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Induction of Heat-Shock Proteins in *Coxiella burnetii*

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INTRODUCTION

All biological organisms studied to date appear to respond to stressful temperature elevation by synthesizing a specific subset of proteins.^{1,2} Known as heat shock proteins (HSP), these proteins range in size from over 100 kilodaltons (kDa) to slightly over 10 kDa.^{1,3} Some of the members of this protein group are referred to as "chaperonins" because of their proven, or sometimes suspected, role in chaperoning or directing the assembly of multisubunit protein complexes during normal growth.⁴ In plants, for example, a protein with an amino acid sequence resembling that of the groEL protein in *Escherichia coli* is believed to aid the assembly of ribulose biphosphate carboxylase/oxygenase (RuBisCO) within chloroplasts. In *E. coli*, the groEL protein functions in bacteriophage assembly and is important in phage nucleic acid synthesis as well.³⁻⁵ When the growth temperature of *E. coli* is raised from 34°C to 42°C, the synthesis of the groEL protein is stimulated, as is the synthesis of 15-20 others, to the extent that it becomes one of the major proteins in the cell. In addition to heat, some of the HSP in *E. coli* can be induced by other stress conditions, such as the presence of ethanol, peroxides, DNA gyrase inhibitors, and application of UV irradiation.¹⁻³ Surprisingly, the induction of a few or several HSPs in *E. coli*, by a means other than heat shock, does not induce thermotolerance to ensuing temperature stress.⁶ Similar observations have been made in eukaryotes.⁷ Furthermore, evidence suggests that some, perhaps most, of the HSPs are indispensable to cell growth at normal or optimum growth temperatures.⁸

One major reason for the interest in HSPs found in pathogenic bacteria is the observation that a so-called common antigen, usually of an apparent molecular

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mass of 58–65 kDa, bears sequence similarity to the *E. coli* groEL protein.^{9–12} In the case of *Coxiella burnetii*, screening of a DNA library (composed of recombinant cosmids) with hyperimmune serum revealed clones which overproduced the protein.⁹ Studies revealed that the cloned *C. burnetii* DNA contained an operon encoding the *htpA* and *htpB* proteins; the promoter possessed a sequence indicative of a heat-shock gene, and the structure of the operon indicated great similarity to the groES-groEL operon in *E. coli*.⁹ Important antigens of similar structure have since been found in *Mycobacterium tuberculosis*,¹⁰ *Mycobacterium leprae*,¹¹ *Treponema pallidum*,¹³ *Borrelia burgdorferi*,¹⁴ *Legionella pneumophila*,¹⁵ *Pseudomonas aeruginosa*,¹⁶ and several non-pathogenic bacteria. A subset of human T cells was recently shown to recognize mycobacterial heat-shock protein.¹⁷ It thus appears that the production of these proteins on the part of pathogenic bacteria during invasion of hosts is a problem relevant to the disease process. The problem is particularly intriguing with respect to thermophilic bacteria which cause zoonoses and are intracellular parasites. If the HSPs are also major immunogens, as appears to be the case, then knowledge regarding the synthesis and regulation of these proteins has both basic and practical importance.

Nothing is known about the expression of HSPs in *C. burnetii*. In order to study these proteins, we used organisms which were grown in tissue culture and then acid activated *in vitro*. During this period of metabolic activity, these organisms synthesize a wide variety of proteins. By using temperature-jump experiments and other means of inducing stress, we were able to show that the *C. burnetii* genes cloned earlier, representing *htpA* and *htpB*, are indeed regulated as HSPs within *C. burnetii*.

MATERIALS AND METHODS

Coxiella burnetii, Nine Mile strain phase I, clone 7 (CB9MIC7),¹⁸ was used to infect fibroblast tissue culture cells as described previously.¹⁹ Infected baby hamster kidney cells (IBHK-21) were grown, as a continuous culture through several passages, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum and containing 4 g/l glucose and 3.7 g/l NaHCO₃, under an atmosphere of 15% CO₂ at 37°C.¹⁹ The infected fibroblasts were trypsinized, diluted threefold with DMEM, and replated (a 1 : 3 split) six days prior to harvest. The medium was changed three days and one day prior to harvest. During the last 12 hr prior to harvest, the infected tissue cultures were removed from the CO₂ incubator (flasks were tightly capped before removal) and further incubated at ambient temperature (20–22°C).

Naturally released *Coxiella burnetii* organisms were harvested from the tissue culture medium after a 24-hr accumulation.¹⁹ Organisms within fibroblasts were released by vortex lysis and then isolated in semipure form via a differential centrifugation method.¹⁹ This latter population of organisms is referred to as mechanically released organisms. The temperature during these operations varied from 4°C to 10°C. The organisms were stored as pellets at 4°C until acid activated (storage usually for 30–90 min).

Acid activation of organisms was initiated by suspending pelleted microbes in phosphate buffer, pH 4.5, (49 mM KH₂PO₄, 169 mM KCl, 16 mM NaCl, 120 mM glycine, 250 mM sucrose) followed by rapid transfer to incubation tubes containing the final reaction components. The final concentrations were 250 mM sucrose, 5.0 mM glucose, 5.0 mM glutamate, 77.0 mM glycine, 0.1 mM of each of the other

18 amino acids, 17.4 mM KH_2PO_4 , 118 mM KCl, 11.9 mM NaCl, 20.0 mM MgCl_2 , and 100 μCi of L-[4,5- ^3H]leucine (145 Ci/mmol; Amersham, Arlington Heights, Ill.) per ml of reaction mixture. Some incubations employed Tran ^{35}S -label (L-[^{35}S]methionine plus L-[^{35}S]cysteine, 1181 Ci/mmol; ICN Radiochemicals, Irvine, Ca.) rather than tritium. The ^{35}S -labeled amino acids were added at a dilution of 200–250 μCi per ml of reaction mixture. Acid-activation incubations were carried out in temperature-controlled shaking water baths at either 20°C or at 42°C for the time periods indicated in RESULTS AND DISCUSSION. Proteins secreted or released into the medium during the acid-activation incubations were assessed after removal of organisms by centrifugation in a microfuge (Beckman Instruments) for 10 min. Incorporation of radioactivity into protein ("global," or overall protein synthesis) was determined as previously described.¹⁹ Radioactive proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) by standard procedures, employing a 4% stacking gel and an 8–15% gradient separating gel under reducing conditions (slight modification of Laemmli's procedure²⁰). Gels were fixed in acetic acid/methanol, impregnated with ^3H -Enlightening (New England Nuclear, Boston, Mass.), and fluorographed using Kodak XAR film.

RESULTS AND DISCUSSION

The protocols for incubations and heat shock were as follows. Infected BHK-21 fibroblasts were incubated at 37°C until the last 12 hr prior to harvest; during that 12-hr period, the cultures were incubated at 21°C. During isolation of naturally released and mechanically released organisms, the temperature was 21°C and then 4°C; this period required 2.5–3 hr. Acid activation was then performed with the freshly prepared organisms. After 6 hr of acid activation, some of the reactions were placed into a 42°C water bath (heat shock), whereas controls were left at 21°C. Some reactions incubated at 21°C received ethanol (6%), or H_2O_2 (60 μM), or NaN_3 or KCN (1.0 mM) after 6 hr and were incubated thereafter at that temperature. Thus, the *C. burnetii* organisms were exposed to ambient temperatures for a period greater than 20 hr prior to either heat or chemical shock; organisms resided within host fibroblasts during the initial part of this period.

Total protein synthesis during acid activation of the organisms, as measured by incorporation of [^3H]leucine into acid-precipitable material, was monitored. As expected, the rate of global protein synthesis showed a significant increase when the incubation temperature was increased from 21°C to 42°C. Thus, the total extent of *de novo* synthesized protein was much greater at 42°C. This result is in agreement with previous observations, which showed that the *C. burnetii* protein synthesis machinery functions optimally at temperatures between 42°C and 45°C.²¹

SDS–PAGE of proteins and fluorography of the resultant gels were done to define the qualitative differences in proteins synthesized during heat shock. FIGURE 1 shows the proteins synthesized during acid activation of metabolism in mechanically released organisms. A comparison of the proteins made at 21°C (lane 1) and those made after a period of heat shock at 42°C (lane 2) suggests that the induction of the 62-kDa groEL-like protein occurred during heat shock. In addition, at least six other protein species appear to be induced by the heat shock. These heat-induced proteins have apparent molecular masses (in kilodaltons) of approximately 100 (2 species), 81, 70, 25, and 14. No proteins were detected in the

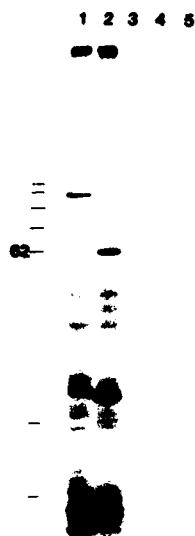


FIGURE 1. Incorporation of [^3H]leucine into mechanically released *C. burnetii* proteins during heat shock. Intracellular organisms were obtained from mechanically disrupted fibroblasts, isolated from host components, and acid activated at pH 4.5 in a medium containing glucose, glutamate, amino acids with [^3H]leucine, and buffered salts. Incubations were carried out for 12 h. Proteins were extracted by boiling in SDS with β -mercaptoethanol and electrophoresed through gradient polyacrylamide gels, which were then subjected to fluorography; a radioautogram is shown. (Lane 1) Proteins labeled during a 12-h incubation at 21°C. (Lane 2) Proteins labeled during incubation at 21°C for 6 h followed by a 6-h incubation at 42°C (heat shock). (Lane 3) Proteins labeled during acid activation in the presence of chloramphenicol (1.5 mM) and heat shocked as in lane 2. (Lane 4) Soluble supernatant proteins from organisms incubated at 21°C. (Lane 5) Soluble supernatant proteins from organisms exposed to heat shock. The position of the 62-kDa heat shock protein, which was identified with specific antibody and antisera, is indicated at the left. The locations of bands representing other proteins induced by heat shock are indicated by the horizontal lines at the left. Note the apparent change in migration of a 30-kDa protein after heat shock (lane 2).

material present in the supernatant after centrifuging the acid-activated organisms previously incubated at 21°C (lane 4) or 42°C (lane 5), suggesting that *de novo* synthesized protein is not secreted or lost from mechanically released organisms.

Similarly, induction of the 62-kDa protein was observed during heat shock of naturally released organisms (obtained free of host fibroblasts in the tissue culture medium). The molecular masses of the proteins made during heat shock (FIG. 2, lane 2) by these organisms appear to be identical to those of the proteins induced within mechanically released organisms (FIG. 1, lane 2). Unlike mechanically released organisms, the naturally released organisms secrete or translocate *de novo* synthesized protein into the medium.²² A protein possessing an apparent molecular mass of 26–28 kDa can be seen in the supernatant (FIG. 2, lanes 4 and 5) and is thus secreted under both control and heat-shock conditions. The secretion of a 28-kDa protein is in approximate agreement with a previous observation.²² Interestingly, the 62-kDa HSP is also secreted at 42°C, as are smaller quantities of the 70-kDa and 81-kDa heat-inducible proteins (FIG. 2, lane 5).

In *E. coli*, some or all of the HSPs can be induced by stress agents other than heat. Ethanol, UV radiation, agents that inhibit DNA gyrase, and hydrogen peroxide all have this effect. We studied the effects of hydrogen peroxide, ethanol, and metabolic poisons on *C. burnetii* protein synthesis at 21°C. In FIGURE 3, ^{35}S -labeled *C. burnetii* were electrophoresed and fluorographed. The profile of heat-induced proteins was similar to that seen with ^3H -labeled organisms (compare FIG. 3, lane 4, with FIG. 1, lane 2). Peroxide had no effect upon HSP induction: the protein pattern was identical to that of the 21°C control (FIG. 3; compare lane 2 with lane 6). Addition of 10% (v/v) ethanol did induce the synthesis of the 62-kDa HSP (FIG. 3, lane 8). Sodium azide and potassium cyanide had no qualitative effect upon the proteins made at 21°C (lanes 10 and 12): these inhibitors did reduce considerably the overall amount of protein synthesis.

Lane 5 in FIGURE 3 again illustrates the presence of newly synthesized protein in the medium after acid activation at 42°C. As with studies utilizing tritium, the ³⁵S-labeled common antigen (62 kDa) appears to have been secreted into the medium, along with a subset of smaller proteins.

Taken collectively, the data argue that the previously cloned *C. burnetii* protein, *htpB*, is indeed a heat-inducible protein. In experiments not shown here, we were able to demonstrate the specific immunoprecipitation of radiolabeled 62-kDa *htpB* protein from a complex of proteins extracted from organisms after acid activation with heat shock. This was done using either monoclonal antibody or hyperimmune serum. *C. burnetii htpA*, whose gene is also located in the same operon as *htpB*, has a deduced molecular mass of 10.5 kDa.⁹ In all of the experiments described here, a smaller polypeptide with an extrapolated molecular mass of 14 kDa is induced by heat (FIGS. 1 and 2, lane 2; FIG. 3, lane 4). It is probable that this is the *htpA* gene product, whose gene lies proximal to the heat shock promoter in the operon.⁹ Presently, there exists no specific antibody with which to verify this identification.

Growth experiments were performed utilizing the *E. coli* clone containing pCS26C1. This is an antibody-positive subclone from the original *C. burnetii* DNA library constructed in cosmids, and it contains a 5.2-kb *Bam*H I/*Cla* I DNA fragment within which lies the 2-kb *htpA/htpB C. burnetii* heat-shock operon.⁹ The experiments were designed to test whether the presence of these *C. burnetii* genes conferred notable thermotolerance or thermostability to the heterologous *E. coli* host. Although these *C. burnetii* genes are overexpressed in this *E. coli* clone grown at 37°C,⁹ we could find no evidence that their presence provided any advantage to *E. coli* grown at normal or elevated temperatures, when compared to the parent strain containing the pH79 cosmid without inserts (data not shown).

FIGURE 2. Incorporation of [³H]leucine into naturally released *C. burnetii* proteins during heat shock. Extracellular organisms were harvested from the medium obtained from infected fibroblast monolayers. They were acid activated, proteins were extracted, and electrophoresis performed as in FIGURE 1. (Lane 1) Acid activation at 21°C. (Lane 2) Proteins labeled during heat shock. (Lane 3) Heat shock in the presence of chloramphenicol. (Lane 4) Soluble supernatant proteins from the 21°C incubation. (Lane 5) Soluble supernatant proteins from the heat-shock incubation. Markings at the left indicate protein bands as described in FIGURE 1. The location of a secreted protein (Lanes 4 and 5) of approximately 28 kDa is indicated at the right. Note the presence of the *htpB* 62-kDa protein in the soluble fraction of the heat-shock incubation (lane 5).



An interesting feature of the present results is the observation that *de novo* synthesized 62-kDa protein somehow becomes dissociated from the organisms after its synthesis. It is now recognized that this protein, the common antigen, is important as an antibody target in a number of bacterial infections. In *E. coli*, the *groEL* protein has been shown to be involved in the translocation process. GroEL associates with both pre- β -lactamase and chloramphenicol acetyltransferase, presumably retaining these in an unfolded state via an ATP-dependent mechanism

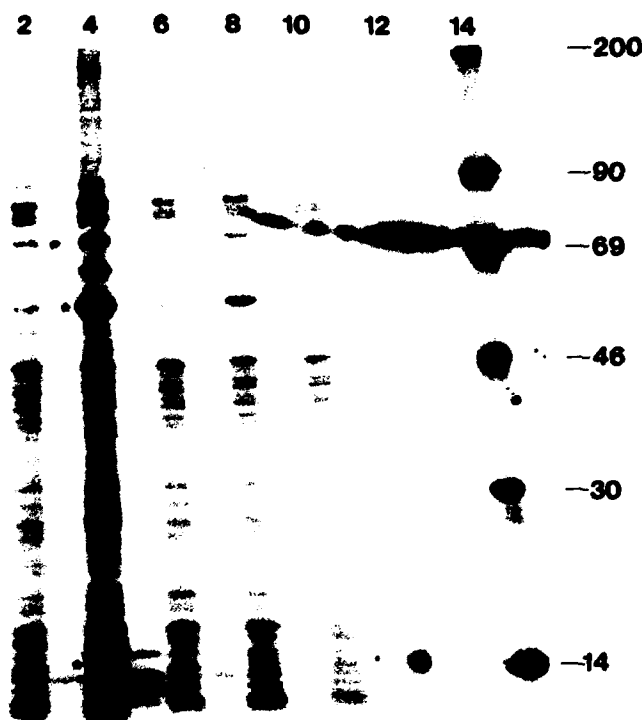


FIGURE 3. Effect of heat shock, peroxide, ethanol, and respiratory poisons on protein synthesis in *C. burnetii*. Intracellular organisms were acid activated and labeled as described in FIGURE 1, except that [35 S]methionine and [35 S]cysteine were used to label proteins. Protein extractions, electrophoresis, and fluorography were done as in FIGURE 1. (Lane 2) Proteins labeled during a 12-h incubation at 21°C. (Lane 4) Proteins labeled during a 12-h incubation, 21°C for 6 h followed by 42°C for 6 h (heat shock). (Lane 6) Acid activation at 21°C with 0.6 mM H_2O_2 . (Lane 8) Acid activation at 21°C with 6% (v/v) ethanol. (Lane 10) Acid activation at 21°C with 1 mM NaCN. (Lane 12) Acid activation at 21°C with 1 mM NaN_3 . (Lane 14) ^{14}C -labeled molecular weight markers (TRK 626, Amersham, Arlington Heights, Illinois: myosin, 22 kDa; phosphorylase B, 90 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14 kDa). The *C. burnetii* *htpB* gene product (62-kDa common antigen) is indicated by the upper asterisk at left of lane 4. Lower asterisk indicates the presumed *htpA* gene product. In the "odd-numbered" lanes (numbers not marked), each immediately to the right of a numbered lane, the soluble proteins, if any, recovered from each incubation were run. Note the soluble proteins released during heat shock (lane 5), one of which is the *htpB* product.

so that they may be competent for translocation.²³ In the present case, the *C. burnetii* groEL-like 62-kDa common antigen appears to be translocated. There is evidence that the groEL-like protein responsible for the assembly of RuBisCo within chloroplasts must also be translocated prior to serving this function.⁴ It is therefore not surprising that its counterpart in *C. burnetii* is translocated. Why the protein is translocated, and presumably secreted to the exterior, is not known, but the question is an intriguing one. The genetic sequence data do not indicate the presence of any obvious signal leader peptide sequence within this protein.⁹

We do not as yet know the identity of the remainder of the proteins made during heat shock in *C. burnetii*. However, since HSPs appear to be so ubiquitous, it is possible to suggest an identity for some of them. In *E. coli*, HSP-70 is the product of the *dnaK* gene. This is a protein necessary for lambda-phage replication, and it is also required for growth at normal temperatures. The *C. burnetii* 70-kDa heat-induced protein may be analogous to this. Likewise, HSP-25 in *E. coli* is the *dnaJ* gene product, which is assumed to be involved in DNA synthesis. Perhaps the *C. burnetii* 25-kDa protein is analogous to it.

In summary, there are three reasons why investigations into thermal regulation of protein synthesis in *C. burnetii* are of interest. First, this is a thermophilic organism. In order to kill a population of *C. burnetii* in an aqueous environment, the temperature must be raised to 145°F (62.8°C) for 30 min. This feature sets the standard for the pasteurization of milk by the holding method.²⁴ Second, the organism is poikilothermic. It is enzootic in ticks, but it is also indigenous in warm-blooded domestic livestock.²⁵ This implies a duality of metabolic activity, perhaps matched by differential gene expression, throughout a two-tiered temperature range.⁹ Third, a dominant antigen, the 62-kDa protein, is analogous in sequence to the groEL (*mopB*) heat-shock protein in *E. coli*.⁹ This antigen is homologous to one found in a number of pathogenic bacteria.^{9,11} Furthermore, it has been suggested that induction of synthesis of these and other stress proteins may confer enhanced survival upon pathogens after their uptake by host phagocytes.^{11,26} It had been previously shown that the protein synthesis machinery of *C. burnetii* possesses curious thermostability characteristics.^{21,27} The organism undergoes a developmental cycle,²⁸ during one stage of which it has a high content of peptidoglycan.²⁹ This wall structure likely confers durable structural properties upon the organism. The 62-kDa *htpB* protein has also been shown to be linked to the *C. burnetii* peptidoglycan fraction³⁰; therefore this protein may serve a structural role as well.

In the present study, we have demonstrated that the groEL-like, 62-kDa "common" antigen, the product of the *htpB* gene, is indeed regulated as a heat-shock protein. At 42°C, some of this product was released from the organism. Additional proteins, whose syntheses are also induced by heat shock, were noted but not positively identified. These proteins, plus *htpB*, were produced over and above a generalized increase in overall protein synthesis due to the higher temperature. The implications of these observations to the intracellular survival and reproduction of *C. burnetii* are presently unclear. It is possible that some of these proteins might be induced at near heat-shock temperatures during the uptake of the organisms by phagocytes, because the process of phagocytosis generates heat in a quantity proportional to the mass of the material engulfed by the phagocyte.³¹ Further, some may be induced by oxidative products within host phagosomes. Although we did not observe induction of any proteins by peroxide in the present study, it is known that HSP-70 (DnaK) is induced by H₂O₂ in *Salmonella typhimurium*.³² These hypotheses need to be tested with a phagocytosis model for *C. burnetii*.

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