INFECTION AND IMMUNITY, Jan. 1995, p. 154–159 Vol. 63, No. 1
0019-9567/95/$04.00+0

Heat Stress Alters the Virulence of a Rifampin-Resistant Mutant of *Francisella tularensis* LVS

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Received 17 August 1994/Accepted 19 October 1994

We have studied the stress response of a rifampin-resistant mutant of *Francisella tularensis* LVS. This mutant, Rif 7, was avirulent with an intraperitoneally administered 50% lethal dose greater than 10^7 CFU in a murine model of infection. Exposure of Rif 7 to heat stress for 5 h in vitro resulted in a 2-log decrease in its 50% lethal dose ($P < 0.02$). The increase in virulence was dependent on the time of exposure to high temperature and was maximal at 5 h. Envelope preparations from heat-stressed cells showed increased levels of several proteins. Notable among these were polypeptides with approximate molecular masses of 16, 60, and 75 kDa. Increases in both virulence and envelope protein levels were reversed when heat-treated cells were subsequently grown at 37°C. Inhibition of protein synthesis by actinomycin D during heat stress blocked the increase in virulence of Rif 7. Cell-free media from the heat-stressed Rif 7 culture or killed heat-stressed cells were not toxic to mice. Hyperimmune serum against Rif 7 reacted with the whole spectrum of bacterial proteins in Western blots (immunoblots), although its reaction with 34- and 45-kDa proteins and two 60- and 75-kDa proteins upregulated during heat stress was weak. Other stress conditions, low iron and low pH, caused similar increases in the virulence of Rif 7. However, examination of the protein profile did not reveal any major common polypeptides induced by different stresses. Heat-treated Rif 7 bacteria were fully able to replicate in macrophages in vitro and in the host tissues, even though heat treatment only partially restored virulence.

Francisella tularensis is a facultative intracellular pathogen with a predilection for cells of the reticuloendothelial system. Infections caused by the wild strains of *F. tularensis* result in a potentially fatal disease called tularemia. The disease is characterized by ulceroglandular infection, pneumonia, and typhoidal symptoms in some cases (24). An attenuated mutant of *F. tularensis*, the live vaccine strain (LVS), with reduced virulence in humans was derived from a wild strain by spontaneous mutation (7). No information is available regarding the mechanism of loss of virulence in this attenuated strain. However, notwithstanding its decrease in virulence in humans, its pathogenicity in mice remains unaffected. The mouse model provides a good in vivo system for studying the pathobiology of virulence of this organism (10).

Earlier, we described the phenotypic properties of rifampin-resistant mutants of the LVS strain, which have reduced virulence in mice (2). These Rif-resistant mutants are temperature-sensitive phenotypes unable to grow at 42°C. One of these mutants, Rif 7, was studied in more detail. The ability of Rif 7 to multiply in host macrophages was found to be greatly diminished (2). Further, inoculation of mice with Rif 7 provided protection against a lethal challenge by LVS that was as good as that provided by the parent LVS (11). Because of its relatively low toxicity in mice and ability to induce protective immunity, the Rif 7 mutant appeared to be a promising candidate for developing an attenuated vaccine against tularemia or a vaccine vector for heterologous antigens. However, in order to use the Rif mutant as a vaccine or vaccine vector, a comprehensive study was required to identify mutations which resulted in the avirulent phenotype. The results of our previous study indicated that the ability of the Rif mutant to respond adequately to the stresses in the host was probably impaired, because it was temperature sensitive and unable to grow in macrophages (2). In the present study, we investigate the heat stress response of the Rif mutant in vitro and its role (if any) in survival of the organism in the host.

Expression of virulence genes in bacterial pathogens is known to be activated by a complex array of environmental signals that are encountered by a pathogen during infection. High temperature, low pH, iron limitation, and low oxygen tension have all been recognized as examples of such environmental stimuli (20) and together constitute a global stress condition for an invading pathogen in the host. Such stresses, including heat shock, induce many well-characterized proteins which prepare the bacteria to face the hostile intracellular environment and thus may act as its virulence factors (18). In *Salmonella typhimurium*, inactivation of a regulatory heat shock gene, *htrA*, by transposon insertion results in the generation of avirulent mutants (13). Abshire and Neidhardt (1) have shown that genes expressed in response to intracellular conditions in *S. typhimurium* are important for survival and/or multiplication of the bacterium within the macrophages. In the same organism, well-known stress proteins such as GroEL and DnaK were expressed upon infection of macrophages (4).

In this study, we show that the Rif 7 mutant, when exposed to simulated stress conditions in vitro, such as high temperature, low pH, and low iron, regains part of its lost virulence through the induction of a number of stress proteins. Its inability to do so inside the host could be partly responsible for its low virulence in mice.

MATERIALS AND METHODS

Animals. Pathogen-free C57BL/6J male mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and used at 5 to 7 weeks of age. Mice were housed in a barrier environment. Animals were periodically screened serologically for murine pathogens and found to be negative.

Bacteria and growth conditions. Rif 7 was derived from *F. tularensis* LVS

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(ATCC 29684; American Type Culture Collection, Rockville, Md.) as a spontaneous mutant in the presence of rifampin (2) and was maintained as glycerol stocks in the presence of 2 μg of rifampin per ml. All subsequent cultures contained 2 μg of rifampin per ml. It was grown on Mueller-Hinton (MH) plates or broth as described previously (2). In some experiments, Chamberlain's defined medium (6) was also used. The plates were incubated at 37°C in 5% CO_2 with 95% humidity for 3 to 5 days. The broth cultures were grown at 37 or 42°C with moderate shaking in a shaker bath for indicated times. Growth was estimated either as an optical density at 700 nm or as viable CFU after dilution in sterile phosphate-buffered saline (PBS) containing 5% heat-inactivated fetal bovine serum, and cells were plated on MH plates with or without rifampin. For heat shock experiments, log-phase cells (15 to 20 h) of Rif 7 were transferred to 42°C for the indicated period of time, at the end of which viable counts were determined. For growth under low-iron or low-pH conditions, the Rif 7 cells were grown in the defined medium (6). For iron-restricted growth, Rif 7 cells were grown in Chamberlain's medium (6) without iron for 20 h, centrifuged, and resuspended in fresh medium without iron and containing 10 μM deferroxamine mesylate (Sigma Chemical Co., St. Louis, Mo.). The cultures were incubated at 37°C for 4 h with moderate shaking. Alternatively, the pH of the defined medium was adjusted to 5.6 with 1 M KH_2PO_4. Rif 7 cells from overnight cultures were resuspended in the above-described medium and incubated at 37°C for 10 h. The cells were washed three times with sterile PBS, plated for viable counts, and injected into mice. To inhibit protein synthesis, Rif 7 was grown for 20 h at 37°C, and actinomycin D (2.5 μg/ml) was added to the culture. The cultures were incubated at 42°C for 5 h with shaking and washed several times with sterile PBS, and viable plate counts were determined before injection of the bacterium into groups consisting of five mice each.

Determination of virulence in mouse model. Using the lethal mouse model described earlier for *F. tularensis* (10), we evaluated the virulence of heat-, low-iron-, or low-pH-treated cells. The bacterial cultures were diluted appropriately in sterile PBS, and mice were inoculated intraperitoneally. The animals were monitored up to 10 days. The 50% lethal dose (LD_{50}) was calculated by the method of Reed and Muench as described by Lennette (17).

Enumeration of bacteria in organs of infected mice. Mice were inoculated intraperitoneally with heat-treated Rif 7 cells (5×10^5 CFU). Progressive growth of the bacterium in the organs was monitored by removing the livers, and the spleens aseptically from the infected mice on indicated days. Tissues were homogenized in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum (Hyclone) with a Ten Brock tissue grinder, serially diluted, and plated on MH plates with or without rifampin as needed.

Preparation of antisera. Mice were inoculated intradermally with 10^4 CFU of LVS, Rif 7 mutant, or heat-treated (for 5 h) Rif 7 mutant. Mice were boosted with the same dose after 3 weeks, sacrificed 2 weeks after the boost, and bled by cardiac puncture. Pooled blood was allowed to clot, and serum was collected and stored at −20°C.

Preparation of bacterial cells and culture fluids for evaluation of toxicity. The heat-treated cells of Rif 7 were separated from the culture fluid by centrifugation at 3,000 × *g* for 15 min. Cells were washed twice with PBS, resuspended in the original volume of sterile PBS, counted, and injected intraperitoneally into each mouse. A corresponding volume of the cell-free supernatant was also injected separately. For evaluating the virulence of killed cells, a culture with a known quantity of CFU was subjected to irradiation with 2.1 megarads of γ rays under frozen conditions; alternatively, cultures were sonicated in a Branson (Danbury, Conn.) sonifier for 6 to 7 min at maximum output in ice and passed through a 0.2-μm-pore-size filter to remove any unbroken cells. A volume corresponding to the number of CFU in the live culture (before killing) was then injected into the mice. For testing the above preparations, each group consisted of five mice.

Preparation of envelopes. Bacterial envelopes were prepared as described previously (2). Briefly, the cells were disrupted by sonication for 5 to 6 min, followed by centrifugation at 10,000 × *g* to remove unbroken cells. The supernatant was then centrifuged at 100,000 × *g*, and the envelopes were obtained in the pellet.

SDS-PAGE and Western blotting (immunoblotting). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (16) in a 12.5% separating gel, and the bands were stained with Coomassie blue. Proteins were electrophoretically transferred to nitrocellulose paper in Tris-glycine buffer containing 10% (vol/vol) methanol, at 30 V overnight and then at 100 V for 1 h. The nitrocellulose paper was blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) (20 mM Tris-HCl, 0.5 M NaCl [pH 7.5]) followed by incubation in antisera diluted 1:100 in 5% skim milk in TBS. Alkaline phosphatase-labelled whole anti-mouse immunoglobulin conjugate (Southern Biotechnology, Inc., Birmingham, Ala.) was diluted 1:2,000 in TBS containing 5% skim milk to develop the color with a nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) substrate (Promega, Madison, Wis.).

Growth of heat-stressed Rif mutant in mouse peritoneal macrophage cultures. Resident peritoneal cells were isolated and cultured as described previously (9). Briefly, mice were killed by decapitation, and the peritoneum of each mouse was washed with 10 ml of DMEM containing 10% fetal bovine serum. The number of macrophages was determined from the total cell counts and a differential count after cytocentrifugation (Shandon Southern Instruments, Camberly, England) and staining with Wright-Giemsa stain. The peritoneal cells were resuspended in DMEM plus fetal bovine serum at a density of 10^6 macrophages per ml and cultured in polypropylene tubes (no. 2063; Falcon, Oxnard, Calif.). The cells were infected with bacteria at a multiplicity of infection of 1 and incubated at 37°C with 5% CO_2. Infected cells were lysed with 0.05% SDS, serially diluted, and plated for viable bacterial counts.

TABLE 1. Change in virulence of Rif 7 mutant with heat stress[a]

Time of incubation at 42°C (h)	Log LD_{50}
0	7.5
1	7.2
3	6.0
5	5.5
18	5.5
5–20[b]	>7

[a] Groups of five mice were inoculated with 10-fold dilutions of the bacterial cultures, heat treated for the indicated times. The number of viable bacterial counts was determined from duplicate plate counts. The LD_{50} values are representative of at least two separate experiments.

[b] Log-phase Rif 7 cells were incubated at 42°C for 5 h and then grown at 37°C for 20 h after dilution (1:10) into fresh medium.

RESULTS

Response of Rif 7 mutant to stresses. The inability of the Rif mutant to grow at 42°C and in the presence of murine macrophages prompted us to study its response to different kinds of stresses in vitro. Table 1 shows that the virulence of Rif 7 was increased after heat stress at 42°C in a time-dependent manner. A 100-fold decrease in the LD_{50} ($P < 0.02$) was observed when the cells were exposed to 42°C for 5 h. Longer exposures, up to 18 h, did not enhance the virulence of the cells any further. Heat stress for shorter periods of 1 and 3 h caused an approximately 10-fold decrease in the LD_{50} of the Rif 7 mutant. To determine if heat stress had permanently changed the phenotype of the Rif 7 mutant or if the increased virulence was dependent on the presence of stress proteins, the cells were heat treated for 5 h, grown at 37°C for 20 h, and then tested for virulence in mice. The LD_{50} of the latter cells was found to be 10^7, which was similar to that for the untreated Rif 7 bacteria, compared with an LD_{50} of 10^5 for heat-treated cells. Heat treatment of the parent LVS for 5 h resulted in a modest 10-fold decrease in the LD_{50}. To study the effect of other stress conditions on the virulence of Rif 7, we subjected the Rif 7 mutant to low-iron levels or low-pH conditions and then tested the virulence of treated cells in mice. The LD_{50}s of the Rif mutant under conditions of low iron or low pH were also reduced to the same extent as those under heat stress. Groups of five mice were inoculated with 10-fold dilutions of the bacterial cultures, and the number of viable cells was determined from duplicate plate counts. The log LD_{50} values (representative of two separate experiments) were 5.3 and 5.2 for low-iron and low-pH cultures, respectively. For low-iron cultures, the cells were grown overnight in synthetic medium without iron, transferred to fresh medium without iron but with 10 μM deferroxamine mesylate, and incubated at 37°C for 5 h. For low-pH cultures, Rif mutant cells were grown in synthetic medium (pH 7) overnight, diluted 1:10 into fresh medium at pH 5.6, and incubated at 37°C for 8 h. As a control, Rif mutant cells were grown in synthetic medium at 37°C and had a log LD_{50} of 7.8.

The envelope protein profile of the heat-treated cells showed an increase in the levels of several polypeptides with approximate molecular masses of 16, 60, and 75 kDa between 1 and 5 h (Fig. 1), which corresponded with the increase in the

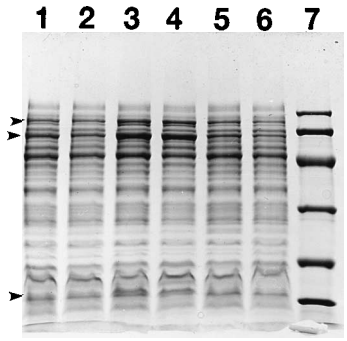

FIG. 1. SDS-PAGE profile of envelopes prepared from the Rif mutant, heat treated at 42°C for different times. Lanes: 1, 1 h; 2, 3 h; 3, 5 h; 4, 18 h; 5, 0 h; 6, 5 h followed by 20 h at 37°C; 7, molecular weight markers (from top to bottom), phosphorylase *b* (97,000), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,000), and lysozyme (14,400). Arrowheads, overexpressed proteins relative to unstressed Rif.

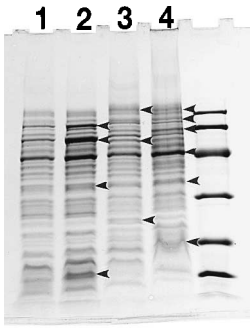

FIG. 2. SDS-PAGE profile of Rif mutant cells grown under different stress conditions. Lanes: 1, grown at 37°C; 2, grown at 42°C; 3, grown in medium without iron and containing 10 μM deferral; 4, grown in medium at pH 5.6. Arrowheads, overexpressed proteins relative to unstressed Rif.

virulence of the Rif 7 cells. Increase in expression of the above-mentioned proteins earlier than 1 h was minimal and was similar to that of the Rif mutant and the parent LVS, as determined by one-dimensional SDS-PAGE (data not presented). Envelope protein expression in the cells subjected to heat stress for 5 h and subsequently cultured at 37°C returned to the levels of unstressed cells (Fig. 1, lane 6). An SDS-PAGE profile of the envelopes prepared from bacteria subjected to growth under iron-restricted conditions or at low pH (Fig. 2) showed upregulation of a number of proteins. Cells exposed to iron-depleted conditions showed increased production of protein bands at about 25, 81, and >97 kDa. The envelopes prepared from cells grown under low-pH conditions, however, did not show a pronounced effect on the protein profile; bands with approximate molecular masses of 20, 45, 66, 80, and >97 kDa showed marginal increases compared with cells grown under normal conditions. Thus, each stress condition appears to induce the expression of a different set of proteins as identified by one-dimensional SDS-PAGE.

Increased virulence dependent on live bacteria and protein synthesis. An increase in the virulence of the Rif mutant during exposure to heat treatment was associated with the synthesis of new proteins. The addition of actinomycin D to the bacterial cultures during incubation at 42°C to block mRNA and protein synthesis caused a 100-fold decrease in the number of viable CFU after 5 h of incubation. Further, the protein profile of the cells did not show increased levels of the above-mentioned proteins and was similar to that of the cells grown at 37°C (data not shown). When these cells were injected into mice at a lethal dose of 10^7 CFU intraperitoneally, five of five mice survived the challenge with actinomycin D-treated bacteria. Toxicity of the heat-treated cells was associated only with live cells. Heat-stressed cells killed by sonication or γ irradiation had no apparent toxicity in mice; five of five mice survived an LD_{100} (10^6 CFU) challenge with sonicated or irradiated cells. Similarly, an equivalent volume of the cell-free supernatant which originally contained 10 times the LD_{100} did not kill any of the five mice challenged.

The growth characteristics of the Rif mutant were studied after exposure to heat stress (Table 2). The cells remained rifampin resistant, and their plating efficiency was the same with or without rifampin. The growth of heat stressed cells at 37°C was similar to that of untreated cells; their generation time was 4 h, and the growth reached stationary phase in about 24 h. When cells were grown at 42°C, the viability of the heat-treated cells remained unaffected up to 5 h but was severely diminished at 24 h; the number of CFU decreased by 3 to 4 orders of magnitude in that period.

Immunogenic response to heat-treated Rif mutant in mice. We prepared hyperimmune antisera against LVS, Rif 7, and heat-treated Rif 7 whole cells in mice and evaluated the resulting antisera in Western blot assays. The Western blots (Fig. 3) revealed a generally weaker reaction of anti-Rif 7 serum (panel B) with high-molecular-weight proteins and a stronger reaction with lower-molecular-weight species (panel B), in contrast to anti-heat-treated Rif mutant serum and anti-LVS serum (panels A and C, respectively). The reactivity of anti-Rif 7 serum was low against three major proteins with masses of 45, 60, and 75 kDa (Fig. 3B). Two of these, the 60- and 75-kDa proteins, were overexpressed in the Rif mutant during heat stress (Fig. 1). The 45-kDa protein, although not upregulated during heat stress, perhaps belonged to the GroEL family of heat shock proteins, as it reacted with a monoclonal antibody against a GroEL-like protein of *Bordetella pertussis* (5) (data

TABLE 2. Growth of heat-stressed Rif mutant cells in MH broth[a]

Bacterial cell	OD_{700}[b] (CFU) at:			
	37°C		42°C	
	0 h	24 h	0 h	24 h
Control (Rif mutant)	0.06 (1×10^7)	0.34 (4×10^8)	0.06 (1×10^7)	0.1 (2×10^5)
Heat-stressed Rif mutant	0.05 (1×10^7)	0.32 (3×10^8)	0.06 (1×10^7)	0.09 (1×10^4)

[a] The Rif 7 was grown in MH broth at 37°C for 20 h, diluted into fresh medium (1:10), and incubated at the indicated temperatures for 24 h. The Rif mutant cells, used as a control, were incubated at 42°C for 5 h and then diluted into fresh medium (1:10); incubation at different temperatures continued for up to 24 h. The results are representative of two separate experiments done in duplicate.

[b] OD_{700}, optical density at 700 nm.

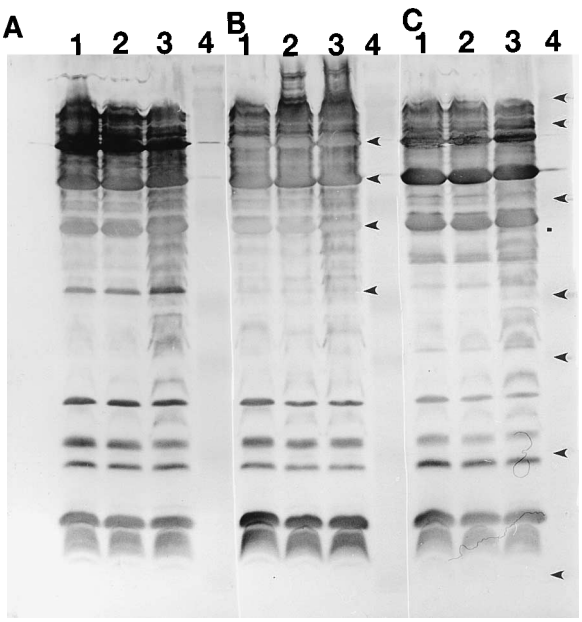

FIG. 3. Western blot of the LVS, Rif 7, and heat-treated (5 h) Rif 7 whole cells with homologous and heterologous antisera. (A) Anti-heat-treated Rif 7 antiserum; (B) anti-Rif serum; (C) anti-LVS serum. Lanes: 1, heat-treated Rif 7; 2, Rif 7; 3, LVS; 4, prestained molecular weight markers (from top to bottom, indicated by arrowheads in lane 4 of panel C [arrowheads in panel B indicate overexpressed proteins]), β-galactosidase (116,500), BSA (80,000), ovalbumin (49,500), carbonic anhydrase (32,500), soybean trypsin inhibitor (27,500), lysozyme (18,500), and aprotinin (6,500).

not shown). The reactivity of the same serum with another protein (34 kDa) was also significantly diminished compared with that of anti-heat-treated Rif serum or anti-LVS serum. Although the proteins were present in the Rif 7 mutant as well as LVS and heat-treated Rif mutant cells (Fig. 3A and C, lanes 1, 2, and 3), their immunogenicity appears to have been altered in the context of different bacterial cells.

Effect of heat treatment on ability of Rif 7 to grow in macrophages. *F. tularensis* LVS has been shown to grow in mouse peritoneal macrophages (9). We have shown previously that the ability of the Rif 7 mutant to grow in the presence of mouse macrophages is greatly diminished both in vivo and in vitro (2). However, when peritoneal macrophage cultures were infected with the heat-treated Rif 7 mutant, these bacteria grew vigorously in the presence of macrophages (Table 3). The viable counts of bacteria associated with the macrophages increased by about 3 orders of magnitude during 72 h of incubation,

TABLE 3. Growth of Rif 7 mutant in cultured mouse peritoneal cells after heat stress

Time (h)	Viable CFU[a]	
	Rif 7	Heat-treated Rif 7
0	7×10^4	4×10^5
24	3×10^5	1×10^7
48	5×10^4	2×10^7
72	8×10^3	1×10^8

[a] Freshly isolated peritoneal macrophages were infected with the indicated bacterial cells at a multiplicity of infection of 1. After the indicated time of incubation the macrophages were lysed with 0.05% SDS and plated, and the viable counts were determined from duplicate cultures. The results presented are the means from one of two replicate experiments.

TABLE 4. Replication of Rif 7 mutant cells in different organs of mice[a]

Days after infection	CFU			
	Rif 7		Heat-treated Rif 7	
	Liver	Spleen	Liver	Spleen
3	4×10	1.6×10^2	4×10^6	4×10^7
4	1×10	6×10	2×10^8	5×10^8
5	0	5	ND[b]	ND

[a] Mice were injected intraperitoneally with 10^6 CFU of differently treated Rif mutant cells. The results are representative of two replicate experiments.
[b] ND, not determined (mice died by day 5).

compared with 0.5 order of magnitude for the untreated Rif 7 mutant (we observed growth of 0.5 to 1 order of magnitude for *F. tularensis* in the DMEM medium in the absence of macrophages). Further, the initial rate of growth of the normally grown Rif 7 cells in the peritoneal cell cultures was significantly lower than that of the heat-treated cells. The latter showed a 100-fold increase in the number of viable counts within 24 h of infection. The number of viable counts of the Rif 7 started declining after 72 h of incubation, while the growth of the heat-treated counterpart was sustained.

Effect of heat treatment on ability of Rif mutant to grow in different organs of mouse. Although the time course of localization of the Rif mutant in organs is same as that of LVS (11), unlike LVS, it is completely cleared from organs within 4 to 5 days after infection. To test whether heat treatment affected localization and growth of bacteria in organs of the mouse, we injected heat-treated Rif 7 cells intraperitoneally at a dose of 10^6 viable cells. Bacteria were isolated from the livers and spleens of mice at day 1 after infection (data not shown). An increase in bacterial burden of 2 log units was observed in both livers and spleens over a period of 4 days postinfection with heat-treated Rif 7, causing death between 4 and 5 days (Table 4). In contrast, when the untreated Rif mutant was injected at the same dose, viable counts of the bacteria isolated from the organs were 5 to 6 log units lower at day 3, and by day 5 the bacteria were almost totally eliminated from the organs.

DISCUSSION

Subjecting the Rif 7 mutant cells to heat stress in vitro appears to make the cells better equipped to face the otherwise lethal host environment. Heat stress resulted in an increase in virulence of the Rif 7 cells through the induction of a set of proteins. The cells totally recovered their ability to grow in cultured murine macrophages and to replicate in vivo. Although the virulence of the Rif mutant after heat stress is still less than that of the parent LVS (LD_{50}, 10^5 cells compared with 10^2 cells of LVS), a decrease of 100-fold in the LD_{50} of these cells after heat shock (10^7 CFU before heat stress) represents a significant ($P < 0.02$) increase in the virulence of the cells. In the Rif mutant, the heat-induced proteins probably represent a part of the whole repertoire constituting its virulence.

Transition of a pathogen from the external biosphere to the body of a host causes a profound change in the biology of the organism, mobilizing the bacterial cellular machinery towards adaptation. The new environmental niche is often characterized by low pH, limited nutrients, low oxygen tension, high temperature, and other hostile conditions. These conditions act as a stimulus, signaling the microbe to respond in self defense (20). Thus, expression of virulence determinants of

bacteria inside the host usually involves a global control system, which assures the coordinate expression of a group of genes in response to a particular stimulus (19).

Since the Rif 7 mutant was not able to grow at 42°C (2), we studied its heat shock response to determine whether inability to express heat shock proteins resulted in its early clearance from the host and, hence, avirulence. Exposure of Rif 7 cells to 42°C for 5 h resulted in increased expression of several proteins, indicating that the Rif mutant had the ability to respond to stress conditions. Whether the same proteins are actually produced in the host is not known at the moment. In order to study their role in bacterial survival, we then subjected the Rif 7 cells to heat stress in vitro, before introducing them in the host. This was based on the rationale that if the protein(s) were important to the survival of Rif 7 but were not expressed in the host when needed, their induction in the bacteria beforehand should put the cells at an advantage in the mice. Indeed, our results show not only that the heat-treated bacterial cells were able to survive and eventually multiply in the host but also that their virulence was also higher than that of the untreated Rif 7 cells.

An increase in the virulence of Rif 7 following heat stress and the inhibition of this increase by actinomycin D, a transcription inhibitor, clearly indicate some role for these proteins in the virulence of Rif 7. Transcriptional control of expression of virulence genes in different microorganisms is well documented (21). Involvement of the proteins is further supported by the observation that when heat-treated Rif 7 cells with increased virulence were grown again at 37°C, they neither showed elevated levels of these proteins nor had increased virulence in mice. Further, the time course of increase in the levels of proteins and decrease in LD_{50}, both reaching their peaks at 5 h, suggest that a threshold level of protein(s) might be required for the cells to manifest increased virulence.

It is a matter of speculation at this stage as to why the stress conditions in the host are not able to cause similar changes in the Rif 7 mutant. One possibility is that Rif 7 mutant cells are unable to express a similar stress response in the host. Analysis of the immunogenic response by Western blotting suggests that the Rif 7 mutant was unable to efficiently elicit antibodies against several proteins upregulated during heat stress in vitro. Although the proteins were present in the Rif 7 cells under in vitro growth conditions, their reduced immunogenicity in the mouse could be a result of altered topology due to masking by other cell surface components or changes in the configuration of the proteins. For instance, we have observed that large aggregates of the 48-kDa protein (identified by their reaction with a monoclonal antibody to the *B. pertussis* GroEL analog) of LVS and the heat-treated Rif 7 but not Rif 7 remained in the sample wells after electrophoresis, reflecting possible differences in extent of or propensity for oligomerization (data not shown). Alternatively, a part of the Rif 7 cells are killed before they can elicit the stress proteins in sufficient quantities, and the remaining cells, even with the proteins induced, are not enough to establish infection. The latter possibility is supported by our earlier observation (2) of a considerably lower growth rate of Rif 7 than that of the parent LVS in association with peritoneal macrophages of mice.

A key component in this process may be the response time of a pathogen after recognizing the host signals. Abshire and Neidhardt (1) have shown that proteins synthesized in the early stages (0 to 2 h) of growth of *Salmonella typhimurium* in response to intracellular conditions are essential for survival within the macrophages and suggested that some of these bacterial proteins might be induced even before the bacteria is internalized by the macrophages. This would result in the bacteria being better prepared for the intracellular conditions within the macrophages (1). Similarly, in a temperature-sensitive avirulent strain of *Histoplasma capsulatum*, expression of a virulence-related protein was found to be delayed compared with the virulent parent (14).

Several reports have suggested that prior adaptation to the stress conditions may be an important step in preparing the organism to tolerate more severe stresses in the host. Brener et al. (3) have shown that meningococci grown at pH 6.6 under iron-limiting conditions in vitro had significantly increased virulence in mice. In *Vibrio cholerae*, expression of the *toxR* gene, a transcriptional activator of the virulence factors, was shown to be modulated by heat shock response (23). In *Salmonella dublin*, in vitro stress conditions were found to be responsible for the induction of a number of genes essential for the development of systemic infection in mice (25). Thus, the in vitro heat shock response of a pathogen is often considered to represent the stress response inside the host, resulting in the synthesis of proteins necessary for survival. Here, in vitro heat treatment of Rif 7 cells apparently improved the initial short-term survival in the host, resulting in efficient establishment of infection and, subsequently, later replication.

The inability to survive and multiply in the host macrophages is a well-accepted cause of avirulence in several microorganisms (8, 12). Although the ability of the Rif 7 cells to grow in cultured murine macrophages was fully recovered after 5 h of heat shock and was comparable to that of the parent LVS (2), the corresponding decrease in the LD_{50} of the cells after heat stress was on the order of 100-fold; this is still 1,000-fold higher than the LD_{50} of the parent LVS. Thus, survival in the macrophages is probably not the only factor needed in order for *F. tularensis* to be fully virulent. Further, in this rifampin-resistant mutant with an altered RNA polymerase (2), interference with expression of other key virulence genes is quite conceivable.

Heat shock proteins such as GroEL, GroES, and DnaK have been reported to be induced during intracellular growth of *Salmonella* and *Legionella* spp. (4, 15). Further work is needed to identify the individual proteins induced in the Rif 7 mutant in response to heat shock, with respect to well-known heat shock proteins of other organisms. Also, the involvement (if any) of the PhoP-PhoQ regulatory system in the Rif 7 mutant, known to control the intracellular survival of *S. typhimurium* (22), is a matter for further investigation. Other stress conditions, such as low-pH and low-iron levels, also caused similar decreases in the LD_{50} of the Rif 7 cells, but the protein profiles of the envelopes do not reveal any major common protein induced under all the stress conditions. Thus, multiple regulons, each regulating a distinct set of genes, are probably involved in the expression of overall virulence of *F. tularensis*. The results presented here clearly show that heat stress acts as an environmental stimulus in vitro to cause the expression of some critical proteins in Rif 7 that are necessary for its survival in the macrophages, contributing to the bacterium's virulence in mice.

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