drawbacks, a safer and more effective vaccine against tularemia is needed.

As a first step toward designing a better vaccine, we sought to identify protein candidates for inclusion in a vaccine. *F. tularensis* is a facultative intracellular bacterium that retards the maturation of its phagosome and subsequently escapes into and multiplies within the cytoplasm of host macrophages (8, 13). In the case of the intracellular pathogens *Legionella pneumophila* and *Mycobacterium tuberculosis*, we have previously shown that proteins that are secreted or abundantly released by these bacteria can be formulated into vaccines that confer immunoprotection in animal models of the diseases caused by the pathogens (5, 15, 26). With this concept in mind, we have sought in this study to identify the major proteins exported by *F. tularensis*. To achieve this, we have employed ³⁵S metabolic radiolabeling, one- and two-dimensional polyacrylamide gel electrophoresis, and N-terminal amino acid sequencing to evaluate the proteins released by *F. tularensis* growing in a defined medium. We have compared the protein profile obtained from a virulent recent clinical isolate (RCI) of *F. tularensis* subsp. *tularensis* with that from the attenuated *F. tularensis* subsp. *holarctica* (LVS). Moreover, we have cloned and expressed genes coding for five of the *F. tularensis* major extracellular proteins that we postulate are promising vaccine candidates as soluble recombinant proteins. One of these, catalase-peroxidase (KatG), one of the most abundant proteins in the culture filtrate of the virulent clinical isolate of *F. tularensis*, exhibits a functional signal peptide and is exported by the recombinant *Escherichia coli* host cells.

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MATERIALS AND METHODS

Bacteria. *F. tularensis* live vaccine strain and a virulent recent clinical isolate (NY 96-3369) were obtained from the Centers for Disease Control and Prevention (Atlanta, GA). Bacteria were cultivated on chocolate II agar (BD BBL,

Sparks, MD), passaged through THP-1 cells, a human monocytic cell line (American Type Culture Collection), and stored at -80°C (8).

Culture media. Chemically defined Chamberlain medium was prepared as described previously (7). All ingredients used in preparation of the medium were purchased from Sigma (St. Louis, MO). The medium was stirred overnight and sterilized by passing through a $0.2\text{-}\mu\text{m}$ filtration unit. The final medium had a pH of 6.3 to 6.5. Mueller-Hinton broth (BD BBL) was supplemented with 0.025% ferric pyrophosphate and 2% IsoVitalX (BD BBL). Brain heart infusion broth (BD BBL) was supplemented with 1% β -cyclodextrin (Sigma). Tryptic soy broth (BD BBL) was supplemented with 0.1% cysteine hydrochloride (Sigma).

Bacterial cultures. Overnight cultures of *F. tularensis* on chocolate II agar plates were scraped and washed twice in normal saline and resuspended in Chamberlain medium to an optical density at 540 nm (OD_{540}) of 0.1. Bacterial cultures were grown at 37°C in Erlenmeyer flasks equipped with $0.2\text{-}\mu\text{m}$ filter caps (Corning Incorporated, Corning, NY), rotated at 200 rpm, and harvested at selected time points by centrifugation at 3,500 rpm and 4°C for 30 min. Culture supernatant was passed sequentially through $0.45\text{-}\mu\text{m}$ and $0.2\text{-}\mu\text{m}$ filters. Culture supernatants with a volume of 200 ml or less were concentrated using 5-kDa cutoff Centricon Plus-20 centrifugal filter devices (Millipore Corporation, Bedford, MA). Culture supernatants with a volume of greater than 200 ml were first concentrated using an Amicon ultrafiltration cell with a YM10 membrane (Millipore) and then further concentrated using a Centricon Plus-20 centrifugal filter device. Bacterial pellets were resuspended in Dulbecco's phosphate-buffered saline (PBS) and subjected to sonication on ice with a W-375 sonication Ultrasonic processor (Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) at 50% duty cycle with a pulse and strength setting of 5 for three 1-min sessions. Insoluble material and unbroken bacteria were removed by centrifugation. Protein concentration in the culture filtrate and in the clear lysate obtained from bacterial sonication was determined by bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL) with albumin as the standard.

Radiolabeling of culture filtrate proteins. *F. tularensis* RCI was cultured in Chamberlain medium for 4 h at 37°C to an OD_{540} of 0.4 to 0.5. The bacteria were pelleted by centrifugation, washed once in normal saline, and suspended in methionine-free Chamberlain medium. [^{35}S]methionine (Amersham Pharmacia Biotech, Little Chalfont, England) was added to the culture to a final concentration of 100 $\mu\text{Ci}/\text{ml}$, the culture was incubated for 1 h, and the radiolabeled culture filtrate was obtained and processed as described above for nonradiolabeled bacterial cultures.

LDH assay. The activity of lactate dehydrogenase (LDH) was measured by a modification of the method of Reeves and Fimognari (29). Concentrated protein samples from 20-ml equivalent of culture filtrate or 1-ml equivalent of bacterial lysate were added to 0.85 ml of PBS, 50 μl of NADH (2.5 mg/ml), and 50 μl of sodium pyruvate (2.5 mg/ml). The decrease in optical density at 340 nm was measured over a 2-min period at room temperature in an optical cuvette with 1.0-cm path length. One unit of LDH activity is defined as the amount of enzyme required to produce 1.0 μmol of lactate within a minute.

Protease assays. Protease activity was examined both by zymogram analysis and a quantitative colorimetric protease assay. Proteins concentrated from 40 ml of culture filtrate were examined by zymogram analysis using polyacrylamide gels embedded with 10% casein or 12% gelatin (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Proteinase K (Epicentric, Madison, WI) (1 μg) was loaded onto the same zymogram gel as a positive control. Culture filtrate proteins harvested from 200-ml bacterial cultures were desalted by passing through an Excellulose GF-5 column (Pierce). Protein effluent was monitored at 280 nm, combined, and concentrated for use in the assay. Protease was quantified using the QuantiCleave protease assay kit (Pierce). A standard curve was prepared using tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK) trypsin at concentrations of 0.5 mg/ml to 0.5 $\mu\text{g}/\text{ml}$.

Two-dimensional polyacrylamide gel electrophoresis (2-DE). Culture filtrate proteins (200 μg) were dissolved in sample buffer containing 8 M urea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), and 0.2% (wt/vol) Bio-Lyte 3/10 ampholytes and separated using an isoelectric focusing (IEF) tube gel (16 cm \times 1.5 mm) (ratio of Biolyte 5/7 ampholytes to Biolyte 3/10 ampholytes of 4:1) at 200 V for 2 h, 500 V for 2 h, and 800 V for 14 h. The IEF gel was equilibrated for 15 min each time with buffer I containing 6 M urea, 2% sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 2% (wt/vol) DTT and buffer II containing 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 2.5% (wt/vol) iodoacetamide. Each equilibrated IEF gel was loaded onto a 12.5% SDS-polyacrylamide gel (16 cm \times 16 cm \times 1 mm). The second-dimension gel system was run at 20 mA for the stacking gel and 30 mA for the separating gel. The gels were fixed in 40% ethanol and 10% acetic acid for 1 h, stained with 0.1% (wt/vol) Coomassie

brilliant blue G, 10% ethanol, 2% phosphoric acid, and 1% (wt/vol) ammonium sulfate for 4 h, and destained with three or four changes of 10% acetic acid.

N-terminal amino acid sequencing. Proteins separated by one- or two-dimensional polyacrylamide gel electrophoresis were transferred to a polyvinylidene difluoride (PVDF) membrane in 10 mM CAPS [3-[cyclohexylamino]-1-propane sulfonic acid], pH 11, and stained with Coomassie brilliant blue R-250. Protein spots cut from the membrane were subjected to N-terminal amino acid sequencing by Edman degradation at the Molecular Structure Facility of the University of California, Davis.

Protein homology search. *F. tularensis* (Schu 4) genome was downloaded from the website <http://artedi.ebc.uu.se/Projects/Francisella/data>. A collection of open reading frames encoding ≥ 50 amino acids were translated using the computer program Artemis (30), and N-terminal amino acid sequences obtained for individual protein spots were searched. We assigned an open reading frame to a particular protein spot only if it met all three of the following criteria. (i) The open reading frame encoded a polypeptide whose N terminus (within the first three methionines of the open reading frame) matched the sequence of the first seven (or more) amino acid residues of the protein spot. (ii) This matched sequence was the only one found in the entire collection of open reading frames. (iii) The calculated molecular mass of the polypeptide predicted by the open reading frame approximated the size of the protein spot observed on SDS-polyacrylamide gels. We then conducted a BLAST search in the GenBank database using the entire protein sequence. We assigned a potential identity to an open reading frame on the basis of its high percentage of identity or similarity in primary sequence to a protein of a known physiological function and/or by virtue of its possessing one or more conserved domain(s) of such a protein.

Amplification of *F. tularensis* genes. Genomic DNA of *F. tularensis* RCI was isolated by the previously published method (28). *F. tularensis* RCI cultured in Chamberlain medium was pelleted by centrifugation and incubated at 50°C for 12 h in a lysis solution containing 10 mM NaCl, 20 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5% SDS, and 0.7 mg/ml protease. Bacterial lysate was treated with sodium perchlorate at room temperature for 1 h and extracted three times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). Nucleic acids were precipitated from the aqueous phase, resuspended, and treated with RNase at 37°C for 1 h. After another round of extraction with phenol-chloroform-isoamyl alcohol, genomic DNA was precipitated and dissolved in buffer containing 10 mM Tris-HCl, pH 8, and 1 mM EDTA. Primer pairs used for amplification of individual genes from the isolated *F. tularensis* RCI genomic DNA were as follows: 5'-CCGCTCGAGCATATGCTAAAGAAAATTGTAACTGCTTTAG GAATGTCTGGAATGCTACTAGC-3' and 5'-CGCTCGAGTTAACAAATT TATTGTTGAACATCAAATCTGCCAAGCATCATAACTTTATGCC-3' for *katG*; 5'-CGGGATCCATATGGCTGCAAAACAAGTTTTATTTTCAGAT GAAGTC-3' and 5'-CGGGATCCCTATTACATCATGCCAGGCATACCG CCCATGGCCACCGCC-3' for *groEL*; 5'-CGGGATCCCATATGTCAAAAAC AGCTGTAGTTTTTCTGGTCAAGGTTTC-3' and 5'-CGGGATCCCTAAAT ATTTTCTAAACTATCAACTACTGTTTGTATCTTTT-3' for *fabD*; 5'-CGGG ATCCGGCTTACCACATGATCTAATTGCGCTGTGCTGCATCGGC-3' and 5'-CGGGATCCAGCATCAAGATCAACAACCACTATTGATAAAACCCCT A-3' for *sodB*; and 5'-CGGGATCCCATCTTTAAAT GTACAGGTTGTATC TAGACTTTCTGC-3' and 5'-CGGGATCCGTCCTACTGAATATCTCG ATAG CGCATCTAGTGAATCCAAG-3' for *bfr*. DNA polymerase from the PfuUltra Hotstart (Stratagene, La Jolla, CA) or FailSafe (Epicentric, Madison, WI) system was used in the amplification reactions under a typical condition of 1 cycle at 95°C for 1 min; 30 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 to 2 min depending on the length of the target gene; and 1 cycle at 72°C for 10 min. The amplified product was gel purified and ligated with pZero (Invitrogen, Carlsbad, CA). The identity of each cloned gene was confirmed by nucleotide sequencing. Nucleotide sequencing was carried out by the sequencing core facility at the University of California, Los Angeles.

Cloning and expression of *F. tularensis* genes. The *groEL* and *fabD* genes were subcloned directly from pZero into pET15b (Novagen, Madison, WI) between the NdeI and BamHI restriction sites. The *sodB* and *bfr* genes were amplified from the corresponding pZero constructs with proper restriction sites and inserted between the NdeI and BamHI restriction sites on pET15b. For expression of the full-length *katG* gene, the gene was released from pZero with NdeI and XhoI and ligated with pET22b (Novagen) treated with the same enzymes. *F. tularensis* genes cloned into the expression vector pET15b produce recombinant proteins with a thrombin-cleavable histidine tag at the N terminus, and the *katG* gene cloned into pET22b produces a recombinant protein with the histidine tag fused to its C terminus. *E. coli* BL21 CodonPlus(DE3)-RIL (Stratagene) transformed with the pET15b or pET22b construct was first grown in LB medium to an absorbance at 540 nm of 0.4 and then induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 5 h before harvesting. The culture filtrate and