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Increased Synthesis of DnaK, GroEL, and GroES Homologs by *Francisella tularensis* LVS in Response to Heat and Hydrogen Peroxide

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The response of the facultative intracellular bacterium *Francisella tularensis* LVS to stress was assayed by pulse-labeling with [^{35}S]methionine followed by two-dimensional gel electrophoresis and autoradiography. A temperature increase from 37 to 42°C or exposure to 5 mM hydrogen peroxide induced increased syntheses of at least 15 proteins. Among these proteins were a 75-, a 60-, and a 10-kDa protein. By N-terminal sequence analysis, these three proteins were found to be extensively homologous to the highly conserved chaperone proteins DnaK, GroEL, and GroES of *Escherichia coli*. Antibodies specific to the DnaK homolog of *E. coli* reacted with the 75-kDa protein, and antibodies to the GroEL homolog of *Legionella micdadei* reacted with the 60-kDa protein. A readiness to respond to hydrogen peroxide with synthesis of the chaperone components may be fundamental to the intracellular survival of pathogens such as *F. tularensis*, which are exposed to oxidative stress while invading the host macrophages.

Tularemia is a zoonotic disease. It occurs among hares, rabbits, and rodents and spreads by various routes to humans. The causative agent, *Francisella tularensis*, is a facultative intracellular bacterium. Histopathological examination of the infected host shows granulomas with abundant macrophages surrounding necrotic centers, a picture similar to that of mycobacterial disease (31). The pathogenicity of the organism is due to its ability to withstand the antimicrobial effects of the macrophages and to proliferate intracellularly into high bacterial numbers (33).

Microorganisms have evolved special mechanisms to facilitate their survival in a hostile environment. When bacteria are exposed to stress in the form of heat, low pH, or hydrogen peroxide, they increase their expression of a wide variety of proteins (5, 19, 24). Among these are the heat shock proteins, including members of the highly conserved hsp70 and hsp60 families. These proteins fulfill functions as molecular chaperones and may protect cellular proteins from denaturation (8, 17, 22).

Bacteria specialized to survive intracellularly are particularly prone to be exposed to harsh stress. Both low pH and hydrogen peroxide are constitutive parts of the antimicrobial defense of the invaded cells. Mycobacteria (16, 37), *Listeria monocytogenes* (30), *Brucella abortus* (18), *Coxiella burnetii* (35), and *Legionella pneumophila* (11) are all intracellular bacteria in which heat shock proteins have been identified.

In the present report, the response of the attenuated vaccine strain *F. tularensis* LVS to elevated temperature and to hydrogen peroxide is described.

MATERIALS AND METHODS

Bacterial strain and culture conditions. The vaccine strain *F. tularensis* LVS was supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md. It was stored at −70°C and cultivated on modified Thayer-Martin agar containing Gc-medium base (29). For each experiment, bacteria were grown overnight at 37°C in the synthetic liquid Chamberlain medium (4). After dilution in the same medium to an optical density of 0.2 at 540 nm, bacteria were harvested in the exponential phase of growth (optical density at 540 nm, 0.6 to 0.7).

Antisera. Rabbit GroEL-specific anti-*Legionella micdadei* antiserum (2) was provided by P. Hindersson, Statens Serum-institut, Copenhagen, Denmark, and rabbit DnaK-specific anti-*Escherichia coli* antiserum (38) was provided by M. Zylicz, Department of Molecular Biology, University of Gdansk, Gdansk, Poland.

Induction of bacterial response to heat and hydrogen peroxide. Bacteria were washed once in phosphate-buffered saline (PBS) (pH 7.3) and suspended at the original density in methionine-free Chamberlain medium. To study the bacterial response to heat, 1-ml portions of the suspension were transferred to appropriately tempered water baths for further incubation under shaking. In other experiments, hydrogen peroxide was added to the tubes to give a final concentration of 50 μM to 500 mM. Before and during various intervals after exposure of the bacteria to heat or hydrogen peroxide, viable counts were determined.

Radioactive labeling of protein. The stress response of *F. tularensis* LVS was assayed by pulse-labeling with L-[^{35}S]methionine (10 μCi/μl; specific activity, 1,000 Ci/mmol; Amersham International). To each 1-ml portion of bacterial suspension in methionine-free Chamberlain medium, [^{35}S]methionine was added to a final concentration of 100 μCi/ml. After a 15-min 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 PBS, suspended in 50 μl of lysis buffer (9 M urea, 2% [vol/vol] Ampholine [pH 3.5 to 10.0] [Bio-Rad Laboratories, Richmond, Calif.], 2% [vol/vol] Triton X-100, and 8 mM phenylmethylsulfonyl fluoride), and frozen at −80°C until subjected to two-dimensional electrophoresis. For quantitation of total protein synthesis, a portion (1 μl) was suspended in 1 ml of Tris-EDTA (pH 7.8), cooled, and precipitated by the addi-

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tion of an equal volume of ice-cold 10% trichloroacetic acid. The precipitate was collected on a glass fiber filter and washed three times with cold 5% trichloroacetic acid and twice with −20°C ethanol for determination of radioactivity.

Two-dimensional electrophoresis. From a bacterial suspension in lysis buffer, a portion (10^8 bacteria in 3 μl) was mixed with 20 μl of sample buffer (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 subjected to two-dimensional electrophoresis by a modification (10) of the method described by O'Farrell (26). First, isoelectric focusing was performed by use of strips of a prefabricated dried gel (Immobiline DryPlate, pH 4.0 to 7.0; Pharmacia LKB Biotechnology, Uppsala, Sweden) according to recommendations of the manufacturer. After storage at −80°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) sodium dodecyl sulfate (SDS) and applied on a uniform 12% acrylamide gel for electrophoresis as described by Laemmli (15).

Western blotting (immunoblotting), autoradiography, and sequencing of proteins. For Western blot analysis, proteins from acrylamide gels were transferred to nitrocellulose filters and probed as described by Swanson et al. (32). An alkaline phosphatase-conjugated secondary antibody system was used. Some filters were probed by use of the ECL Western blotting protocol RPN 2108 (Amersham International). Autoradiography was performed by exposure of the nitrocellulose filters to Hyperfilm-MP (Amersham International) for 1 to 3 days at −80°C.

For amino acid sequencing, proteins from acrylamide gels were transferred to polyvinylidenedifluoride transfer membrane (Millipore Corp., Bedford, Mass.). After being stained with Coomassie brilliant blue, protein-containing spots were excised and subjected to N-terminal sequencing by the Edman degradation technique. Sequences were analyzed for homology to known protein sequences in the SWISS protein data base (version 32).

In vitro translation. The plasmid pOF39 (6), containing the GroES and GroEL genes, was provided by Costa Georgopoulos, Centre Médical Universitaire, Geneva, Switzerland. In vitro translation of the *groE* operon was performed by use of the Procaryotic DNA-directed translation kit according to the manual of the manufacturer (Amersham International). L-[^{35}S]methionine was used for labeling. Translated material was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), blotted on nitrocellulose filters, and autoradiographed.

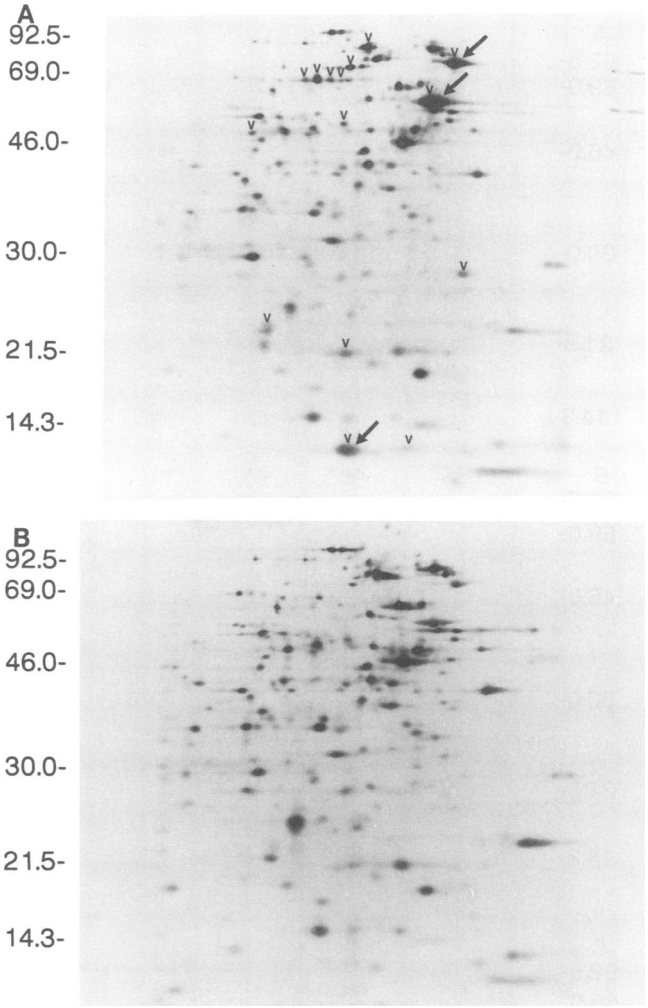

FIG. 1. Heat shock-induced proteins of *F. tularensis* LVS. Cells grown at 37°C were labeled with [^{35}S]methionine (100 μg/ml) during the 5- to 20-min interval of incubation at 42°C (A) or 37°C (B). Cell extracts were separated by isoelectric focusing in the first dimension (pH 4 to 7 from right to left) and by SDS-PAGE in the second dimension. Heat shock proteins are indicated by arrowheads; the DnaK (75 kDa), GroEL (60 kDa), and GroES (10 kDa) homologs are also indicated by arrows. The numbers on the left are molecular masses in kilodaltons.

RESULTS

Response of *F. tularensis* LVS to heat. *F. tularensis* LVS was grown overnight at 37°C and thereafter incubated for 60 min at 42, 45, or 48°C. Survival was not affected by incubation at 42°C but decreased at higher temperatures. At 45 and 48°C, the survival was 27% and <0.1%, respectively. After a temperature shift to 42°C, the bacteria were pulse-labeled with [^{35}S]methionine during four successive 15-min periods, starting 5 min after the shift. The rate of total protein synthesis was roughly the same during all periods. When protein synthesis was analyzed by two-dimensional gel electrophoresis and autoradiography, the most pronounced effect was seen within 5 to 20 min (Fig. 1A) and 20 to 35 min (data not shown) after the temperature shift. Compared with the case of control cultures incubated at 37°C (Fig. 1B), at least 15 polypeptides with molecular masses of 10 to 80 kDa were synthesized at an increased rate (Fig. 1A). Among these were a 75-, a 60-, and a 10-kDa protein, which were also induced by hydrogen peroxide (see below). In autoradiography of bacteria incubated at 42°C, the densitometric volumes of the 75-, 60-, and 10-kDa proteins were 3.1, 1.5, and 11.4 times higher, respectively, than those of cultures incubated at 37°C. There was also a concomitant decrease in synthesis of several proteins.

Response of *F. tularensis* LVS to hydrogen peroxide. When hydrogen peroxide (50 μM to 500 mM) was added to cultures exponentially growing at 37°C, a sharp decrease in viability occurred at concentrations higher than 10 mM. At 20 mM hydrogen peroxide, more than 99.9% of the bacteria were killed. To study the capacity of *F. tularensis* LVS to adapt to hydrogen peroxide, bacteria were first incubated at a 5 mM concentration for 60 min and then exposed to 10, 20, 50, and 500 mM concentrations. In contrast to results from studies

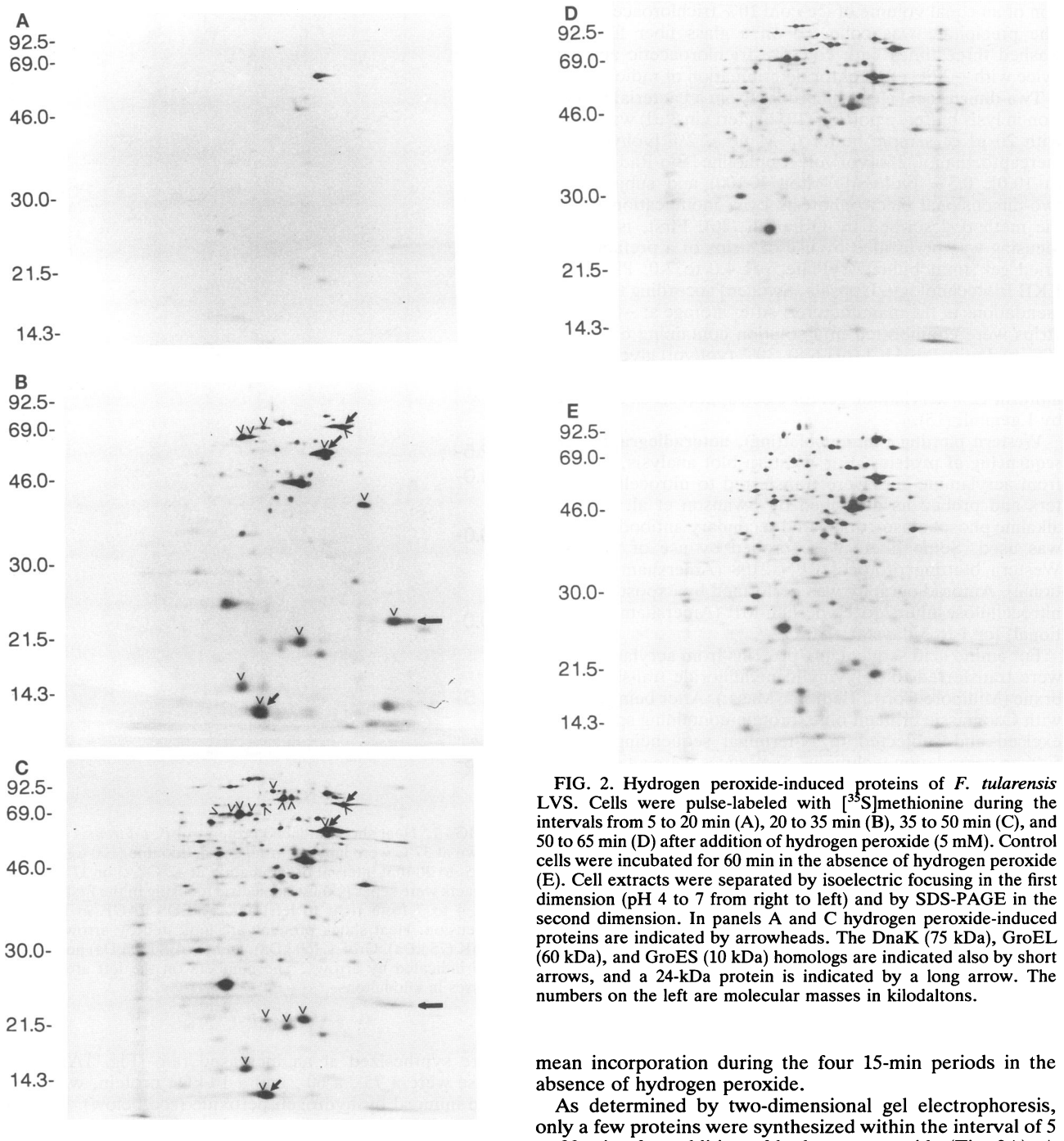

FIG. 2. Hydrogen peroxide-induced proteins of *F. tularensis* LVS. Cells were pulse-labeled with [^{35}S]methionine during the intervals from 5 to 20 min (A), 20 to 35 min (B), 35 to 50 min (C), and 50 to 65 min (D) after addition of hydrogen peroxide (5 mM). Control cells were incubated for 60 min in the absence of hydrogen peroxide (E). 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. In panels A and C hydrogen peroxide-induced proteins are indicated by arrowheads. The DnaK (75 kDa), GroEL (60 kDa), and GroES (10 kDa) homologs are indicated also by short arrows, and a 24-kDa protein is indicated by a long arrow. The numbers on the left are molecular masses in kilodaltons.

on members of the family *Enterobacteriaceae* (5), no adaptation occurred (data not shown).

The effect of hydrogen peroxide on protein synthesis was studied. Hydrogen peroxide (5 mM) was added to bacteria growing at 37°C in exponential phase, and [^{35}S]methionine labeling was performed during four successive 15-min periods. Within the period of 5 to 20 min after addition of hydrogen peroxide, the incorporation of radioactivity was only 6% of that in control cultures lacking hydrogen peroxide. Within the periods of 20 to 35, 35 to 50, and 50 to 65 min, the incorporation was 57, 146, and 167%, respectively, of the mean incorporation during the four 15-min periods in the absence of hydrogen peroxide.

As determined by two-dimensional gel electrophoresis, only a few proteins were synthesized within the interval of 5 to 20 min after addition of hydrogen peroxide (Fig. 2A). A 60-kDa protein, corresponding to a heat-inducible major 60-kDa protein (Fig. 1A), was predominant. When the relative amounts of incorporated radioactivity were determined by densitometry, approximately 80% was found in the 60-kDa protein.

During the interval of 20 to 35 min after the addition of hydrogen peroxide, 10 proteins were strongly expressed (Fig. 2B), including the heat-inducible 75-, 60-, and 10-kDa proteins. In autoradiography of bacteria harvested after this period of incubation, the densitometric volumes of the 75-, 60-, and 10 kDa-proteins were 5.3, 1.6, and 43 times higher, respectively, than those of bacteria incubated for 65 min in

the absence of hydrogen peroxide (Fig. 2E). During the interval of 35 to 50 min, renewed synthesis of most of the native proteins had started (Fig. 2C). Fifteen proteins were synthesized at a rate higher than that seen within 65 min of incubation in the absence of hydrogen peroxide. Other proteins were synthesized at a decreased rate. For example, a 24-kDa protein was synthesized at a high rate within 20 to 35 min but was only barely visible by pulse-labeling during the 35- to 50-min period. During the interval of 50 to 65 min, there was a decrease in number of proteins labeled (Fig. 2D).

Relatedness of *F. tularensis* stress proteins to conserved stress proteins. Three proteins of *F. tularensis* LVS with molecular masses of 75, 60, and 10 kDa were studied in more detail. All three proteins were synthesized at increased rates after exposure to elevated temperature (Fig. 1) or to hydrogen peroxide (Fig. 2). To obtain the N-terminal amino acid sequences of the proteins, spots were excised from blotted two-dimensional PAGE gels and sequenced. From the 75-, 60-, and 10-kDa proteins, N-terminal sequences containing 13, 19, and 15 amino acids, respectively, were obtained. These sequences were analyzed for homology to known protein sequences in the SWISS protein data base (version 32).

The 75-kDa protein showed complete identity in the N-terminal sequence with the DnaK protein of *E. coli* as well as the cognate protein of *Chlamydia trachomatis* (Fig. 3A). The 60-kDa protein showed 63% homology with the GroEL protein of *E. coli* (Fig. 3B), and the 10-kDa protein showed 73% homology with the GroES homolog of both *L. micdadei* and *C. burnetii* (Fig. 3C). To further identify the 75- and 60-kDa proteins as DnaK and GroEL homologs, their reactivities with specific antisera were determined. Rabbit antiserum against the DnaK homolog of *E. coli* reacted with the 75-kDa protein (Fig. 4A), and a serum against the GroEL homolog of *L. micdadei* reacted with the 60-kDa protein of *F. tularensis* LVS (Fig. 4B). In a control experiment, the reactivity of the antiserum with the GroEL protein of *E. coli*, expressed as an in vitro translation product of the plasmid pOF39 (5), was demonstrated (data not shown).

A) DnaK

	1									10			
F. tularensis LVS	G	K	I	I	G	I	D	L	G	T	T	N	S
E. coli	•	•	•	•	•	•	•	•	•	•	•	•	•
C. trachomatis	•	•	•	•	•	•	•	•	•	•	•	•	•
B. burgdorferi	A	•	•	•	•	H	•	•	•	•	•	•	•
M. tuberculosis	M	A	R	A	V	•	•	•	•	•	•	•	•
M. leprae	M	A	R	A	V	•	•	•	•	•	•	•	•
M. bovis BCG	A	R	A	V	•	•	•	•	•	•	D	L	G

B) GroEL

	1									10						
F. tularensis LVS	A	A	K	Q	V	L	F	S	D	G	A	R	A	K	M	*	D	G	V
E. coli	•	•	•	D	•	K	•	G	N	D	•	•	V		•	L	•	•	•
B. abortus	M	•	•	D	•	K	•	G	R	T	•	•	E	•	•	L	R		
C. burnetii	•	•	•	V	L	K	•	•	H	E	V	L	H	A	•	S	R	•	•
M. tuberculosis	M	•	•	T	I	A	Y	D	E	E	•	•	R	G	L	E	R	•	L
M. leprae	M	•	•	T	I	A	Y	D	E	E	•	•	R	G	L	E	R	•	L

C)GroES

	1									10				
F. tularensis LVS	M	N	I	R	P	L	Q	D	R	V	L	V	R	R	A
L. micdadei	•	K	•	•	•	•	H	•	•	•	V	•	•	•	M
C. burnetii	•	K	•	•	•	•	H	•	•	•	V	•	•	•	L
B. abortus	I	K	F	•	•	•	H	•	•	•	V	•	•	•	L
E. coli	•	•	•	•	•	•	H	•	•	•	I	•	K	•	K
M. tuberculosis	V	•	•	K	•	•	E	•	K	I	•	•	Q	A	N
M. bovis	V	•	•	K	•	•	E	•	K	I	•	•	Q	A	N

FIG. 3. Comparison of the N-terminal amino acid sequences of the 75-kDa DnaK homolog (A), 60-kDa GroEL homolog (B), and 10-kDa GroES homolog (C) of *F. tularensis* LVS with corresponding published sequences (SWISS data base version 32) of other bacterial species. The single-letter code is used to denote amino acids. Dots indicate identities between corresponding amino acid residues, and the asterisk indicates a gap inserted by the alignment program to maximize identity between sequences.

DISCUSSION

Intracellular bacteria induce in professional phagocytes a more or less pronounced increase in oxygen consumption (25). By the respiratory burst, oxygen is converted to such toxic products as superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. Studies of *F. tularensis* have shown that human polymorphonuclear leukocytes respond with a respiratory burst to both a wild strain and the vaccine strain *F. tularensis* LVS (20). In spleens of mice infected with *F. tularensis* LVS, hydrogen peroxide production has been demonstrated (13). According to the present results, hydrogen peroxide causes a strong stress response in *F. tularensis*, involving a wide variety of bacterial proteins.

Exposure of *F. tularensis* LVS to 5 mM hydrogen peroxide led to a prompt arrest of protein synthesis. When the synthesis gradually recurred during the first hour after exposure, the 75-kDa DnaK homolog, the 60-kDa GroEL homolog, and the 10-kDa GroES homolog, i.e., three chaperone proteins, were among the predominant proteins. Hydrogen peroxide may induce oxidative modifications of cellular proteins. The *groE* operon has been found to be induced in response to formation of denaturated or abnormal proteins (9, 27). A GroEL homolog of eucaryotic cells has recently been shown to bind intracellular proteins and protect them from denaturation (22). The DnaK and GroE proteins seem to function in an ordered protein-folding reaction (17), a function which may be responsible for the renaturation of denatured cellular proteins (7). The transient arrest of protein synthesis in *F. tularensis* after hydrogen peroxide exposure may provide the time required to mobilize the chaperone response necessary to preserve the integrity of the proteins during oxidative stress.

The present observation of a pronounced synthesis of GroE proteins by *F. tularensis* in response to hydrogen peroxide was not completely expected. Heat shock, but not hydrogen peroxide, has been found to cause increased

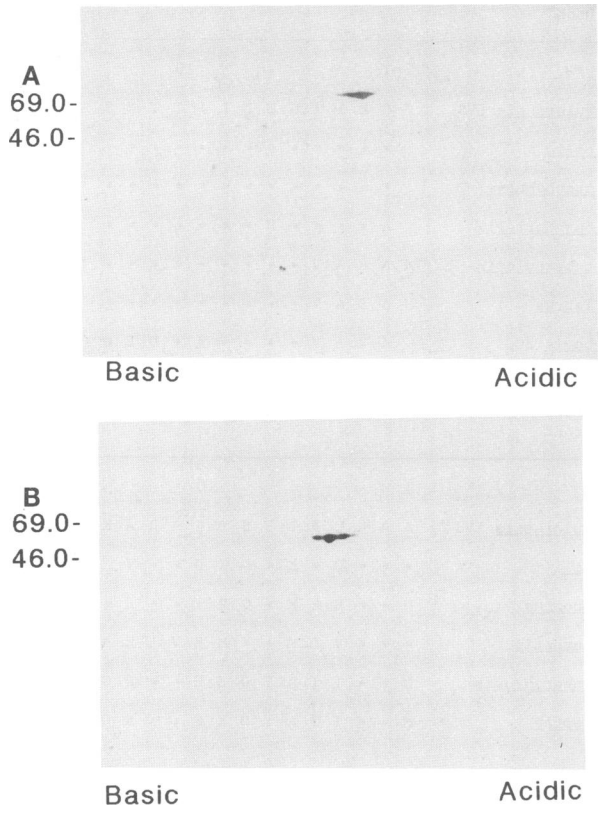

FIG. 4. Identification of DnaK and GroEL homologs of *F. tularensis* LVS by immunoblotting. Cell extracts were separated by isoelectric focusing in the first dimension (pH 4 to 7) and by SDS-PAGE in the second dimension. Western blotting was performed with polyclonal rabbit antiserum against the DnaK homolog of *E. coli* (A) and the GroEL homolog of *L. micdadei* (B). The numbers on the left are molecular masses in kilodaltons.

expression of GroEL in *E. coli* (34, 36) and of GroEL homologs in *Salmonella typhimurium* (24) and *Mycobacterium tuberculosis* (37). There is some evidence indicating that the GroES protein may be induced in *E. coli* by hydrogen peroxide, even though this induction is not under the control of *oxyR* (34). Genes involved in the responses to heat and hydrogen peroxide are believed to be regulated by at least partly different mechanisms. The induction of several proteins synthesized in response to hydrogen peroxide is regulated by the *oxyR* gene, whereas the control of the *groE* response to heat exposure is regulated by other, less completely understood, mechanisms. The reason why *F. tularensis*, in apparent contrast to members of the *Enterobacteriaceae*, showed a GroEL response to hydrogen peroxide is unknown. *F. tularensis* may be especially sensitive in detecting denaturing effects of hydrogen peroxide which lead to induction of a chaperone response. There may also be a difference among bacterial species in the regulation of the *groE* genes. In members of the *Enterobacteriaceae*, the GroEL and GroES proteins are encoded within a single operon (6), whereas *Mycobacterium leprae* contains, beside this operon, a separately arranged *groEL* gene (28). A more trivial explanation, finally, would be that the difference is simply due to differences in experimental conditions between the present study of *F. tularensis* and previous studies of other bacterial species.

Although the GroEL and DnaK homologs of various bacterial species are highly conserved in procaryotic and eucaryotic organisms, they are strongly immunogenic in mammals (12, 21, 23, 37). If these proteins are indeed induced as a result of host-parasite interaction in the infected macrophages, they may serve as important T-cell antigens and have a role in the induction of host protection. An identification of stress proteins during endocytosis will require dissociation of the microbial response from the response of the host cell. In studies of the interaction between *S. typhimurium* or *L. pneumophila* and macrophages, this has been possible (1, 3, 14). The involvement of stress proteins of *F. tularensis* in the host response to the organism remains to be studied.

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