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A Mutant of *Francisella tularensis* Strain SCHU S4 Lacking the Ability To Express a 58-Kilodalton Protein Is Attenuated for Virulence and Is an Effective Live Vaccine

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***Francisella tularensis* subsp. *tularensis* (type A) strain SCHU S4 is a prototypic strain of the pathogen that is highly virulent for humans and other mammals. Its intradermal (i.d.) 50% lethal dose (LD_{50}) for mice is <10 CFU. We discovered a spontaneous mutant, designated FSC043, of SCHU S4 with an i.d. LD_{50} of $>10^8$ CFU. FSC043 effectively vaccinated mice against challenge with a highly virulent type A strain, and the protective efficacy was at least as good as that of *F. tularensis* LVS, an empirically attenuated strain which has been used as an efficacious human vaccine. Comparative proteomics was used to identify two proteins of unknown function that were identified as defective in LVS and FSC043, and deletion mutants of SCHU S4 were created for each of the two encoding genes. One mutant, the ΔFTT0918 strain, failed to express a 58-kDa protein, had an i.d. LD_{50} of $\sim10^5$ CFU, and was found to be less capable than SCHU S4 of growing in peritoneal mouse macrophages. Mice that recovered from sublethal infection with the ΔFTT0918 mutant survived when challenged 2 months later with >100 LD_{50}s of the highly virulent type A strain FSC033. This is the first report of the generation of defined mutants of *F. tularensis* subsp. *tularensis* and their use as live vaccines.**

Francisella tularensis is a pathogenic intracellular bacterium capable of causing infectious disease in more than 150 mammalian species. It can be transmitted via arthropod vectors and also via contaminated water and as an aerosol (19). There are four subspecies of *F. tularensis*, but only two, subsp. *tularensis* (type A) and subsp. *holarctica* (type B), are commonly infectious for humans (27). Generally, only type A strains of *F. tularensis* cause lethal infection in humans. Because of its high infectivity, ease of dissemination by aerosol, and capacity to cause severe morbidity and mortality, type A *F. tularensis* has long been considered a potential biological warfare agent (8). To date no specific virulence factors that explain the high virulence of type A strains have been identified (16, 26).

Live attenuated *F. tularensis* vaccines were developed in Russia in the 1950s based on type B strains. One of these strains, designated as the live vaccine strain (LVS), afforded effective albeit not complete protection against laboratory-acquired tularemia (3, 10, 22). However, the nature of the genetic lesion responsible for its attenuation, the protective antigens, and the immunological basis for its efficacy remain unknown (5). Moreover, in both human and animal studies, systemic vaccination with LVS provided suboptimal protection against aerosol challenge with type A *F. tularensis* (9, 13). For these reasons, LVS has never been fully licensed as a vaccine and, in turn, this has motivated a search for better-defined vaccines of equal or greater efficacy (5, 26). In the present study, we have identified genes that may explain the attenuation of spontaneous vaccine strains of *F. tularensis* and demonstrated that a defined gene deletion mutant of virulent type A strain SCHU S4 elicited protection against subsequent exposure to a fully virulent type A isolate.

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MATERIALS AND METHODS

Bacteria. *F. tularensis* LVS was originally obtained from the American Type Culture Collection (ATCC 29684). The *F. tularensis* subsp. *tularensis* strain FSC033/SnMF was originally isolated from a squirrel in Georgia (14). *F. tularensis* subsp. *tularensis* strains SCHU S4 (FSC237) and a spontaneous mutant of the SCHU S4 strain, FSC043, were all obtained from the *Francisella* Strain Collection (FSC) of the Swedish Defense Research Agency, Umeå. The ΔFTT0918, ΔFTT0919, and Δ*iglC* mutant strains were all derived from the SCHU S4 strain as detailed below. For the present study, stock cultures of all strains were prepared by growing them as confluent lawns on cysteine heart agar supplemented with 1% (wt/vol) hemoglobin (CHAH). Bacteria were harvested after 48 to 72 h of incubation at 37°C in an atmosphere of 5% CO_2 into freezing medium consisting of modified Mueller-Hinton broth (1) containing 10% (wt/vol) sucrose. Stocks were aliquoted in a volume of 1 ml and stored at −80°C.

Construction of mutagenesis plasmids. The suicide vector for deletion of the *iglC* gene was described previously (12). For construction of pPV-ΔFTT0918 or pPV-ΔFTT0919 regions, approximately 1,500 bp upstream and downstream of each targeted gene was amplified by PCR. The PCR fragments for each gene construct contained complementary sequences in the 3′ end of the upstream fragment and the 5′ end of the downstream fragment and were therefore annealed during a second round of PCR. The 5′ ends of the resulting fragments contained SalI or NheI restriction sites, and the 3′ ends contained SalI or XbaI sites. After restriction enzyme digestion and purification, the PCR fragments were cloned to XbaI/SalI-digested suicide vector pPV (12).

Conjugal transfer of plasmids. Early log cultures of *Escherichia coli* S17-1 carrying pPV-ΔFTT0918, pPV-ΔFTT0919, or pPV-Δ*iglC* and *F. tularensis* SCHU S4 were concentrated by centrifugation and resuspended in 50 μl of culture medium, mixed, and plated on either Luria agar or modified GC agar base plates. After incubation, cells were resuspended in phosphate-buffered saline and plated

on modified GC agar base plates containing 50 μg/ml of polymyxin B for counterselection of the donor *E. coli* strain (12) and 8.0 μg/ml of chloramphenicol. To select for a second recombination event, recombinant bacteria were plated on medium containing 5% sucrose. All sucrose-resistant colonies that were sensitive to chloramphenicol were selected for further analysis. The entire procedure was repeated once more for the *iglC* mutant. All deletion mutants were verified by sequencing 1,500 bp on each side of the deleted region.

Infection of macrophages. Peritoneal exudate cells (PEC) were obtained from mice 3 days after intraperitoneal injection of 2 ml of 10% Proteose Peptone. PEC were washed with Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Islands, N.Y.) and resuspended at a density of 3×10^6 cells/ml in culture medium consisting of DMEM with 10% heat-inactivated fetal calf serum. The suspension was aliquoted in 100-μl volumes in 96-well tissue culture plates. After incubation for 2 h at 37°C, nonadherent cells were removed by washing and after an additional 24 h, *F. tularensis* bacteria were added to give a multiplicity of infection (MOI) of 50 bacteria/PEC. The actual MOI was determined by retrospective plating; thus, there were slight variations between experiments. After allowing uptake of bacteria to occur for 1.5 h, the macrophages were washed to remove extracellular bacteria. Macrophages were reconstituted in culture medium supplemented with 2 μg/ml of gentamicin to kill any remaining extracellular bacteria and incubated for the indicated periods of time. Then, PEC were lysed with 0.1% dodeoxycholate and the number of intracellular bacteria was determined by plating 10-fold serial dilutions. Release of lactate dehydrogenase (LDH) in the cell cultures was determined according to a previously published method (15). The levels in lysed cell cultures were designated as 100%.

Proteomic analysis of strains SCHU S4, FSC043, and ΔFTT0918. *Francisella* strains were plated for single-colony growth on CHAH agar. At 72 h of incubation, 200 colonies of each strain were resuspended in 12 times the estimated pellet volume of lysis solution: 7 M urea, 2 M thiourea, 1% (wt/vol) dithiothreitol (DTT), 4% (wt/vol) CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and 0.5% (wt/vol) ASB-14. Cell pellets were resuspended by vortexing and then shaken for 30 min at room temperature and incubated for at least 4 h at 4°C. Unlysed cells and cell debris were removed by centrifugation at $14,000 \times g$ for 10 min. Protein concentrations of the extracts were determined using a modified Bradford assay (20).

The extracted proteins were separated in the first dimension using either linear pH 4 to 7 gradient Ready Strips (17 cm; Bio-Rad, Calif.) or linear pH 6 to 11 gradient Immobiline drystrips (18 cm; Amersham Biosciences, Uppsala, Sweden). In each case, 100 to 300 μg of each protein solution was diluted in 350 μl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% ASB-14, 1% DTT, 1% [vol/vol] Pharmalyte, pH 3 to 10 or 6 to 11, 0.003% Orange G). For isoelectric focusing in the basic pH range (pH 6 to 11), protein solutions were treated with Destreak rehydration solution (Amersham Biosciences) prior to rehydration of the immobilized pH gradient (IPG) strips. The samples were incubated for 1 h with shaking and then centrifuged at $10,000 \times g$ for 10 min. Proteins were loaded onto the IPG strips by in-gel rehydration overnight. Isoelectric focusing was conducted using a Protean IEF cell (Bio-Rad). Proteins were focused at 200 V for 1 h, 500 V for 1 h, 5,000 V for 5 h, and then 5,000 V for a total of 80 kVh. Next, IPG strips were equilibrated in 6 M urea, 50 mM Tris, pH 8.8, 30% (wt/vol) glycerol, 2% (wt/vol) sodium dodecyl sulfate (SDS), and 1% (wt/vol) DTT for 20 min. The IPG strips were then equilibrated for another 20 min in the same solution containing 4% (wt/vol) iodoacetamide instead of DTT. Strips were then embedded on top of an SDS-polyacrylamide gel electrophoresis (PAGE) gel (12% polyacrylamide; 190- by 190- by 1.5-mm gel) using 0.5% (wt/vol) agarose and 0.003% (wt/vol) bromophenol blue. Electrophoresis was then carried out using the Protean IIxi system with the XL conversion kit (Bio-Rad) at 24 mA per gel for 5 h. Following two-dimensional (2D) electrophoresis, gels were fixed for 1 h in 10% (vol/vol) methanol plus 7% (vol/vol) acetic acid and then stained overnight with Sypro Ruby (Bio-Rad). Background staining was removed by two 30-min washes in 10% (vol/vol) methanol plus 7% (vol/vol) acetic acid, prior to imaging with the Fluor-S MultiImager (Bio-Rad). The gels were then stained with silver nitrate, scanned, and analyzed a second time (25).

Images of the scanned gels were made using PDQuest software (Bio-Rad). At least four replicate gel sets were run for each bacterial strain. Spot positions were matched between replicate gel sets, and both matched and unmatched spots were checked manually. Spots were considered absent if unmatched in all gel sets. Protein spots of interest were excised, cut into 1-mm cubes, destained with a mixture of 30 mM ferricyanide and 100 mM sodium thiosulfate for 5 min, and then washed three times with water. The gel pieces were dehydrated repeatedly with 100% acetonitrile, until the pieces blanched and became hard. Acetonitrile was then removed, and gel pieces were air-dried under a laminar flow hood. Then, 20 μl of 20-ng/ml trypsin in 50 mM ammonium bicarbonate was added to each tube and gel pieces were incubated at 37°C for 16 h. Peptides were extracted from the gel pieces by sonication for 10 min.

TABLE 1. Viable counts of *F. tularensis* strains in PEC[a]

Bacterial strain[a]	No. of bacteria ($\log_{10}$ CFU/well)[b]	
	0 h	15 h
SCHU S4	3.91 ± 0.04	6.21 ± 0.03[c]
FSC043	4.21 ± 0.10	4.67 ± 0.04
ΔFTT0918	4.13 ± 0.07	5.63 ± 0.03[d]

[a] The *F. tularensis* strain was allowed to infect the cells at a multiplicity of infection of 50.

[b] Data represent the mean $\log_{10}$ CFU ± standard deviation of three cultures. One representative experiment out of three is shown.

[c] Significantly different ($P < 0.05$) from the CFU of strain FSC043 and the ΔFTT0918 strain by Wilcoxon's nonparametric test.

[d] Significantly different ($P < 0.05$) from the CFU of strain FSC043 by Wilcoxon's nonparametric test.

The in-gel digests were analyzed by nano-liquid chromatography tandem mass spectrometry (nLC-MS/MS) using a "CapLC" capillary chromatography system (Waters) coupled to a "QTOF Ultima" hybrid quadrupole time-of-flight mass spectrometer (Waters). Peptide extracts were injected on a 75-μm internal diameter by 150-mm PepMap C_{18} nanocolumn (Dionex/LC packings) and resolved by gradient elution (5 to 75% acetonitrile, 0.12% formic acid in 30 min at 350 nl/min). MS/MS spectra were acquired on doubly, triply, and quadruply charged ions. The experimentally collected MS/MS spectra were matched against the *Francisella* strain SCHU S4 genome sequence using Mascot Daemon. Results were evaluated according to the Mascot score, number of peptides identified, and quality of MS/MS matching. A protein identification was considered positive if at least one peptide with a Mascot score greater than 30 was matched.

Infection of mice. Specific-pathogen-free female BALB/c mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). Mice were maintained and used in accordance with the recommendations of the Canadian Council on Animal Care *Guide to the Care and Use of Experimental Animals* (3a). In our hands, type A strain FSC033 is more virulent for mice than SCHU S4 in that the same dose of the former kills mice 1 to 2 days earlier than the latter. Therefore, FSC033 was used as the challenge strain to assess the efficacy of the various live vaccines employed. For aerosol exposure, thawed bacteria were diluted in Mueller-Hinton broth containing 20% (wt/vol) glycerol to a concentration of approximately 10^8 CFU/ml; for intradermal (i.d.) inoculations, stocks of the strains were diluted in sterile saline. Actual concentrations of inocula were determined by plating 10-fold serial dilutions on CHAH.

Intradermal inocula (50 μl/mouse) were injected into a fold of skin in the shaved mid-belly, and the blister that formed was circumscribed with indelible marker. For subsequent bacteriology, an area of skin encompassing the marked region was excised, chopped into small pieces with scissors, and then homogenized in aerosol-proof homogenizers and plated on CHAH. In some experiments, lungs, livers, and spleens were similarly processed for bacteriology. Aerosols containing strain FSC033 were generated with a Lovelace nebulizer as described previously (6). This protocol results in the delivery of ~20 CFU to the lower airways of BALB/c mice (6). Aerosol exposures and i.d. challenges were performed in a federally licensed small animal containment level 3 facility. In the present study, mice were examined daily for signs of infection. Whenever feasible, mice were euthanized by CO_2 asphyxiation as soon as they displayed signs of irreversible morbidity. In our experience, such mice were at most 24 h from death, and the time to death of these animals was estimated on this premise.

Histology. For histology, mice were killed by CO_2 asphyxiation. Next, the site of skin infection was removed en bloc, placed in 10% buffered formalin overnight, and then processed by standard paraffin-embedding methods. Sections were cut 4 μm thick, stained with hematoxylin-eosin (HE), and examined by light microscopy.

RESULTS

A spontaneous mutant, FSC043, of strain SCHU S4 is attenuated in vitro and in vivo but affords effective protection against challenge with virulent type A strain, FSC033. Screening of the FSC revealed an attenuated strain, FSC043, derived from the prototypic *F. tularensis* subsp. *tularensis* strain, SCHU

TABLE 2. Growth of *F. tularensis* strains in host tissues following i.d. inoculation of the pathogen

F. tularensis strain and i.d. inoculum	Log_{10} CFU of *Francisella* on day 4 of infection ($n = 3$)[a]		
	Skin	Liver	Spleen
10^6 CFU			
FSC043	4.00 ± 0.48	3.81 ± 0.08	4.57 ± 0.09
LVS	6.28 ± 0.21	5.76 ± 0.50	5.88 ± 0.47
Δ*iglC*	5.31 ± 0.43	2.30 (1/3)[b]	3.09 ± 0.69
10^2 CFU			
SCHU S4	7.37 ± 0.40	7.09 ± 0.83	7.77 ± 0.91
LVS	6.65 ± 0.58	3.65 ± 1.10	4.37 (2/3)[c]
ΔFTT0918	5.66 ± 0.73	3.50 ± 0.29	4.36 ± 1.33

[a] In separate experiments, mice were inoculated intradermally with either 10^6 or 10^2 CFU of the stated *F. tularensis* strain. Mice were killed on day 4 of infection, and bacterial burdens were determined. Values are means ± standard deviation.
[b] Bacteria only detected in one of three organs.
[c] Bacteria only detected in two of three organs (lower detection limit = 200 CFU/organ).

S4. This strain was found to be markedly attenuated for multiplication in PEC (Table 1) and J774 cells (data not shown). In fact, no net growth of the bacterium was observed during a 15-h period. In contrast, the number of intracellular SCHU S4 bacteria increased >100-fold during the same incubation period (Table 1). Since the intracellular infection with *F. tularensis* results in a cytopathogenic effect (15), we assessed the effect of the various infections on the PEC by measuring the release of LDH. We found no difference between the levels in cultures infected with SCHU S4 and the ΔFTT0918 mutant (36.3% ± 1.4% and 38.0% ± 6.5%, respectively), whereas levels were lower in cell cultures infected with FSC043 (24.6% ± 0.7%).

In several separate experiments, BALB/c mice were challenged intradermally with 10^2 to 10^8 CFU of FSC043 or LVS. All LVS-challenged BALB/c mice displayed overt signs of illness between days 4 and 11 (hunched gait, pilo-erection, lethargy), and some mice inoculated with 10^7 or 10^8 CFU died by day 8 of infection. No other mice died during the next 28 days. In contrast, only mice challenged with $>10^6$ CFU of FSC043 displayed obvious signs of infection and no mice inoculated with this strain died at any test dose.

To further examine the relative virulence of LVS and FSC043, BALB/c mice were intradermally challenged with 10^6 CFU of one or other strain and then killed on day 4 of infection, and bacterial burdens in the skin, liver, and spleen were determined (Table 2). By this time, LVS was present at 100-fold-higher levels than FSC043 in the skin and liver and 20-fold-higher levels in the spleen. Moreover, large macroscopic skin lesions were visible at the site of inoculation of LVS (Fig. 1A) but not of FSC043 (Fig. 1B). Histologically, these LVS-induced lesions (Fig. 1D) consisted of large areas of degenerative and necrotic dermatitis containing degenerating and necrotic epidermal cells with detachment of the epidermis from the subdermal layer and, in places, the complete disappearance of the epidermal layer. Large numbers of mixed inflammatory cells, predominantly polymorphonuclear leukocytes, had infiltrated the adjacent subdermal region, which also contained large numbers of necrotic inflammatory cells and debris. The epidermis adjacent to these severely affected areas was thickened with increased

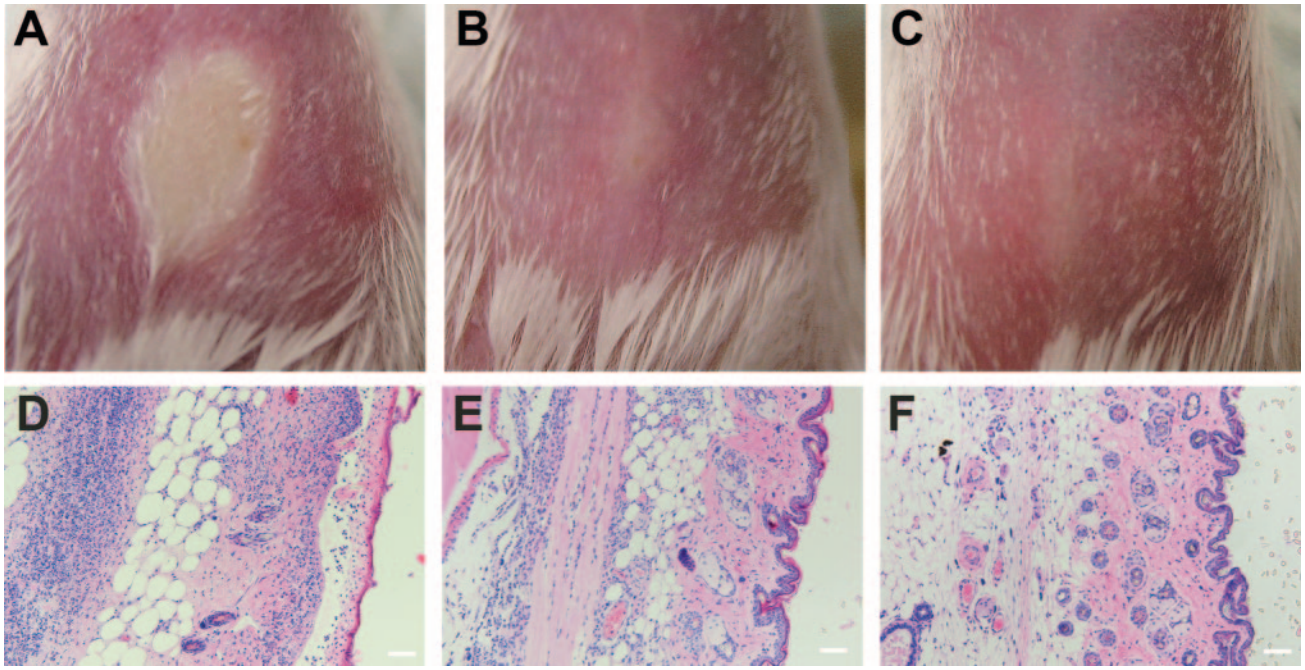

FIG. 1. Skin lesion development at sites of intradermal inoculation of *F. tularensis* strains. Shown are representative skin reactions observed in mice inoculated with (A) 10^2 CFU of SCHU S4 or 10^6 CFU of LVS; (B) 10^2 CFU of the ΔFTT0918 mutant or 10^6 CFU of FSC043; or (C) nothing or 10^2 CFU of LVS or 10^6 CFU of the Δ*iglC* mutant. Panels D, E, and F represent hematoxylin-eosin staining of skin samples to histologically characterize the skin reactions shown in panels A, B, and C, respectively. Bar, 40 μm.

TABLE 3. Growth of *F. tularensis* strains in host tissues following intravenous inoculation of the pathogen

F. tularensis strain	Log_{10} CFU of *F. tularensis*/organ ($n = 3$)[a]			
	Lung	Liver	Spleen	Blood[b]
FSC033 (type A)	>8.0	>9.0	>9.0	>8.0
FSC108 (type B)	3.97 ± 0.34	7.26 ± 0.32	7.35 ± 0.20	3.58 ± 0.77
LVS (attenuated type B)	2.31 ± 1.02	5.03 ± 0.12	5.18 ± 0.12	<2.00 (0/3)
FSC043 (attenuated type A)	<1.3 (0/3)	<2.3 (2/3)	3.16 ± 0.66	<2.00 (0/3)

[a] Approximately 100 CFU of each the indicated strains of *F. tularensis* were intravenously inoculated into BALB/c mice ($n = 3$ per group), and bacterial burdens in organs on day 3 of infection were determined. Values are means ± standard deviation. Numbers in parentheses indicate the proportion of organs infected.
[b] CFU/ml of blood.

amounts of keratin. Similar, but less extensive destruction of skin and inflammation was observed at the site of inoculation of FSC043 (Fig. 1E), whereas the skin in mice inoculated with an equal volume of saline appeared histologically normal (Fig. 1F).

When inoculated intravenously, FSC043 grew less well than virulent type A or B strains or LVS in the livers, spleens, and lungs of mice (Table 3). Thus, the lower level of FSC043 versus LVS in the liver and spleen in the preceding experiment (Table 2) was not due solely to an inability of the former to disseminate from its site of implantation in the skin.

Next, BALB/c mice were intradermally inoculated with 10^6 CFU of LVS or FSC043. The former mice showed overt signs of infection before recovering, whereas the latter mice remained healthy. All immunized mice survived and were challenged 77 days later intradermally or by aerosol with type A strain FSC033 (Table 4). We have previously shown that immunization of BALB/c mice with LVS leads to excellent protection against intradermal challenge but only weak protection against low-dose aerosol challenge (4, 7, 24), as confirmed here (Table 4). The FSC043 immunization afforded a similar degree of protection to LVS against intradermal challenge and somewhat better protection against aerosol challenge.

In a complementary experiment, groups of mice ($n = 3$) were challenged intradermally with 150 CFU of strain FSC033 120 days after intradermal immunization with LVS or FSC043. Mice were killed on day 3 of infection, and *Francisella* burdens in livers, spleens, and lungs were determined (Table 5). Again, it was found that FSC043 immunization was at least as effective as LVS immunization at controlling disseminated infection with a type A strain.

TABLE 4. Protective immunity against strain FSC033 elicited by i.d. immunization with LVS or FSC043[a]

Immunizing strain (challenge route and dose in CFU)	Time to death (days)	
	Individual	Median
None (i.d., 10)	5, 5, 6, 6, 6	6
LVS (i.d., 1,000)	5, >35, >35, >35, >35	>35[b]
FSC043 (i.d., 1,000)	11, 12, >35, >35, >35	>35[b,c]
None (aerosol, ~10)	5, 5, 5, 5, 6	5
LVS (aerosol, ~10)	6, 6, 11, 13, >35	11[b]
FSC043 (aerosol, ~10)	5, 7, >35, >35, >35	>35[b,c]

[a] Mice immunized 77 days earlier by intradermal inoculation with 10^6 CFU of LVS or FSC043 and age-matched controls were challenged intradermally or by aerosol with various doses of virulent type A *F. tularensis* strain 33, and survival was monitored.
[b] Significantly longer survival than controls ($P < 0.05$) by log-rank analysis of survival curves.
[c] Not significantly different from mice immunized with the same dose of LVS ($P > 0.05$) by log-rank analysis of survival curves.

Proteomic comparison of strains SCHU S4 and FSC043. We used 2D-PAGE to compare the proteomes of the virulent strain SCHU S4 and the attenuated strain FSC043. The majority of the protein spots resolved in the pH range 4 to 7. Six spots comprising eight proteins were undetectable in FSC043 compared to SCHU S4, and the putative identities of these proteins are shown in Table 6. In contrast, one spot (no. 35) was observed only in FSC043 (Fig. 2). The protein spot was found to contain peptides corresponding to parts of proteins FTT0918 and FTT0919 found in the parental strain. Both are identified as hypothetical proteins with no known homology to other proteins and no assigned function. FTT0918 was also identified as spot 30 in the parental strain (Fig. 2). MS analysis of spot 30 identified peptides spanning the entire predicted protein sequence (Fig. 2). In contrast, MS analysis of FSC043 spot 35 identified peptides confined to the first half of the predicted amino acid sequence of protein FTT0919 and the second half of that predicted for protein FTT0918. The genes corresponding to these two proteins are in close proximity on the chromosome: FTT0918 is encoded by bp 927,667 to 929,340, and FTT0919 is encoded by bp 929,357 to 930,802. Thus, it appears that a deletion mutation overlapping these genes resulted in the creation of a novel gene coding for a hybrid protein consisting of the N terminus of FTT0918 and the C terminus of FTT0919. We have found evidence for the presence of a similar hybrid protein in LVS (data not shown), and the hybrid gene for this protein is recorded in the current LVS genome sequence database (http://greengenes.llnl.gov/bbrp/html/microbe.html).

Characteristics of defined mutants of strain SCHU S4. The aforementioned two genes found to be partially absent from

TABLE 5. Growth of virulent strain FSC033 in organs of control mice and mice vaccinated with LVS or FSC043[a]

Immunizing strain	*Francisella* burden on day 3 of infection (mean log_{10} CFU ± SD)[b]		
	Lungs	Liver	Spleen
None	5.18 ± 2.03	7.49 ± 0.86	8.33 ± 1.13
LVS	<1.30 (0/3)	2.91 ± 0.41	3.62 ± 0.25
FSC043	<1.30 (0/3)	<2.25 (1/3)	2.63 ± 1.74

[a] Mice ($n = 3$/group) immunized i.d. 120 days earlier with 10^6 CFU of LVS or FSC043 were challenged by the same route with 150 CFU of *F. tularensis* type A strain FSC033. Mice were killed on day 3 of infection, and *Francisella* burdens in livers, spleens, and lungs were determined.
[b] Numbers in parentheses show the proportion of organs infected.

TABLE 6. Proteins observed to be differentially expressed when comparing the proteome maps of strains SCHU S4 and FSC043

Spot no. (FTT no.)[a]	Mol mass in kDa (pI)[b]		Mascot score[c]	% Sequence coverage[d]	Protein product[e]	Protein identification no.[f]
	Theoretical	Observed				
2 (1355)	22.1 (6.77)	18.2 (6.05)	132	20	Conserved hypothetical protein	YP_170307.1
6 (0049/0192)	55.1 (4.49)	61.4 (4.85)	774	41	N utilization substance protein A	YP_169124.1
	66.2 (5.55)	61.4 (4.85)	60	3	Lysyl-tRNA synthetase	YP_169253.1
22 (0655)	26.8 (4.68)	34.9 (4.70)	450	41	Hypothetical protein	YP_169673.1
28 (0409)	49.6 (5.77)	50.0 (6.16)	108	18	Glycine cleavage system P protein, subunit 1	YP_169454.1
30 (0918)	58.7 (4.75)	56.6 (4.45)	263	14	Hypothetical protein	YP_169915.1
31 (0007/1129c)	66.9 (5.49)	67.0 (5.85)	826	42	Aspartyl-tRNA synthetase	YP_169088.1
	63.0 (5.46)	67.0 (5.85)	493	35	Cyanophycin synthetase	YP_170102.1
35 (0918/0919)	—[g]	55.0 (5.02)	463	25[h]	Fusion protein (FTT0918/0919)	NA[i]

[a] Proteins were identified by LC-MS/MS. Mascot (Matrix Science, London, United Kingdom) was then used to match the MS/MS spectra against the translated *F. tularensis* SCHU S4 genome sequence (Refseq: NC_006570). The indicated number is an arbitrary designation of the annotated spots on the 2DE proteome maps. The FTT no. represents the *F. tularensis* SCHU S4 genome locus tag (designated as FTTxxxx). Note that protein spots 2, 6, 22, 28, 30, and 31 were not observed in FSC043 and protein spot 35 was uniquely observed in FSC043.

[b] The theoretical molecular mass and pI were calculated from the amino acid sequence of the translated open reading frame. The observed experimental molecular mass and pI were estimated using PDQuest software.

[c] Total Mascot score for peptides identified. A score of >30 was required for positive identification each polypeptide.

[d] Percentage of sequence coverage based on the peptides identified.

[e] Name of identified protein based upon the *F. tularensis* SCHU S4 genome sequence.

[f] Accession number according to the NCBI.

[g] —, no protein corresponding to the fusion protein is found within SCHU S4 genome sequence in FSC043 estimated to be 58.5 kDa (28).

[h] Composite sequence coverage for fusion protein. Peptides detected by nLC-MS/MS gave 11% coverage of the FTT0918 amino acid sequence and 14% coverage of the FTT0919 amino acid sequence. Peptides were detected corresponding to the N-terminal region of FTT0918 and the C-terminal region of FTT0919.

[i] NA, no accession number available for fusion protein.

both strains LVS and FSC043 were subjected to the deletion strategy using plasmid pPV (12), and both mutants were obtained in strain SCHU S4. Additionally, the Δ*iglC* gene that we had previously deleted from LVS, resulting in a mutant unable to grow in mouse macrophages and in mice (12), was deleted from SCHU S4.

The mutant missing the gene annotated as FTT0919 remained highly virulent for mice by the i.d. route (i.d. 50% lethal dose [LD_{50}], <50 CFU) and so was not further evaluated as a live vaccine candidate. In contrast, the mutant missing FTT0918 was highly attenuated compared to the parental strain (i.d. LD_{50}, $\sim 10^5$ CFU versus <10 CFU, respectively, based on accumulated data from four separate experiments). Proteomic analysis confirmed that it lacked the expected protein spot 30 in SCHU S4 (Fig. 2) and failed to elaborate the hybrid protein represented by spot 35. When inoculated intradermally at a dose of 100 CFU, the mutant multiplied less than the parental strain, caused a much less overt tissue reaction at this site, and disseminated less to internal organs (Table 2; Fig. 1). Indeed, at this test dose, the ΔFTT0918 mutant appeared as attenuated as LVS (Table 2), although its LD_{50} ($\sim 10^5$) indicates it is more virulent than LVS (LD_{50}, $\sim 10^6$) or FSC043 (LD_{50}, $>10^8$) at higher doses. Likewise, the fact that the ΔFTT0918 mutant persisted and multiplied slightly in PEC whereas FSC043 was unable to grow in these host cells again suggests that the former is more virulent than the latter (Table 1). At the opposite end of the spectrum from the ΔFTT0919 mutant, the Δ*iglC* mutant of SCHU S4 appears to be totally avirulent in that it failed to induce any overt disease in mice even at an i.d. dose of 10^8 CFU. Interestingly, this mutant persisted at least as well as FSC043 in the skin but appeared less able to disseminate to internal organs (Table 2). Mice immunized intradermally with defined mutants of *F. tularensis* were challenged 8 to 9 weeks later with virulent strain FSC033 by the intradermal or aerosol route, respectively, and their survival was monitored (Table 7). The ΔFTT0918 mutant was at least as effective a live vaccine as LVS in these studies to provide protection against a systemic challenge with virulent type A *F. tularensis*, whereas immunization with the Δ*iglC* mutant resulted in no significant protection (Table 7). Against an aerosol challenge, all mice immunized with the ΔFTT0918 mutant survived longer than any of those immunized with LVS or the Δ*iglC* mutant.

DISCUSSION

The identities and characteristics of the virulence factors and protective antigens of the subspecies of *Francisella* are essentially unknown. Although recent comparative genomic analyses have begun to demonstrate genetic differences among the subspecies, these alone have been insufficient to explain their relative virulence (2, 14, 21, 28). It was recently shown that sublethal infection of mice with *F. tularensis* subsp. *novicida*, unlike similar infection with LVS or FSC043, failed to confer protection against subsequent challenge with *F. tularensis* subsp. *holarctica* or *tularensis*, suggesting that the protective antigens of the latter two are highly restricted (23). This could also explain why only certain mouse strains can be protected by LVS (4, 24), since major histocompatibility complex haplotype restriction should not be an issue if many of the 1,800 genes

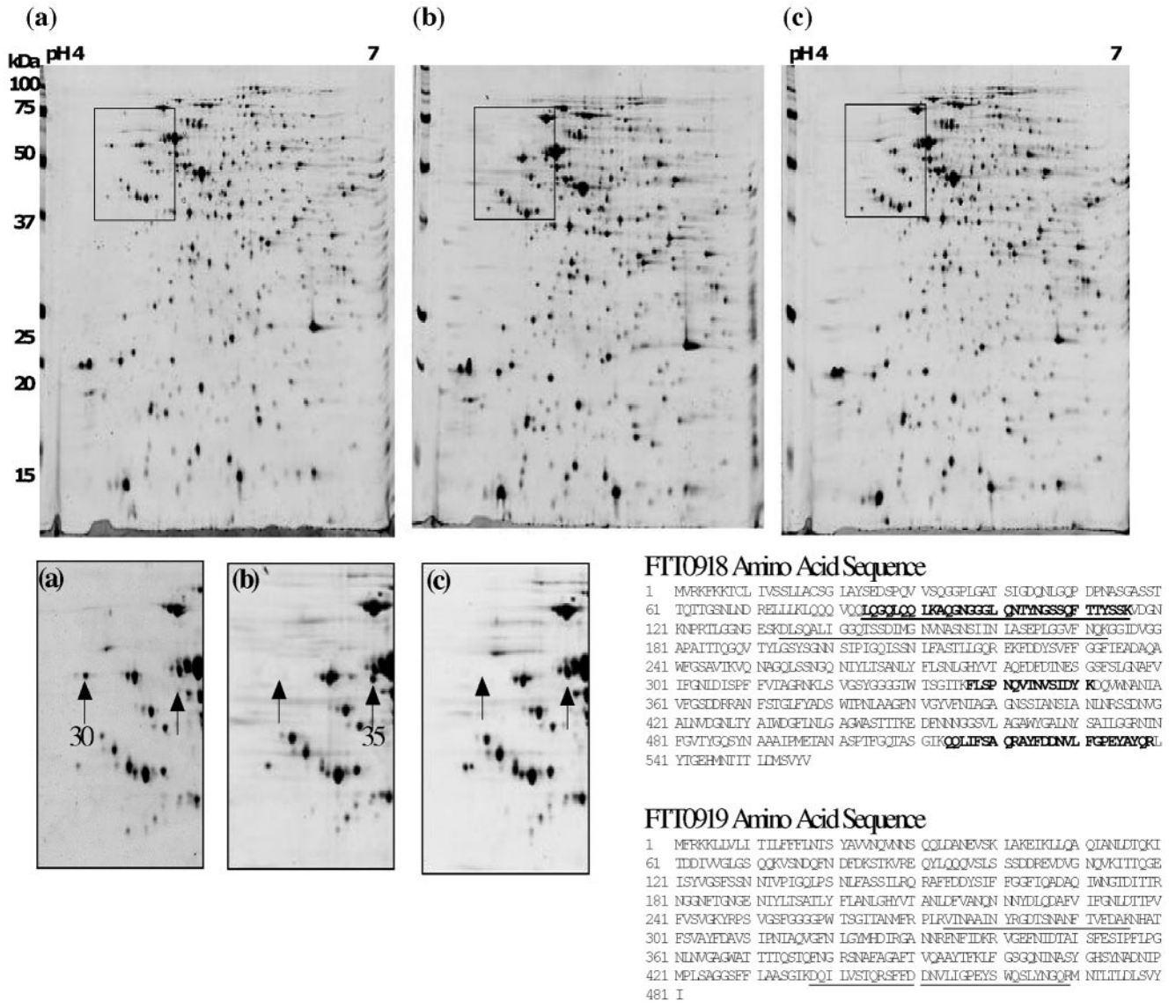

FIG. 2. Proteomic comparisons of *F. tularensis* strains. Shown are the results of 2D-PAGE of the *F. tularensis* (a) SCHU S4, (b) SCHUAV, and (c) ΔFTT0918 strains visualized by staining with Sypro Ruby. Replicate gels within experimental groups were compared. Boxed regions in the large gels correspond to the enlarged areas below. Protein sequences for FTT0918 and FTT0919 are shown on the bottom right. Amino acid sequences underlined correspond to those peptides detected by LC-MS/MS of a tryptic digest of spot 35 from FSC043, while those in bold were detected from a tryptic digest of spot 30. No spot corresponding to FTT0919 was detected on any gel.

encode protective antigens. One way to ensure the expression of all protective antigens in a vaccine is to develop a live, attenuated vaccine strain lacking the minimum number of critical virulence genes to guarantee its safety. In addition, if the vaccine strain is based on a type A strain, it will also possess more than 20 additional genes unique to *F. tularensis* subsp. *tularensis* that might code for protective antigens missing from *F. tularensis* subsp. *holarctica*, from which the current LVS vaccine was derived (10). To this end, the present study aimed to create defined, attenuated type A mutant strains.

The discovery of a naturally attenuated type A isolate in our strain collection allowed the demonstration that *F. tularensis* subsp. *tularensis* can be attenuated to a greater degree than LVS to yield a less reactogenic vaccine strain with improved efficacy against both systemic and aerosol challenge. In contrast, earlier studies abandoned attenuated strains of SCHU S4 on the basis that they were more virulent than LVS for mice (10). The FSC043 strain identified in the present study suffers all of the same regulatory drawbacks as LVS: most importantly, the reason(s) for its attenuation remain(s) unknown. Nevertheless, FSC043 demonstrates the feasibility of deriving safe and effective live vaccines from virulent type A strains of the pathogen. A proteomic comparison of FSC043 and the wild-type virulent parental strain, SCHU S4, revealed the absence of eight proteins from the former versus the latter (Table 6). Intriguingly, FSC043 expressed a specific protein (spot 35 in Fig. 2b) not expressed by the parental strain. A proteomic analysis of this protein revealed it to be a hybrid

TABLE 7. Protective immunity against strain FSC033 elicited by i.d. immunization with LVS or defined mutants of SCHU S4[a]

Immunizing strain (dose in CFU)	Challenge route and dose in CFU	Time to death (days)	
		Individual	Median
None	i.d., 10	4, 4, 5, 5, 5	5
LVS (10^6)	i.d., 500	>35, >35, >35, >35, >35	>35[b,c]
ΔFTT0918 (10^5)	i.d., 500	>35, >35, >35, >35, >35	>35[b,c,d]
Δ*iglC* mutant (10^6)	i.d., 500	4, 7, 7, 7, 8	7[b]
None	Aerosol, ~10	4, 5, 5, 5, 5	5
LVS (10^7)	Aerosol, ~10	5, 7, 7, 7	7
ΔFTT0918 (10^5)	Aerosol, ~10	9, 11, 11, 19, >35, >35	15[b,c]
Δ*iglC* mutant (10^7)	Aerosol, ~10	5, 5, 6, 6, 6	6

[a] Mice (4 to 6/group) were immunized by intradermal inoculation with the indicated strain and together with age-matched control mice challenged intradermally 8 weeks later with ~500 CFU of type A strain FSC033 or by aerosol 9 weeks later with ~10 CFU of FSC033, and survival was monitored.
[b] Survived significantly longer ($P < 0.05$) than control mice (rank-sum test).
[c] Survived significantly longer ($P < 0.05$) than mice immunized with the Δ*iglC* mutant.
[d] Group survival not different from that of LVS-immunized mice.
[e] Survived significantly longer ($P < 0.05$) than all other aerosol-challenged test groups.

protein consisting of the N-terminal domain of FTT0918 and the C-terminal domain of FTT0919. In agreement with this finding, a previously published genomic analysis identified a deletion in the region in strains FSC043 and LVS encompassing the downstream region of FTT0918 and upstream region of FTT0919 (28). To better assess the potential role of the two wild-type genes in virulence, they were individually targeted for deletion from SCHU S4 using an allelic replacement method we had previously used to generate defined mutations in LVS (12). The method incorporates a counterselection step to ensure that the antibiotic resistance genes as well as all other DNA present in the plasmid used to generate the crossover mutations are absent from the ensuing mutant strain. Deletion of one of the targeted genes, FTT0919, had no obvious effect on virulence, and this mutant is unlikely, therefore, to be acceptable as a vaccine candidate.

In contrast to the ΔFTT0919 strain, the ΔFTT0918 mutant showed significantly reduced virulence for mice. Moreover, mice that recovered from infection with this mutant were protected from subsequent challenge with a highly virulent type A strain. Despite its attenuation, the ΔFTT0918 mutant retained a greater residual virulence for mice than either LVS or FSC043. This is unsurprising, since the latter two spontaneous mutants are missing additional genes, some of which must encode additional virulence factors. By selectively deleting some of the latter genes from the ΔFTT0918 mutant, it should be possible to attenuate it to the same degree as FSC043 to thereby produce a rationally attenuated strain with superior vaccine properties (safer and more effective) compared to LVS.

FTT0918 encodes a 58-kDa protein with no close homology to any proteins in the present versions of the databases. When the genome sequence of SCHU S4 was published, it was noted that using the TribeMCL method, FTT0918 and FTT0919, together with three other proteins, constitute a predicted protein family (2, 16). In fact, of all predicted families, this was the largest. None of the members showed any similarity to sequences in protein data banks. Both FTT0918 and FTT0919 are predicted to contain signal peptides and coiled-coil domains and therefore are likely membrane-associated proteins. Attenuation of the mutant strain might be directly and solely due to the absence of the specific protein FTT0918. This is supported by the finding that no other proteomic differences were noted between the mutant and the wild-type strain. However, as the entire proteome was not examined, and in the absence of complementation studies, the possibility that other changes, indirectly related to FTT0918, occurred in the mutant to contribute to its attenuation cannot be precluded at this time.

There are several recent publications on the roles of the protein encoded by the *iglC* gene. It was originally identified as one of a few proteins that were differentially expressed by LVS growing intracellularly versus in broth culture (11). When a Δ*iglC* mutant of the LVS strain was investigated, it was found that the mutant was unable to effectively replicate intracellularly and, unlike the parent strain, was unable to escape from the phagolysosome of murine macrophages (12, 18). Similarly, a Δ*iglC* mutant of *F. tularensis* subsp. *novicida* was found to lack the ability of the parent strain to multiply in amoebae (17). Thus, expression of the IglC protein appears to be essential for *F. tularensis* to behave as an intracellular pathogen. Although the original findings were derived from studies on relatively low-virulence strains of *F. tularensis*, our present findings on the marked attenuation of strain SCHU S4 carrying this mutation further validate the general conclusion. Thus, an inability to express the IglC protein is sufficient to render even wild-type type A strains avirulent for an otherwise highly susceptible host. Furthermore, our results indicate that the mere ability of attenuated *F. tularensis* isolates to persist in the host is an insufficient stimulus for priming of an effective, long-lasting protective immune response, as evidenced by the marginal protection afforded by strain Δ*iglC* versus the other mutant strains of SCHU S4 examined herein. Nor is overt reactogenicity at the site of inoculation in the skin a prerequisite for eliciting an effective immune response, since FSC043 is a better vaccine than LVS in the absence of this phenomenon.

In conclusion, by a comparative proteomic approach, we have identified a number of candidate proteins that may explain the attenuation of spontaneously mutated *F. tularensis* strains. By making deletion mutants, we demonstrate that one of the genes, ΔFTT0918, contributes significantly to the virulence of the SCHU S4 strain in vitro and in vivo. This work demonstrates the feasibility of producing an effective genetically defined live *F. tularensis* vaccine from a type A strain of *F. tularensis*.

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REFERENCES

1. **Baker, C. N., D. G. Hollis, and C. Thornsberry.** 1985. Antimicrobial susceptibility testing of *Francisella tularensis* with a modified Mueller-Hinton broth. J. Clin. Microbiol. **22:**212–215.
2. **Broekhuijsen, M., N. Larsson, A. Johansson, M. Byström, U. Eriksson, E. Larsson, R. G. Prior, A. Sjöstedt, R. W. Titball, and M. Forsman.** 2003. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. J. Clin. Microbiol. **41:**2924–2931.
3. **Burke, D. S.** 1977. Immunization against tularemia: analysis of the effectiveness of live *Francisella tularensis* vaccine in prevention of laboratory acquired tularemia. J. Infect. Dis. **135:**55–60.

3a. **Canadian Council on Animal Care.** 1993. Guide to the care and use of experimental animals. Canadian Council on Animal Care, Ottawa, Ontario, Canada.

4. **Chen, W., H. Shen, A. Webb, R. KuoLee, and J. W. Conlan.** 2003. Tularemia in BALB/c and C57BL/6 mice vaccinated with *Francisella tularensis* LVS and challenged intradermally, or by aerosol with virulent isolates of the pathogen: protection varies depending on pathogen virulence, route of exposure, and host genetic background. Vaccine **21:**3690–3700.
5. **Conlan, J. W.** 2004. Vaccines against *Francisella tularensis*—past, present and future. Expert Rev. Vaccines **3:**307–314.
6. **Conlan, J. W., W. Chen, H. Shen, A. Webb, and R. KuoLee.** 2003. Experimental tularemia in mice challenged by aerosol or intradermally with virulent strains of *Francisella tularensis*: bacteriologic and histopathologic studies. Microb. Pathog. **34:**239–248.
7. **Conlan, J. W., H. Shen, R. KuoLee, X. Zhao, and W. Chen.** 2005. Aerosol-, but not intradermal-immunization with the live vaccine strain of *Francisella tularensis* protects mice against subsequent aerosol challenge with a highly virulent type A strain of the pathogen by an alphabeta T cell- and interferon gamma-dependent mechanism. Vaccine **23:**2477–2485.
8. **Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, A. M. Friedlander, J. Hauer, M. Layton, S. R. Lillibridge, J. E. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, and K. Tonat.** 2001. Tularemia as a biological weapon: medical and public health management. JAMA **285:**2763–2773.
9. **Eigelsbach, H., J. Tulis, E. Overholt, and W. Griffith.** 1961. Aerogenic immunization of the monkey and guinea pig with live tularemia vaccine. Proc. Soc. Exp. Biol. Med. **108:**732–734.
10. **Eigelsbach, H. T., and C. M. Down.** 1961. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. J. Immunol. **87:**415–425.
11. **Golovliov, I., M. Ericsson, G. Sandström, A. Tärnvik, and A. Sjöstedt.** 1997. Identification of proteins of *Francisella tularensis* induced during growth in macrophages and cloning of the gene encoding a prominently induced 23-kilodalton protein. Infect. Immun. **65:**2183–2189.
12. **Golovliov, I., A. Sjostedt, A. Mokrievich, and V. Pavlov.** 2003. A method for allelic replacement in *Francisella tularensis*. FEMS Microbiol. Lett. **222:**273–280.
13. **Hornick, R. B., and H. T. Eigelsbach.** 1966. Aerogenic immunization of man with live tularemia vaccine. Bacteriol. Rev. **30:**532–538.
14. **Johansson, A., A. Ibrahim, I. Göransson, U. Eriksson, D. Gurycova, J. E. Clarridge III, and A. Sjöstedt.** 2000. Evaluation of PCR-based methods for discrimination of *Francisella* species and subspecies and development of a specific PCR that distinguishes the two major subspecies of *Francisella tularensis*. J. Clin. Microbiol. **38:**4180–4185.
15. **Lai, X. H., I. Golovliov, and A. Sjöstedt.** 2001. *Francisella tularensis* induces cytopathogenicity and apoptosis in murine macrophages via a mechanism that requires intracellular bacterial multiplication. Infect. Immun. **69:**4691–4694.
16. **Larsson, P., P. C. F. Oyston, P. Chain, M. C. Chu, M. Duffield, H.-H. Fuxelius, E. Garcia, G. Hälltorp, D. Johansson, K. E. Isherwood, P. D. Karp, E. Larsson, Y. Liu, S. Michell, J. Prior, R. G. Prior, S. Malfatti, A. Sjöstedt, K. Svensson, N. Thompson, L. Vergez, J. K. Wagg, B. W. Wren, L. E. Lindler, S. G. E. Andersson, M. Forsman, and R. W. Titball.** 2005. The complete genome sequence of *Francisella tularensis* the causative agent of tularemia. Nat. Genet. **37:**153–159.
17. **Lauriano, C. M., J. R. Barker, S. S. Yoon, F. E. Nano, B. P. Arulanandam, D. J. Hassettt, and K. E. Klose.** 2004. MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoebae and intramacrophage survival. Proc. Natl. Acad. Sci. USA **101:**4246–4249.
18. **Lindgren, H., I. Golovliov, V. Baranov, R. K. Ernst, M. Telepnev, and A. Sjöstedt.** 2004. Factors affecting the escape of *Francisella tularensis* from the phagolysosome. J. Med. Microbiol. **53:**953–958.
19. **Oyston, P. C., A. Sjöstedt, and R. W. Titball.** 2004. Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat. Rev. Microbiol. **2:**967–978.
20. **Ramagli, L. S.** 1999. Quantifying protein in 2-D PAGE solubilization buffers. Methods Mol. Biol. **112:**99–103.
21. **Samrakandi, M. M., C. Zhang, M. Zhang, J. Nietfeldt, J. Kim, and T. Benson.** 2004. Genome diversity among regional populations of *Francisella tularensis* subspecies *tularensis* and *Francisella tularensis* subspecies *holarctica* isolated from the US. FEMS Microbiol. Lett. **237:**9–17.
22. **Saslaw, S., H. Eigelsbach, H. Wilson, J. Prior, and S. Carhart.** 1961. Tularemia vaccine study. II. Respiratory challenge. Arch. Intern. Med. **107:**702–714.
23. **Shen, H., W. Chen, and J. W. Conlan.** 2004. Mice sublethally infected with *Francisella novicida* U112 develop only marginal protective immunity against systemic or aerosol challenge with virulent type A or B strains of *F. tularensis*. Microb. Pathog. **37:**107–110.
24. **Shen, H., W. Chen, and J. W. Conlan.** 2004. Susceptibility of various mouse strains to systemically- or aerosol-initiated tularemia by virulent type A *Francisella tularensis* before and after immunization with the attenuated live vaccine strain of the pathogen. Vaccine **22:**2116–2121.
25. **Shevchenko, A., O. N. Jensen, A. V. Podtelejnikov, F. Sagliocco, M. Wilm, O. Vorm, P. Mortensen, H. Boucherie, and M. Mann.** 1996. Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proc. Natl. Acad. Sci. USA **93:**14440–14445.
26. **Sjöstedt, A.** 2003. Virulence determinants and protective antigens of *Francisella tularensis*. Curr. Opin. Microbiol. **6:**66–71.
27. **Sjöstedt, A. B.** 2005. *Francisella*, p. 200–210. *In* D. J. Brenner, N. P. Krieg, J. T. Staley, and G. M. Garrity (ed.), The proteobacteria, part B. Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. Springer, New York, N.Y.
28. **Svensson, K., P. Larsson, D. Johansson, M. Byström, M. Forsman, and A. Johansson.** 2005. Evolution of subspecies of *Francisella tularensis*. J. Bacteriol. **187:**3903–3908.

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