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Identification of Proteins of *Francisella tularensis* Induced during Growth in Macrophages and Cloning of the Gene Encoding a Prominently Induced 23-Kilodalton Protein

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The adaptation of facultative intracellular bacteria to host macrophages involves regulation of the synthesis of bacterial proteins. We analyzed the protein synthesis of *Francisella tularensis* LVS growing intracellularly in the macrophage-like murine cell line J774 and extracellularly in culture medium. After pulse-labeling with [^{35}S]methionine and separation by one- and two-dimensional polyacrylamide gel electrophoresis, induction of a few proteins during intracellular growth was demonstrated. One of them, a 23-kDa protein, was prominently induced in the macrophages and also when extracellularly growing *F. tularensis* was exposed to hydrogen peroxide. After isolation of the 23-kDa protein from a preparative two-dimensional gel, a 22-amino-acid N-terminal peptide and two peptides obtained by trypsin digestion were sequenced. Based on the sequences, degenerate oligonucleotides were constructed for use as primers in a PCR. Hybridization of amplified DNA to *Xba*I-digested LVS DNA identified the gene of the 23-kDa protein in a 1.3-kb DNA fragment. Nucleotide sequence analysis revealed an open reading frame encoding a putative protein of a calculated molecular mass of 22.2 kDa. The open reading frame was preceded by a sequence typical of ribosome-binding sites in *Escherichia coli*. The amplified gene was successfully expressed by the pTrc99A vector in *E. coli* under control of the *trc* promoter. The gene product showed the same mobility and immunoreactivity as the 23-kDa protein of *F. tularensis*. The deduced amino acid sequence showed no significant homology with protein sequences in current data banks. Thus, intracellular growth of *F. tularensis* in macrophages was associated with prominent upregulation of a novel 23-kDa protein.

Although most intracellular organisms live in a symbiotic relationship with their hosts, some have developed an ability to multiply excessively within cells and become true parasites. The genera *Mycobacterium*, *Legionella*, *Listeria*, and *Salmonella* all include species of facultative intracellular bacteria pathogenic to humans. After ingestion by macrophages, the microorganisms may end up in phagolysosomes, where they become exposed to various oxygen-dependent and -independent killing mechanisms. To survive within or escape from this hostile environment, intracellular microorganisms have developed an array of strategies. Some microorganisms avoid initiating the microbicidal oxidative burst when ingested in phagocytes, whereas others prevent phagosome-lysosome fusion or escape from the phagosome into the cytoplasm (8, 18, 19).

The adaptation of facultative intracellular bacteria to various hostile environments involves modulation of their protein synthesis. By use of two-dimensional polyacrylamide gel electrophoresis (PAGE), a general picture of this modulation can be obtained. When facultative intracellular pathogens are exposed to various noxious agents, the expression of a large number of proteins is induced or repressed. Among proteins induced are the so-called heat shock proteins, stress proteins which fulfill functions as molecular chaperones and stabilize protein conformation (26, 35).

To better understand mechanisms of adaptation of facultative intracellular bacteria to the intracellular environment, two-dimensional electrophoresis has been used for analysis of protein regulation during growth in macrophage cell lines. *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Listeria monocytogenes*, and *Salmonella typhimurium* have all been shown to modulate their protein synthesis while growing in macrophages (1, 6, 16, 21, 23). Among proteins induced are both well-characterized stress proteins and previously unrecognized proteins.

Francisella tularensis, the etiological agent of the zoonotic disease tularemia, has a well-documented ability to invade and multiply inside various eucaryotic cells, including both professional phagocytes and nonphagocytic cells (3, 12, 25). In experimental infection, the attenuated live vaccine strain *F. tularensis* LVS is used. The strain is virulent for mice, and murine tularemia caused by the vaccine strain is an established model of human tularemia (4, 13, 31).

In this study, we examined the translational response of *F. tularensis* LVS during growth within the murine macrophage-like cell line J774 by two-dimensional gel electrophoresis. Compared to extracellular growth, intracellular growth resulted in increased synthesis of a few proteins. The gene encoding a strongly upregulated 23-kDa protein was cloned, sequenced, and expressed in *Escherichia coli.*

MATERIALS AND METHODS

Bacterial strains, cell lines, and plasmids. *F. tularensis* LVS was supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md. It was stored at −70°C and cultivated on modified Thayer-Martin agar containing GC medium base (30). For each experiment, bacteria were grown overnight at 37°C in the synthetic liquid Chamberlain medium (7). After dilution in the same medium, bacteria were grown to exponential phase. The murine macrophage line J774 was grown in cell medium containing Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Grand Island, N.Y.) with 10% (vol/vol) fetal calf serum (FCS). *E. coli* DH5α has been previously described (15).

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Plasmids pTrc99A (Pharmacia AB, Uppsala, Sweden) and pBluescript II KS$^+$ (Stratagene, La Jolla, Calif.) were purchased.

DNA manipulations. All DNA manipulations, including purification, digestion with restriction endonucleases, ligations, and gel electrophoresis, were performed according to methods described by Sambrook et al. (28). Purification of DNA fragments was done by β-agarase treatment as instructed by the manufacturer (Boehringer, Mannheim, Germany). Southern blot analysis was carried out on nylon membranes (Hybond-N; Amersham International, Amersham, England) as described by Southern (33). Double-stranded DNA probes were labeled with [α-^{32}P]dATP (Amersham International) by use of random hexanucleotide primers (Pharmacia) according to the manufacturer's instructions.

Radioactive labeling of extracellularly growing *F. tularensis* LVS. Exponentially growing *F. tularensis* LVS was pelleted and suspended at a density of 10^7 organisms/ml in methionine-free Chamberlain medium or methionine-free DMEM with 10% FCS. To 1-ml portions, 10 μl of L-[^{35}S]methionine (10 μCi/μl; specific activity, 1,000 Ci/mmol; Amersham International) was added to a final concentration of 100 μCi/ml. After a 2-h labeling period, an excess of cold methionine (0.4 g/liter) was added, and the tubes were placed on ice. Each suspension was transferred to an Eppendorf tube, and the cells were pelleted, washed twice in phosphate-buffered saline (PBS), suspended in 50 to 100 μl of distilled H_2O, and frozen at −70°C until subjected to two-dimensional electrophoresis.

Intracellular growth of *F. tularensis* LVS in macrophages and radiolabeling of bacterial proteins. After overnight incubation of 1-ml cultures of J774 cells (10^6 cells per well in a six-well tissue culture plate) in cell medium at 37°C, 1.0 ml of DMEM containing 10^7 cells of *F. tularensis* LVS was added to each culture. After incubation for 2 h at 37°C in 5% CO_2, the wells were washed twice to remove extracellular bacteria. To each well, 1.0 ml of cell medium with gentamicin (50 μg/ml) was added, and incubation was continued for 1 h. Thereafter, the monolayer was washed once, and cell medium containing 2 μg of gentamicin per ml was added. After various periods of incubation at 37°C, the medium was changed to methionine-free DMEM. After 1 h of further incubation, cycloheximide (200 μg/ml) and L-[^{35}S]methionine (100 μCi/ml) were added for a 2-h period of pulse-labeling. The monolayer was washed twice with ice-cold PBS and lysed with 0.1% sodium deoxycholate in PBS. Bacteria were pelleted by centrifugation, washed twice in PBS, and resuspended in 100 μl of distilled H_2O. The suspension was stored at −70°C until gel electrophoresis was performed. From parallel wells, viable counts were performed by lysis of the monolayers and incubation of samples of the suspensions on Thayer-Martin agar plates. In a control experiment, the bacterial growth rate in macrophages was found to be essentially the same irrespective of whether methionine was present (data not shown).

For quantitation of total protein synthesis, a portion of the bacterial suspension was dissolved in 1 ml of Tris-EDTA (pH 7.8), cooled, and precipitated by the addition of an equal volume of ice-cold 10% trichloroacetic acid (TCA). The precipitate was collected on a glass fiber filter and washed three times with cold 5% TCA and twice with −20°C ethanol for determination of radioactivity in a liquid scintillation counter.

One- and two-dimensional gel electrophoresis. Samples from the stored bacterial suspensions, containing 10^5 cpm, were concentrated to a minimal volume in a Speed Vac Concentrator (Savant Instruments, Holbrook, N.Y.) and subjected to one- or two-dimensional gel electrophoresis. For one-dimensional sodium dodecyl sulfate (SDS)-PAGE, the samples were dissolved in a buffer containing 62.5 mM Tris (pH 6.8), 1% SDS, 5% β-mercaptoethanol, and 10% glycerol. For two-dimensional electrophoresis, the samples were suspended in 20 μl of lysis buffer (9 M urea, 2% [vol/vol] 2-β-mercaptoethanol, 2% [vol/vol] Ampholine [Bio-Rad Laboratories, Richmond, Calif.], pH 3.5 to 10.0, 2.0% [vol/vol] Triton X-100) and mixed with 10 μl of a buffer containing 8 M urea, 2% (vol/vol) 2-β-mercaptoethanol, 2% (vol/vol) Ampholine (Bio-Rad), pH 3.5 to 10.0, 0.5% (vol/vol) Triton X-100, and 0.01% bromophenol blue and separated according to a modification (14) of the method described by O'Farrell (27). First, isoelectric focusing was performed by use of strips of a prefabricated dried gel (Immobiline DryPlate, pH 4.0 to 7.0; Pharmacia) as recommended by the manufacturer. After storage at −70°C, the strips were equilibrated in a solution containing 6 M urea, 10% (vol/vol) Tris-HCl (pH 6.8), 30% (vol/vol) glycerol, and 2% (wt/vol) SDS and applied on a uniform 14% acrylamide gel for electrophoresis as described by Laemmli (22). For quantitation of protein synthesis, protein spots were assayed by an InstantImager (Packard Instrument Company, Inc., Meriden, Conn.). The ratios were expressed as the means of three separate experiments.

Identification of proteins in membrane and cytoplasmic fractions of *F. tularensis*. By ultrasonic disintegration of *F. tularensis* LVS cells (29), a cytoplasmic fraction and a membrane fraction were obtained. Proteins of the cytoplasmic fraction were precipitated by addition of 10% TCA at 4°C. Each fraction was analyzed by one-dimensional gel electrophoresis and blotted. By blotting of the filters with a monoclonal antibody specific to a 17-kDa membrane protein (32), we confirmed that this protein was exclusively localized in the membrane fraction.

Antisera. After separation by two-dimensional PAGE, a gel piece containing the intracellularly induced 23-kDa protein was excised, washed, and suspended in complete Freund's adjuvant. The suspension was used for subcutaneous immunization of rabbits according to a previously used schedule (30). A monoclonal antibody specific to a 17-kDa membrane lipoprotein of *F. tularensis* LVS was produced as previously described (32).

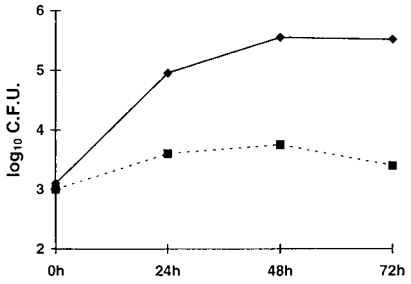

FIG. 1. Enumeration of *F. tularensis* LVS organisms after various intervals of growth in the murine cell line J774 (solid line) or cell medium, i.e., DMEM with 10% FCS (broken line).

Western blotting, autoradiography, and sequencing of peptides. For Western blot analysis, proteins in acrylamide gels were transferred to nitrocellulose filters and probed as described by Swanson et al. (34). An alkaline phosphatase-conjugated secondary antibody system was used.

Sequence determination of peptides. For amino acid sequencing, proteins of polyacrylamide gels were transferred to a polyvinylidene difluoride transfer membrane (Millipore Corp., Bedford, Mass.). After staining with Coomassie brilliant blue, the spot containing the 23-kDa protein was excised and subjected to N-terminal sequencing by the Edman degradation technique. To generate internal fragments of the protein, trypsin degradation was kindly performed by Bo Ek, Biomedical Center, Uppsala, Sweden.

DNA sequencing. An *Xba*I-cleaved fragment, shown by Southern blot analysis to contain the gene of the 23-kDa protein of *F. tularensis*, was ligated into pBluescript II KS$^+$ phagemid vector DNA. The recombinant vector DNA was used for transformation of competent cells of *E. coli* DH5α. After plating, clones were screened by PCR using degenerate primers deduced from the sequenced peptides. Plasmids from recombinant clones were purified by the alkaline-lysate procedure (28) and sequenced in both directions by the dideoxynucleotide chain termination method, using an AmpliCycle sequencing kit (Perkin-Elmer, Norwalk, Conn.). Universal primers and specific primers, based on the sequenced DNA, were synthesized by SGS, Köping, Sweden, and used for PCR amplification and initiation of the reaction. [α-^{32}P]dATP was used for labeling.

The software package GCG, developed by the Genetics Computer Group (University of Wisconsin, Madison), was used to assemble and analyze the sequences. The FASTA and BLAST programs of the GCG package were used for homology searches in the GenBank and SWISS PROT databases.

Expression of a 23-kDa protein of *F. tularensis* LVS in *E. coli*. Oligonucleotide primers complementary to the ends of the gene encoding the 23-kDa protein were synthesized. The primer complementary to the 5′ end was extended by an *Nco*I restriction site, and that complementary to the 3′ end was extended by an *Xba*I restriction site. Due to exchange of a nucleotide, the procedure resulted in substitution of the second amino acid (serine to glycine) of the 23-kDa protein. The DNA fragment was amplified by PCR and ligated to plasmid pTrc99A. After transformation into *E. coli* DH5α and induction of the *lac* promoter of the plasmid, the bacterial lysate was probed with a polyclonal rabbit serum specific to the 23-kDa protein.

Nucleotide sequence accession number. The nucleotide sequence of the gene encoding the 23-kDa protein was submitted to the EMBL database and given accession no. Y08861.

RESULTS

Survival and multiplication of *F. tularensis* LVS in J774 macrophages. *F. tularensis* LVS invaded and replicated intracellularly in the macrophage-like cell line J774. During a period of 48 h, exponential growth occurred and bacterial numbers increased approximately 100-fold (Fig. 1). In repeated experiments, the doubling time was 5 to 7 h. After 2 days of incubation, the macrophage monolayers began to detach and bacterial growth ceased.

In preliminary experiments, limited growth of *F. tularensis* LVS was found to occur in cell medium lacking macrophages (Fig. 1). To arrest extracellular bacterial growth of the organism, gentamicin was added to the medium. Since gentamicin might affect intracellular bacterial growth (9), we assessed its effect on *F. tularensis* incubated under various conditions. In

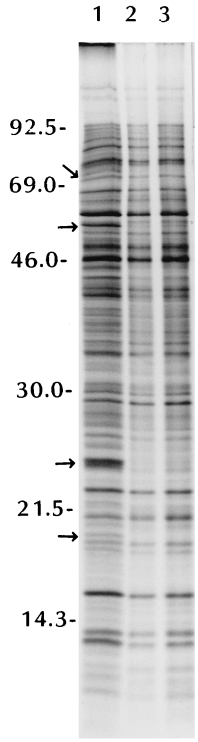

FIG. 2. Protein synthesis of *F. tularensis* LVS during intracellular growth in the J774 cell line analyzed by one-dimensional SDS-PAGE. After 24 h of growth, bacteria were pulse-labeled for 2 h with [^{35}S]methionine. Cell extracts were separated by SDS-PAGE and subjected to autoradiography. The numbers on the left are molecular masses in kilodaltons. Lane 1, intracellularly growing *F. tularensis* LVS, ~10^5 cpm; lane 2, *F. tularensis* LVS grown in Chamberlain medium, 10^5 cpm; lane 3, *F. tularensis* LVS grown in Chamberlain medium, 2×10^5 cpm.

Chamberlain medium, the MIC was 0.1 to 0.2 μg/ml. In the presence of macrophages, however, bacterial growth was not completely inhibited even at 50 μg/ml. Under conditions used in the experiments, i.e., incubation for 1 h at a concentration of 50 μg/ml followed by washing and continued incubation in the presence of 2 μg of gentamicin per ml, intracellular replication of *F. tularensis* was not affected.

Pattern of protein synthesis of *F. tularensis* LVS during infection of macrophages. After 2, 6, 24, or 48 h of growth of *F. tularensis* intracellularly in J774 macrophages or extracellularly in cell medium or Chamberlain medium, [^{35}S]methionine was added for a 2-h period of pulse-labeling. Proteins were separated by one- or two-dimensional PAGE, and the patterns of protein synthesis were analyzed by autoradiography of the blotted gels.

One-dimensional electrophoresis of bacteria pulse-labeled at 24 h of intracellular growth showed increased synthesis of at least four proteins (approximate molecular masses of 20, 23, 55, and 70 kDa) compared to bacteria incubated in Chamberlain medium (Fig. 2). After incubation in cell medium without macrophages, the pattern of protein synthesis was quite similar to that in cell medium (data not shown). Two-dimensional gels, analyzed by a bioimager for computer-aided quantification, showed similar results in three separate experiments. Of 24 major proteins present at 24 h of intracellular growth, 4 were synthesized at a rate at least 1.5 times higher than that of bacteria growing in cell medium (proteins 7, 8, 12, and 17 in Fig. 3A and C and Table 1). Notably, DnaK (protein 2), GroEL (protein 3), and GroES (protein 10) were only marginally or not at all induced. In comparison to logarithmically growing bacteria in Chamberlain medium, a number of proteins were up- and downregulated (Table 1). The most marked upregulation was demonstrated for three of the four proteins indicated above (proteins 7, 8, and 17 in Fig. 3A and B and Table 1) and also for protein 15 and DnaK (protein 2).

Upregulation during intracellular growth of a 23-kDa protein with a pI of 5.8 (protein 7 in Fig. 3 and Table 1) was observed at 6, 24, and 48 h but not at 2 h postinfection (data not shown). At 24 h, a 3.7-fold increase was recorded (Fig. 3). Of proteins found by one-dimensional electrophoresis to be induced during intracellular growth, only the 23-kDa protein was shown to be induced also by use of two-dimensional gels. Possibly, the pI of the other proteins might have been outside the pH range (4 to 7).

As expected (6), cycloheximide effectively arrested eucaryotic protein synthesis; no labeled proteins were detected in J774 cells inoculated without bacteria. In other control experiments, the presence of cycloheximide was found not to alter bacterial growth rate, viability, or pattern of protein synthesis (data not shown).

In further control experiments, we addressed the possibility that gentamicin used to inhibit extracellular bacterial growth affected the synthesis of the 23-kDa protein or other proteins of LVS. Irrespective of whether gentamicin was added immediately after addition of the bacteria to the macrophage culture or at 22 h postinfection, bacteria labeled at 24 h showed the same pattern of protein synthesis (data not shown). Moreover, when gentamicin was not added, thereby allowing both extra- and intracellular growth to occur, pronounced induction of the 23-kDa protein was observed. Finally, at 2 h postinfection, no induction of the protein was observed, irrespective of the presence of gentamicin.

Subcellular localization of the 23-kDa protein in *F. tularensis*. Ultrasonic disintegration of *F. tularensis* resulted in a supernatant containing the cytoplasmic fraction and a pellet containing the membrane-bound proteins. The 23-kDa protein was predominantly found in the cytoplasmic fraction (Fig. 4). By quantitative analysis, more than 90% of the protein was found in the cytoplasmic fraction.

Induction of the 23-kDa protein in response to hydrogen peroxide. We tested whether the 23-kDa protein may be upregulated in response to defined stress conditions. To this end, hydrogen peroxide (5 mM) was added to exponentially growing *F. tularensis*. Five minutes after the addition, [^{35}S]methionine was added to the culture. The induction of the 23-kDa protein was prominent (Fig. 5). A temperature shift to 42°C, however, did not result in a significant induction.

In most experiments, induction by exposure to hydrogen peroxide (Fig. 5) as well as to macrophages (Fig. 3A and C) caused a slight decrease in the pI of the 23-kDa protein. Western blot analysis consistently verified the identity of the protein. Similar observations have been made for other bacterial proteins. For example, the ribosomal protein L7 of *S. typhimurium* and its N-terminally acetylated form L12 vary in pI but not in relative molecular mass (1).

Sequence determination of the gene encoding the 23-kDa protein. To obtain the N-terminal sequence of the 23-kDa protein, a preparative two-dimensional gel was electroeluted to polyvinylidene fluoride membranes and the protein spot was excised. Twenty-two amino acids of the N-terminal sequence were determined by automated Edman degradation. Moreover, after trypsin digestion of the 23-kDa protein and separation of the resulting peptides by high-pressure liquid chromatography, two additional peptides were sequenced. Altogether, 55 amino acid residues were thus identified.

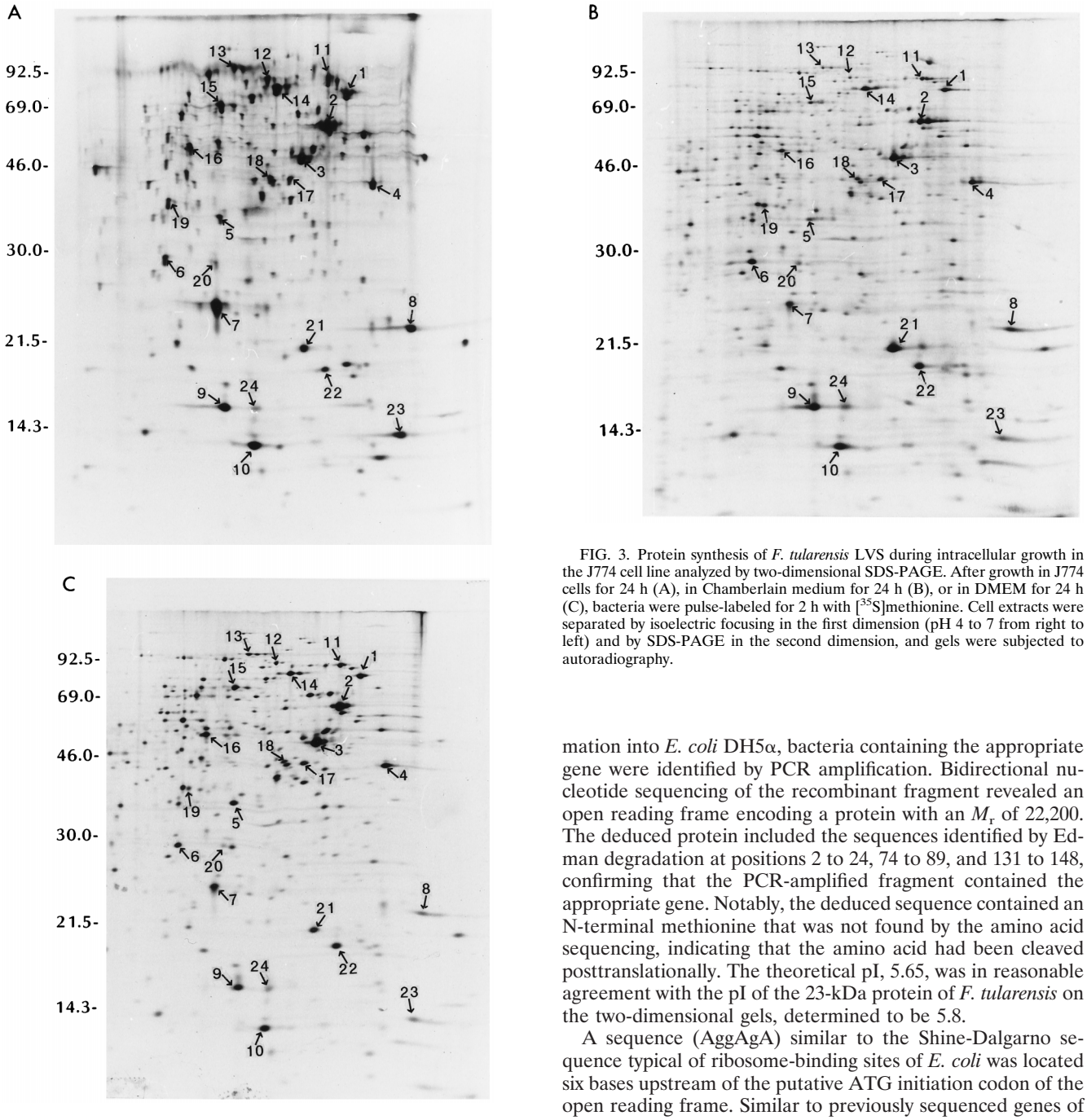

FIG. 3. Protein synthesis of *F. tularensis* LVS during intracellular growth in the J774 cell line analyzed by two-dimensional SDS-PAGE. After growth in J774 cells for 24 h (A), in Chamberlain medium for 24 h (B), or in DMEM for 24 h (C), bacteria were pulse-labeled for 2 h with [^{35}S]methionine. Cell extracts were separated by isoelectric focusing in the first dimension (pH 4 to 7 from right to left) and by SDS-PAGE in the second dimension, and gels were subjected to autoradiography.

Based on the three amino acid sequences, degenerate oligonucleotide probes were designed. To minimize the degeneration, sequence information on the codon usage in *F. tularensis* was utilized (32). By use of PCR, three DNA fragments of different sizes were successfully amplified, confirming the successful annealing of the oligonucleotides to *F. tularensis* DNA.

Chromosomal DNA of *F. tularensis* was digested with restriction enzymes. The digested DNA was separated on an agarose gel and probed by a radiolabeled DNA fragment amplified by PCR. A 1.3-kb fragment, shown to hybridize to the probe, was excised, eluted from the gel by β-agarase treatment, and ligated into the pBluescript vector DNA. After transformation into *E. coli* DH5α, bacteria containing the appropriate gene were identified by PCR amplification. Bidirectional nucleotide sequencing of the recombinant fragment revealed an open reading frame encoding a protein with an M_r of 22,200. The deduced protein included the sequences identified by Edman degradation at positions 2 to 24, 74 to 89, and 131 to 148, confirming that the PCR-amplified fragment contained the appropriate gene. Notably, the deduced sequence contained an N-terminal methionine that was not found by the amino acid sequencing, indicating that the amino acid had been cleaved posttranslationally. The theoretical pI, 5.65, was in reasonable agreement with the pI of the 23-kDa protein of *F. tularensis* on the two-dimensional gels, determined to be 5.8.

A sequence (AggAgA) similar to the Shine-Dalgarno sequence typical of ribosome-binding sites of *E. coli* was located six bases upstream of the putative ATG initiation codon of the open reading frame. Similar to previously sequenced genes of *F. tularensis*, the overall A+T content was high. This resulted in a highly biased codon usage with a pronounced predominance of A+T, 83%, at the third and most degenerate position of the translated codons.

Expression of the 23-kDa protein in *E. coli*. To express the 23-kDa protein in *E. coli*, DNA of *F. tularensis* LVS comprising the open reading frame was PCR amplified. Primers complementary to the 5′ and 3′ ends had been extended to contain *Nco*I and *Xba*I restriction sites, respectively. A fragment of 620 bp was amplified, cleaved, and ligated into plasmid pTrc99A. By transformation into *E. coli* DH5α and induction of the *trc* promoter of the plasmid, the gene was successfully translated. The gene product showed the same mobility as the 23-kDa protein of *F. tularensis*, and a polyclonal rabbit antiserum ob-

TABLE 1. Ratios of synthesis of various proteins of *F. tularensis* LVS during intracellular growth in macrophages and extracellular growth in cell medium or Chamberlain medium

Protein no.[a]	Ratio[b]	
	Intracellular/cell medium	Intracellular/Chamberlain
1	0.52 ± 0.31	0.80 ± 0.45
2	1.08 ± 0.05	2.63 ± 1.06
3	0.73 ± 0.10	1.02 ± 0.71
4	0.69 ± 0.83	0.69 ± 0.25
5	1.00 ± 0.18	0.85 ± 0.45
6	0.89 ± 0.13	0.54 ± 0.23
7	3.70 ± 0.08	3.70 ± 0.05
8	1.89 ± 0.56	1.06 ± 0.35
9	1.14 ± 0.03	0.74 ± 0.06
10	0.95 ± 0.23	0.75 ± 0.13
11	0.53 ± 0.08	0.78 ± 0.35
12	1.88 ± 0.87	2.51 ± 0.92
13	0.86 ± 0.10	1.00 ± 0.57
14	0.66 ± 0.04	1.04 ± 0.08
15	1.43 ± 0.39	1.66 ± 0.63
16	0.79 ± 0.14	0.85 ± 0.28
17	1.67 ± 0.23	1.67 ± 0.49
18	0.92 ± 0.07	0.80 ± 0.07
19	1.29 ± 0.39	0.91 ± 0.07
20	0.83 ± 0.49	0.83 ± 0.35
21	1.00 ± 0.39	0.63 ± 0.06
22	0.46 ± 0.18	0.43 ± 0.10
23	1.50 ± 0.28	0.92 ± 0.07
24	0.67 ± 0.21	0.45 ± 0.16

[a] Numbers refer to protein designations in Fig. 3.
[b] Ratio of incorporation of [^{35}S]methionine (percentage of total detected disintegration per minute) during intracellular growth and growth in cell medium (DMEM with 10% FCS) or in Chamberlain medium. Ratios are means ± standard errors of the means of three experiments.

tained by immunization with the 23-kDa protein of *F. tularensis* reacted with the recombinant protein (Fig. 6).

DISCUSSION

F. tularensis is a potent pathogen. As few as 10 organisms of the biovar *F. tularensis tularensis* (type A) given subcutaneously are sufficient to cause infection in humans, and if antibiotic treatment is not given, type A tularemia is associated with a significant mortality. A few days after onset of disease, a septic phase occurs and the bacteria are spread to various organs.

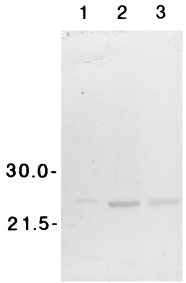

FIG. 4. Subcellular localization of a 23-kDa protein of *F. tularensis* LVS. After separation by ultracentrifugation of a lysate of *F. tularensis* LVS, 10 μg of the cytoplasmic or the outer membrane fraction was separated by electrophoresis in a 14% polyacrylamide gel and transferred to a nitrocellulose filter. Filters were probed with a polyclonal rabbit antiserum specific to the 23-kDa protein at a dilution of 1:100. Lane 1, outer membrane preparation; lane 2, cytoplasmic preparation; lane 3, total lysate of *F. tularensis* LVS. Sizes are indicated in kilodaltons.

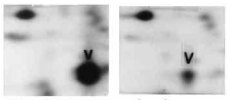

FIG. 5. Induction by hydrogen peroxide of a 23-kDa protein of *F. tularensis* LVS. *F. tularensis* LVS was grown to logarithmic phase in Chamberlain medium, exposed to hydrogen peroxide (5 mM) for 35 min, and pulse-labeled for 15 min with [^{35}S]methionine. Cell extracts were separated by isoelectric focusing in the first dimension (pH 4 to 7 from right to left) and SDS-PAGE in the second dimension, and gels were subjected to autoradiography.

There is no evidence of production of any potent bacterial toxin. Instead, symptoms and lesions are believed to be secondary to host-derived responses to the vigorously multiplying bacteria. An event central to the pathogenesis of tularemia is the adaptation of the pathogen to macrophages and other target cells of the host. Obviously, modulation of protein synthesis may be crucial to this adaptation. This study seems to pinpoint one of the proteins regulated. The 23-kDa protein was the most prominently upregulated protein during intracellular growth in macrophages.

Two-dimensional electrophoresis is useful in studies of the adaptation of facultative intracellular bacteria to their intracellular environment. Besides providing a general picture of the regulation of bacterial protein synthesis, it allows comparison of changes caused by macrophages to those caused by direct exposure to agents known to be active intracellularly. Usually, only some of the proteins induced under a defined stress condition are induced also in cultured macrophages (1, 6, 20, 23). The prominent induction of the 23-kDa protein of *F. tularensis* LVS both during intracellular growth in the macrophage line J744 and after exposure of extracellularly growing bacteria to hydrogen peroxide suggests that it is important to the bacteria during host-derived stress. The lack of upregulation of the protein when bacteria were growing in cell medium or during logarithmic growth in Chamberlain medium indicates that the induction is related to the adaptation of *F. tularensis* to the intracellular compartment.

The 23-kDa protein showed no significant homology with any sequence in current databases, and thus, no hints regarding a possible function of the protein were obtained. The lack of genetic systems for the generation of specific mutants in *F. tularensis* is another obstacle in delineation of its function. The 23-kDa protein was localized predominantly in the cytoplasmic fraction of the bacterial cells. Although computer analysis of the amino acid sequence did identify a putative transmembrane region in the protein, a cytoplasmic localization of the protein was nonetheless supported by a lack of putative signal peptide. It showed no similarity to the superoxide dismutase of *F. tularensis* (accession no. U35670), a protein with a deduced M_r similar to that of the 23-kDa protein. Proteins which, like the 23-kDa protein, are regulated in response to diverse stimuli have been called universal stress proteins. Such a protein of similar molecular weight has been identified in *L. pneumophila* (21), though with no homology in the N-terminal sequence to the 23-kDa protein of *F. tularensis*. In *M. tuberculosis*, a 23-kDa stress protein has been found, the sequence of which was not reported (2).

Compared to *S. typhimurium*, *L. pneumophila*, or *M. tuberculosis* (1, 6, 21, 23), the number of proteins found to be induced in *F. tularensis* LVS during growth in macrophages was quite low. In fact, only four proteins were here shown to be expressed at notably increased levels. In contrast, *F. tularensis* LVS responded to heat or hydrogen peroxide with increased

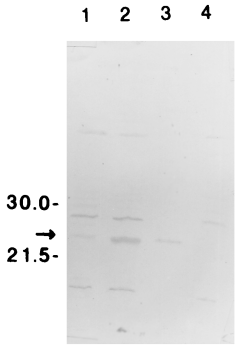

FIG. 6. Western blot analysis of the expression of the 23-kDa protein of *F. tularensis* LVS by *E. coli. E. coli* DH5α was transformed with a recombinant plasmid derived from pTrc99A containing the 23-kDa-protein gene. The bacterial extract was separated by SDS-PAGE and blotted onto a nitrocellulose filter. Filters were probed with an adsorbed polyclonal rabbit antiserum, diluted 1:100, specific to the 23-kDa protein. The background reactivity visible originated from the polyclonal serum. Lane 1, lysate of recombinant strain before induction with isopropylthiogalactopyranoside (IPTG); lane 2, lysate of recombinant strain after induction with IPTG; lane 3, native 23-kDa protein; lane 4, lysate of *E. coli* DH5α harboring the wild-type pTrc99A plasmid. Sizes are indicated in kilodaltons.

synthesis of at least 15 proteins (10). The low response to macrophages was apparently not due to unfavorable conditions for bacterium-host cell interaction per se. *F. tularensis* LVS replicated in the macrophages with a doubling time of 5 to 7 h, which is similar to previous observations on the organism (11) and similar also to results on the replicative rates of *S. typhimurium* and *L. pneumophila* in macrophages (1, 21). Our control experiments indicated that gentamicin and cycloheximide, used to inhibit extracellular bacterial growth and protein synthesis of host cells, respectively, did not interfere with intracellular synthesis of bacterial proteins.

Also, in *L. monocytogenes*, the induction of proteins by intracellular infection in macrophages has been reported to be modest (16). Consistent with the present observations in *F. tularensis*, *L. monocytogenes* showed a pronounced upregulation of the DnaK and GroEL homologs in response to heat or oxidative stress but not when grown intracellularly in macrophages. In this respect, *F. tularensis* and *L. monocytogenes* seem to differ from *M. tuberculosis* and *Brucella* species, both of which have been observed to markedly upregulate the synthesis of DnaK and GroEL during growth in macrophages (20, 23, 24).

The stress response of an intracellular parasite may be related to which intracellular compartment is inhabited. In vitro, *F. tularensis* LVS replicates in endosomes of murine macrophages (4), a compartment which is acidified, thereby making iron available to the pathogen (11). The ability of *F. tularensis* to replicate in this environment without inducing a vigorous stress protein response may seem remarkable. In this respect, it differs not only from *S. typhimurium*, *L. pneumophila*, *M. tuberculosis*, and *Brucella* species (1, 6, 20, 21, 23, 24), agents which mobilize a strong stress protein response while localized in the endosome, but also from *L. monocytogenes*, an agent which escapes the phagosome and replicates in the cytoplasm (8). In fact, this localization has accordingly been suggested to make *L. monocytogenes* less dependent on its stress proteins (16).

A bacterium which is well adapted to the macrophage may be able to survive intracellularly without a need for dramatic change in protein synthesis. Actually, this would be a matter of virulence, and again, observations on *L. monocytogenes* are of interest. When an attenuated mutant of the species was ingested by macrophages, it showed a much stronger increase in bacterial protein synthesis than did a virulent strain (17). *F. tularensis* is a highly virulent intracellular pathogen causing rapidly developing infection in the susceptible mammalian host, indicating that it may be well adapted to intracellular growth. The present finding of a modest stress response of *F. tularensis* to macrophages would be in line with such an adaptation. Interestingly, an attenuated mutant of *F. tularensis* LVS has been found to depend on its stress protein response in order to express any degree of virulence. After exposure to various forms of stress in vitro, the mutant strain showed a transient increase in both virulence in mice and capacity to infect and proliferate intracellularly in macrophages (5). Possibly, an ability to mobilize a stress response is of special importance to attenuated, less well adapted strains of facultative intracellular bacteria.

In essence, the stress proteins produced by *F. tularensis* in response to macrophages, as demonstrated in this study, and the 23-kDa protein in particular, may be worth focusing on when one is trying to understand the mechanisms involved in the adaptation of the organism to the host macrophages and inherent to its pathogenic potency. The lack of homology of the 23-kDa protein to known proteins suggests that it is not a previously characterized virulence factor.

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REFERENCES

1. **Abshire, K. Z., and F. C. Neidhardt.** 1993. Analysis of protein synthesized by *Salmonella typhimurium* during growth within a host macrophage. J. Bacteriol. **175:**3734–3743.
2. **Alavi, M. R., and L. F. Affronti.** 1994. Induction of mycobacterial proteins during phagocytosis and heat shock: a time interval analysis. J. Leukocyte Biol. **55:**633–641.
3. **Anthony, L. S. D., and P. A. L. Kongshavn.** 1987. Experimental murine tularemia caused by *Francisella tularensis*, live vaccine strain: a model of acquired cellular resistance. Microb. Pathog. **2:**3–14.
4. **Anthony, L. S. D., R. D. Burke, and F. E. Nano.** 1991. Growth of *Francisella* spp. in rodent macrophages. Infect. Immun. **59:**3291–3296.
5. **Bhatnagar, N. B., K. L. Elkins, and A. H. Fortier.** 1995. Heat stress alters the virulence of a rifampin-resistant mutant of *Francisella tularensis* LVS. Infect. Immun. **63:**154–159.
6. **Buchmeier, N. A., and F. Heffron.** 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science **248:**730–732.
7. **Chamberlain, R. E.** 1965. Evaluation of live tularemia vaccine prepared in a chemically defined medium. Appl. Microbiol. **13:**232–235.
8. **Dabri, G. A., J. M. Sanger, D. A. Portnoy, and F. S. Southwick.** 1990. *Listeria monocytogenes* moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. Proc. Natl. Acad. Sci. USA **87:**6068–6072.
9. **Drevets, D. A., B. P. Canono, P. J. M. Leenen, and P. A. Campbell.** 1994. Gentamicin kills intracellular *Listeria monocytogenes*. Infect. Immun. **62:** 2222–2228.
10. **Ericsson, M., A. Tärnvik, K. Kuoppa, G. Sandström, and A. Sjöstedt.** 1994. Increased synthesis of DnaK, GroEL, and GroES homologs by *Francisella tularensis* LVS in response to heat and hydrogen peroxide. Infect. Immun. **62:**178–183.
11. **Fortier, A. H., D. A. Leiby, R. B. Narayanan, E. Asafoadjei, R. M. Crawford, C. A. Nacy, and M. S. Meltzer.** 1995. Growth of *Francisella tularensis* LVS in macrophages: the acidic intracellular compartment provides essential iron required for growth. Infect. Immun. **63:**1478–1483.
12. **Fortier, A. H., T. Polsinelli, S. J. Green, and C. A. Nacy.** 1992. Activation of macrophages for destruction of *Francisella tularensis*: identification of cytokines, effector cells, and effector molecules. Infect. Immun. **60:**817–825.
13. **Fortier, A. H., M. V. Slayter, R. Ziemba, M. S. Meltzer, and C. A. Nacy.** 1991. Live vaccine strain of *Francisella tularensis*: infection and immunity in mice. Infect. Immun. **59:**2922–2928.

14. **Görg, A., W. Postel, and S. Günther.** 1988. Two-dimensional electrophoresis. Electrophoresis **9:**531–546.
15. **Hanahan, D.** 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. **166:**557–580.
16. **Hanawa, T., T. Yamamoto, and S. Kamiya.** 1995. *Listeria monocytogenes* can grow in macrophages without the aid of protein induced by environmental stresses. Infect. Immun. **63:**4595–4599.
17. **Hévin, B., M. Morange, and R. M. Fauve.** 1993. Absence of an early detectable increase in heat-shock protein synthesis by *Listeria monocytogenes* within mouse mononuclear phagocytes. Res. Immunol. **144:**679–689.
18. **Holzer, T. J., K. E. Nelson, V. Schauf, R. G. Crispen, and B. R. Andersen.** 1986. *Mycobacterium leprae* fails to stimulate phagocytic cell superoxide anion generation. Infect. Immun. **51:**514–520.
19. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:**2108–2126.
20. **Köhler, S., J. Teyssier, A. Cloeckaert, B. Rouot, and J.-P. Liautard.** 1996. Participation of the molecular chaperone DnaK in intracellular growth of *Brucella suis* within U937-derived phagocytes. Mol. Microbiol. **20:**701–712.
21. **Kwaik, Y. A., B. I. Eisenstein, and N. C. Engleberg.** 1993. Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. Infect. Immun. **61:**1320–1329.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
23. **Lee, B.-Y., and M. A. Horwitz.** 1995. Identification of macrophage and stress-induced proteins of *Mycobacterium tuberculosis*. J. Clin. Invest. **96:**245–249.
24. **Lin, J., L. G. Adams, and T. A. Ficht.** 1992. Characterization of the heat shock response in *Brucella abortus* and isolation of the genes encoding the GroE heat shock proteins. Infect. Immun. **60:**2425–2431.
25. **Merriott, J., A. Shoemaker, and C. M. Downs.** 1961. Growth of *Pasteurella tularensis* in cultured cells. J. Infect. Dis. **108:**136–150.
26. **Morgan, R. W., M. F. Christman, F. S. Jacobsson, G. Storz, and B. N. Ames.** 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proc. Natl. Acad. Sci. USA **83:**8059–8063.
27. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sandström, G., A. Tärnvik, and H. Wolf-Watz.** 1987. Immunospecific T-lymphocyte stimulation by membrane proteins from *Francisella tularensis*. J. Clin. Microbiol. **25:**641–644.
30. **Sandström, G., A. Tärnvik, H. Wolf-Watz, and S. Löfgren.** 1984. Antigen from *Francisella tularensis*: nonidentity between determinants participating in cell-mediated and humoral reactions. Infect. Immun. **45:**101–106.
31. **Sjöstedt, A., G. Sandström, and A. Tärnvik.** 1992. Humoral and cell-mediated immunity in mice to a 17-kilodalton lipoprotein of *Francisella tularensis* expressed by *Salmonella typhimurium*. Infect. Immun. **60:**2855–2862.
32. **Sjöstedt, A., G. Sandström, A. Tärnvik, and B. Jaurin.** 1990. Nucleotide sequence and T cell epitopes of a membrane protein of *Francisella tularensis*. J. Immunol. **143:**311–317.
33. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
34. **Swanson, J., L. W. Mayer, and M. R. Tam.** 1982. Antigenicity of *Neisseria gonorrhoeae* outer membrane protein(s) III detected by immunoprecipitation and Western blot transfer with a monoclonal antibody. Infect. Immun. **38:**668–672.
35. **Young, D. B., and T. R. Garbe.** 1991. Heat shock proteins and antigens of *Mycobacterium tuberculosis*. Infect. Immun. **59:**3086–3093.

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