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Identification and Comparison of Macrophage-Induced Proteins and Proteins Induced under Various Stress Conditions in *Brucella abortus*

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***Brucella abortus* is a facultative intracellular pathogen of cattle and humans that is capable of survival inside macrophages. In order to understand how *B. abortus* copes with the conditions during intracellular growth in macrophages, the protein synthesis pattern of the bacteria grown inside bovine macrophages has been compared with that of bacteria grown in the cell culture medium by two-dimensional polyacrylamide gel electrophoresis. Approximately 24 new proteins that are not detected in the bacteria grown in the cell culture medium have been induced during intracellular growth in macrophages. In contrast, approximately 50 proteins that were expressed during growth in cell culture medium were completely repressed during intracellular growth. The level of expression of 19 proteins increases while that of 54 proteins decreases during intracellular growth. To understand these results, the protein synthesis pattern of *B. abortus* during intracellular growth was compared with those during other stress conditions. Under each stress condition studied, several new proteins were induced that were not present during regular growth conditions. Comparison of the protein synthesis pattern of *B. abortus* during intracellular growth with those obtained under various stress conditions has indicated that the response to intracellular growth was not just a simple sum of stress conditions studied so far.**

Brucella abortus is a facultative intracellular pathogen that survives ingestion by macrophages and neutrophils (21, 30). The ability to survive inside macrophages allows *B. abortus* to interfere with the function of macrophages and also provides the bacteria with protection from the immune system and the factors in blood which are capable of killing *B. abortus* (12). After uptake of *B. abortus* by macrophages, bacteria are found in a membrane-bound vacuole referred to as a phagosome. The phagosome may then fuse with a lysosome, forming a structure called a phagolysosome and thereby exposing the phagocytosed bacteria to a diverse collection of hydrolytic enzymes. The antimicrobial function of phagocytic cells has been classified into an oxygen-dependent mechanism and an oxygen-independent mechanism. The oxygen-dependent mechanism results in the generation of reactive oxygen molecules such as superoxide anion, hydroxyl radicals, hypochlorite ion, hydrogen peroxide, and singlet oxygen within a phagosome. Examples of the oxygen-independent mechanism include acidification of the phagosome to a pH of 5.5 and release of hydrolytic enzymes and small cationic peptides called defensins (11). In addition, intracellular bacteria have to cope with other conditions present inside the host cell which may include nutritional deprivation, at least initially (26). The mechanisms and virulence factors that allow *B. abortus* to cope with the hostile environment inside the phagocytic cells are not well understood. Kreuzer et al. (21) and Riley and Robertson (30) examined the ability of human and bovine neutrophils to ingest and kill smooth and rough strains of *B. abortus*. Both bacterial strains were ingested, and both resisted killing by neutrophils; however, the smooth strain was more resistant to intraphagocytic killing than the rough strain. Electron microscopy showed that the degranulation of both primary and secondary granules was inhibited by *B. abortus* and that viable organisms were not required for the inhibition of granule fusion (9). Canning et al. (9) subjected the culture medium in which *B. abortus* was grown to high-performance liquid chromatography and isolated two nucleotide-like substances which inhibited the ability of neutrophils to iodinate proteins, a measure of neutrophils' myeloperoxidase-H_2O_2 activity, which requires primary granule fusion.

The objective of the experiments described here was to determine the pattern of gene expression in terms of defined responses to specific environmental and stress conditions which may resemble the environment in macrophages during intracellular growth. Two-dimensional polyacrylamide gel electrophoresis (PAGE) was used as a tool to study the physiological responses of the bacteria to intracellular growth and various stress conditions. To achieve this goal, first, the protein synthesis pattern of *B. abortus* during intracellular growth was determined. Second, proteins that were expressed during oxidative, low pH, nutritional, and heat stresses were identified. Finally, the pattern of protein synthesis under each stress condition was carefully compared with that of intracellular growth. This study has revealed that although there may be some overlap among the protein synthesis patterns of *B. abortus* exposed to various stress conditions and those grown intracellularly, the overall patterns are not similar. Therefore, the response to intracellular growth is not just a simple sum of the responses to various stress conditions that may resemble intracellular conditions.

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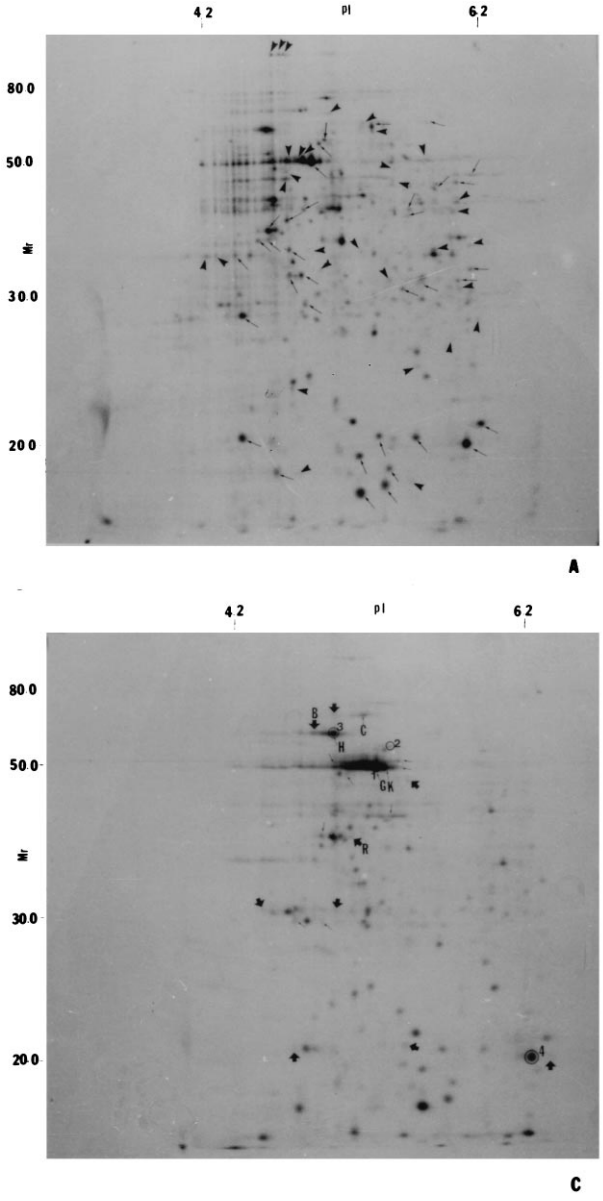


FIG. 1. Two-dimensional protein synthesis pattern of *B. abortus* during intracellular survival in bovine macrophages. (A) *B. abortus* labeled in methionine-free RPMI 1640 medium with 15% serum in the presence of cycloheximide. Proteins whose expression has decreased or those that are not expressed during 60 or 120 min of intracellular survival have been marked with ➞ or ➤, respectively. (B and C) *B. abortus* labeled during growth in macrophages in the presence of cycloheximide and streptomycin for 60 and 120 min, respectively. Proteins induced during intracellular growth have been marked with ◗. Proteins whose expression has increased during intracellular growth are marked with →. Proteins which have been induced during both intracellular survival and under any of the stress conditions studied have been marked with letters. The numbers and circles present GroEL (no. 1), HtrA (no. 2), Dnak (no. 3), and SodC (no. 4).

MATERIALS AND METHODS

Bacterial strain. *B. abortus* 2308 was used for all experiments. *B. abortus* 2308 was obtained from the Oklahoma Animal Disease Diagnostic Laboratory. Bacterial cells were maintained as glycerol stocks at −80°C and grown on tryptose (Difco) agar plates prior to the experiments.

Isolation of monocytes from bovine blood. Peripheral blood was isolated from the jugular vein of healthy heifers into 60-ml syringes containing EDTA (1 ml of 180-mg/ml Na_2EDTA per 60 ml of blood). Aliquots of 30 ml of anticoagulant-treated blood were centrifuged for 30 min at 2,500 rpm in a Sorvall SH-3000 rotor at 23°C. The interface between the erythrocytes and the plasma containing the leukocytes was harvested and diluted 1:3 with Hanks' balanced salt solution (Sigma), underlaid with Ficoll-Paque (Pharmacia), and centrifuged for 20 min at 2,000 rpm (18) in the Sorvall SH-3000 rotor. Mononuclear cells were isolated from the interface between diluted plasma and Ficoll-Paque and were washed with Hanks' balanced salt solution twice (5). Mononuclear cells were resuspended in Dulbecco's modified Eagle medium (DMEM; Sigma) containing 15% heat-inactivated fetal bovine and equine serum (HyClone) and were cultured at 5×10^7 cells in 100-mm-diameter tissue culture-treated petri dishes (Corning) in a humidified incubator at 37°C and 5% CO_2 for 2 h. Nonadherent cells were removed by being washed once with DMEM containing 15% serum and then being washed twice with Hanks' balanced salt solution. Adherent cells were cultured in DMEM with 15% serum in an atmosphere containing 5% CO_2. The culture medium was changed every 4 days. Monocytes were allowed to mature into macrophages for 1 week (4).

Radiolabeling of proteins synthesized in response to intracellular growth. To identify proteins induced in response to macrophages by *B. abortus*, the protocol described by Buchmeier and Heffron (7) was followed. *B. abortus* 2308 cells grown on tryptose agar plates for 72 h were harvested by centrifugation at 2,080 × *g*. *Brucella* cells were resuspended in DMEM containing 15% heat-inactivated serum and added to plates containing macrophages at a density of 50 bacteria per macrophage. These plates were incubated at 37°C in 5% CO_2 and a humidified atmosphere for 60 min to allow infection of macrophages and phagocytosis of bacteria. Extracellular bacteria were removed by washing the plates twice with methionine-free RPMI 1640 medium (ICN) containing 15% serum. Methionine-free RPMI 1640 medium (5 ml) containing 15% serum, 20 μg of streptomycin per ml, 50 μg of cycloheximide per ml, and 50 μCi of ^{35}S protein labeling mix (DuPont) per ml was added to each plate. Bacterial proteins were labeled for 60 and 120 min and chased with L-methionine at a final concentration of 200 μg/ml for 2 min. At the end of the labeling period, the medium was removed, and the macrophages were lysed with 1% (vol/vol) ice-cold Triton X-100 for 30 min. The plates were washed, and bacterial cells were collected by centrifugation at 2,080 × *g* with a benchtop centrifuge for 15 min. The pellet was washed twice with saline and was resuspended in 1 volume of saline. As a control, *Brucella* cells were labeled in RPMI 1640 medium containing 15% serum. Furthermore, to account for the differences in the protein synthesis of *B. abortus* cells grown intracellularly and in the tissue culture medium due to treatment of macrophages with cycloheximide, *B. abortus* cells were labeled in the tissue culture medium in the presence of 50 μg of cycloheximide per ml and in the absence of macrophages. To ensure that macrophage protein synthesis was effectively inhibited by cycloheximide, uninfected macrophages were labeled in the presence and absence of 50 μg of cycloheximide per ml, and their protein patterns were analyzed.

Radiolabeling of *B. abortus* proteins under various stress conditions. For all experiments, an untreated culture in the same medium in which the experiment was conducted and in the absence of the stress was labeled at 37°C for 60 min. The bacterial culture Klett reading was adjusted to 100 to 120 Klett units (green filter). Cells were labeled with 50 μCi of ^{35}S protein labeling mix per ml for 20 or 60 min. Labeled cells were chased with 200 μg of cold methionine per ml. All of the stress conditions were chosen so that they would induce a response rather than completely inhibit growth. For the heat treatment, the protocol described by Lin et al. (23) was followed. *B. abortus* was grown in minimal medium (17)

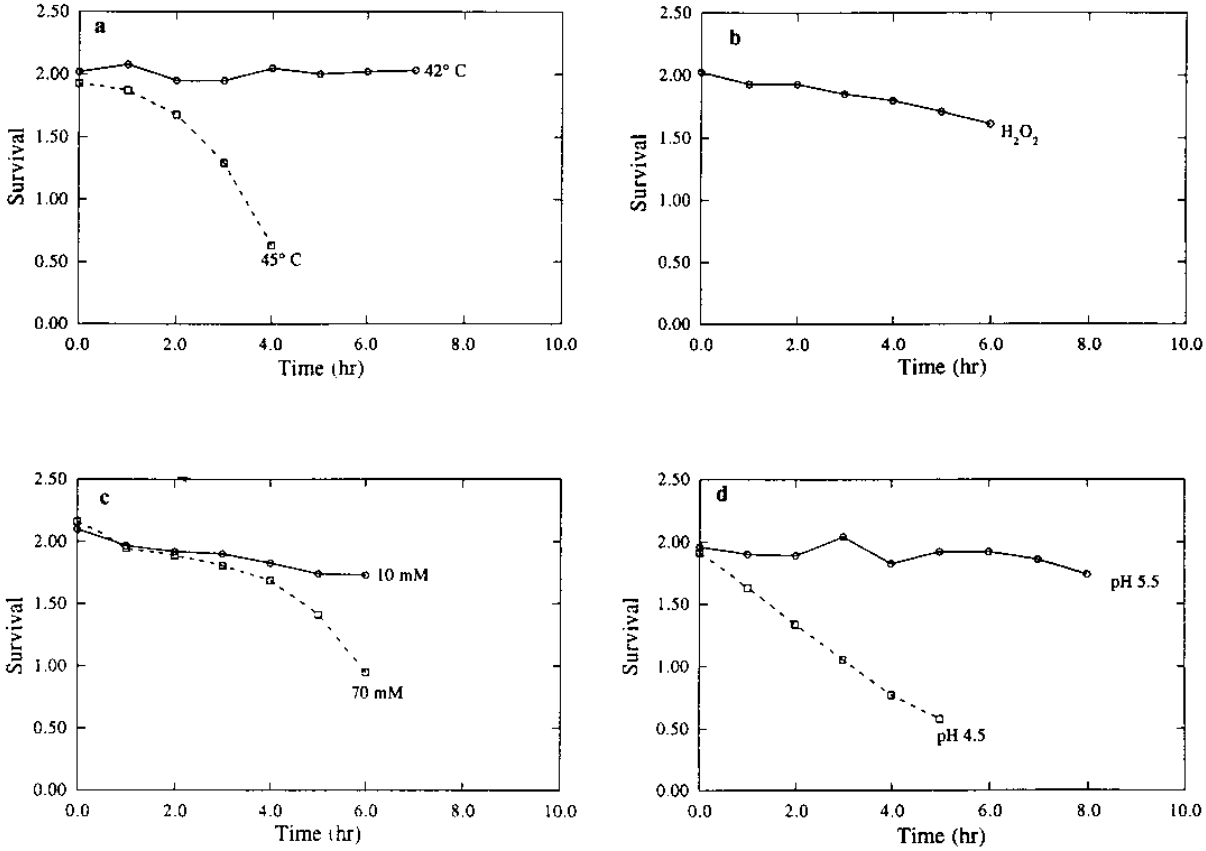


FIG. 2. Survival of *B. abortus* under the following stress conditions: different temperatures (a), presence of H_2O_2 (b), presence of menadione sodium bisulfite (c), and reduced pH (d). Bacteria were grown and were subjected to the appropriate stress conditions as described in the text. After the shift in the condition, the number of viable cells was determined by making serial dilutions in saline and plating on tryptose plates. Survival was determined in three different experiments, and each time duplicate samples of bacteria were plated for each experiment. Survival was determined as log [(number of CFU present under any given stress condition at any time/number of CFU present under the appropriate control at the same time) × 100%].

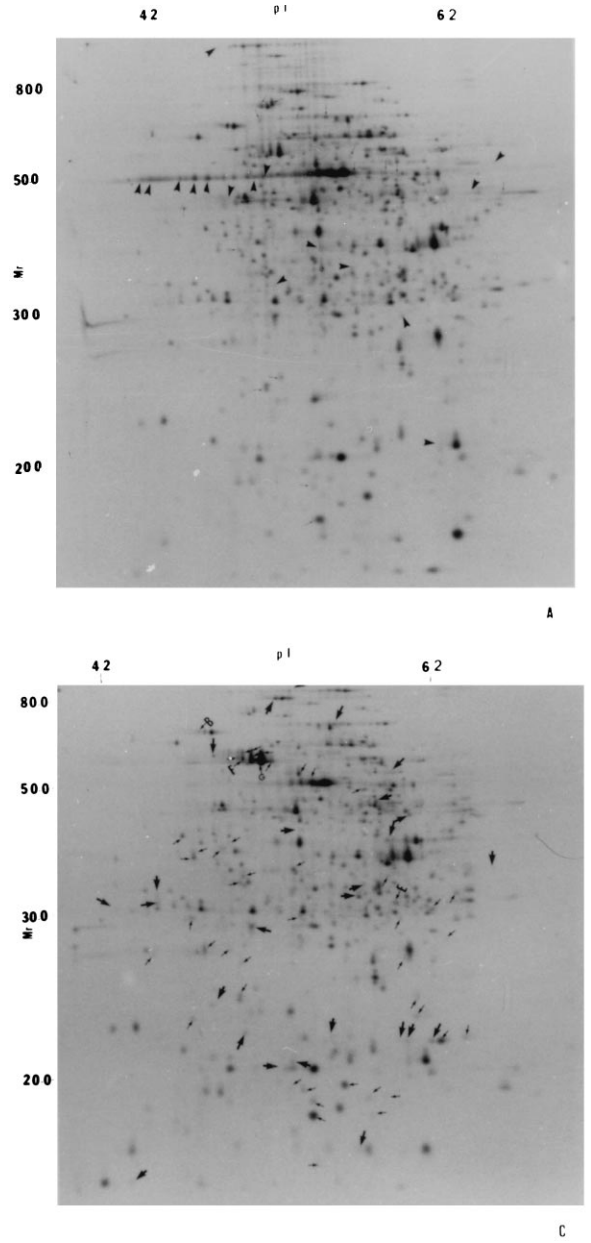
overnight at 37°C to the mid-log growth phase. The bacterial culture was centrifuged at 2,080 × *g*, and the pellet was resuspended in an equal volume of minimal medium prewarmed to 42°C. For reduced pH, *B. abortus* was grown in minimal medium containing citrate (2 mg/ml) at pH 7.0 overnight. This culture was centrifuged, and the pellet was resuspended in an equal volume of minimal medium with citrate at pH 5.5 and prewarmed to 37°C. For the oxidative stress, the procedure of Fitzgeorge et al. (14) was followed with a few minor modifications. *B. abortus* was grown on tryptose agar plates for 18 h. Bacterial cells were harvested and transferred to saline. The concentration of H_2O_2 (Sigma) in the culture was adjusted to 0.005%, and the cells were immediately labeled. The same procedure as that described for H_2O_2 was used for labeling of proteins in the presence of menadione sodium bisulfite, a redox recycling agent that generates superoxides (19). However, the concentration of menadione sodium bisulfite in the culture was adjusted to 10 mM. In the case of nutritional deprivation, *B. abortus* cells grown on tryptose plates were harvested with saline and were washed twice with saline. The culture was divided into three equal aliquots, and the cells were centrifuged. The pellets were resuspended in methionine-free RPMI 1640 medium with 15% serum, minimal medium (17), or saline. The cells were labeled for 60 min.

Survival plots. The survival rate of *B. abortus* during the oxidative stress, reduced pH, heat stress, and nutritional deprivation was measured by subjecting 1 to 5 ml of bacterial culture to one of the stress conditions as described above for radiolabeling. Samples (10 μl) were removed from each treatment every hour and diluted to 10^{-6} in saline. Cells were spread on tryptose plates, which were then incubated at 37°C for 72 h. The number of viable cells was determined by counting the number of colonies on the tryptose plates (10).

Two-dimensional PAGE analysis of proteins. To prepare labeled proteins for two-dimensional PAGE, pulse-labeled cells were mixed with 1 volume of 10% trichloroacetic acid, and the precipitate was collected by centrifugation. The precipitate was washed once with 5% trichloroacetic acid and once with acetone (34). The precipitate was solubilized in sodium dodecyl sulfate (SDS) and urea solubilization buffer (3). Two-dimensional PAGE was performed as described by O'Farrell (27) with the Investigator system of Millipore. Isoelectric focusing (IEF) gels were cast in capillary tubes (Millipore Co.). The IEF gels were prepared with ampholyte solution (Millipore Co.) at pH 3 to 8. The IEF gels were prefocused for about 1 h, and then 10^5 cpm of labeled proteins was loaded on each IEF gel. The first-dimensional gels were run at 18,000 v · h. After focusing, the IEF gels were equilibrated for 2 min in equilibration buffer consisting of 0.3 M Tris-base, 0.075 M Tris-HCl, 3.0% SDS, 50 mM dithiothreitol, and 0.01% bromophenol blue (Millipore) and were loaded on top of the polyacrylamide gels (12.5%). Polyacrylamide gels were electrophoresed at 1,200 mW. Along with each gel, standards (Bio-Rad) for pI and molecular mass were run. Gels were fixed and then dried at 80°C under vacuum. The dried gels were exposed to X-ray film (Kodak-XAR-5 or Fuji Medical X-ray) for 60 days. Autoradiographs were scanned and analyzed with the PDQuest (PDI, Huntington Station, N.Y.) software package designed for the analysis and databasing of two-dimensional gels and films. Each sample was electrophoresed twice, and each experiment was performed twice, except for that to determine intracellular growth, which was performed twice, although each sample was electrophoresed only once. The changes in the protein expression were accepted if they were observed in three of four autoradiographs for each stress condition studied and in both autoradiographs for intracellular survival experiments.

Immunization of rabbits with DnaK. Female New Zealand White rabbits were immunized with purified recombinant *Escherichia coli* DnaK protein (Epicentre Technologies) emulsified in Hunter's TiterMax (CytRx Corporation). The emulsion was prepared according to the manufacturer's instructions and contained 100 μg of protein per ml. Rabbits received 0.25-ml injections intramuscularly in four sites: in the posterior aspect of the upper portion of each front leg and in the posterior aspect of the upper portion of each rear leg.

Rabbits were given secondary immunizations of similar doses with the same adjuvant 4 and 8 weeks after the primary immunization. Twelve weeks after the primary immunization, the rabbits were anesthetized with parental ketamine (35 mg/kg of body weight) and xylazine (5 mg/kg) and exsanguinated by cardiac puncture. The blood was allowed to clot at room temperature and kept at 4°C overnight to allow the clot to retract. Serum was separated from the clotted blood by centrifugation (1,000 × *g*) for 20 min at 4°C, aliquoted, and stored at −80°C.



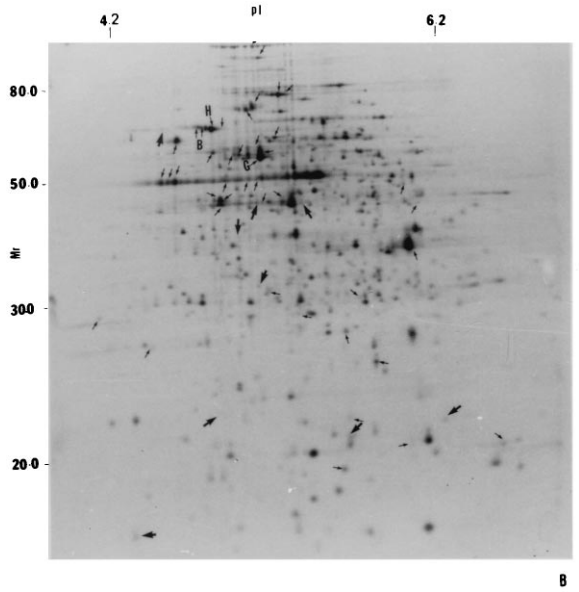


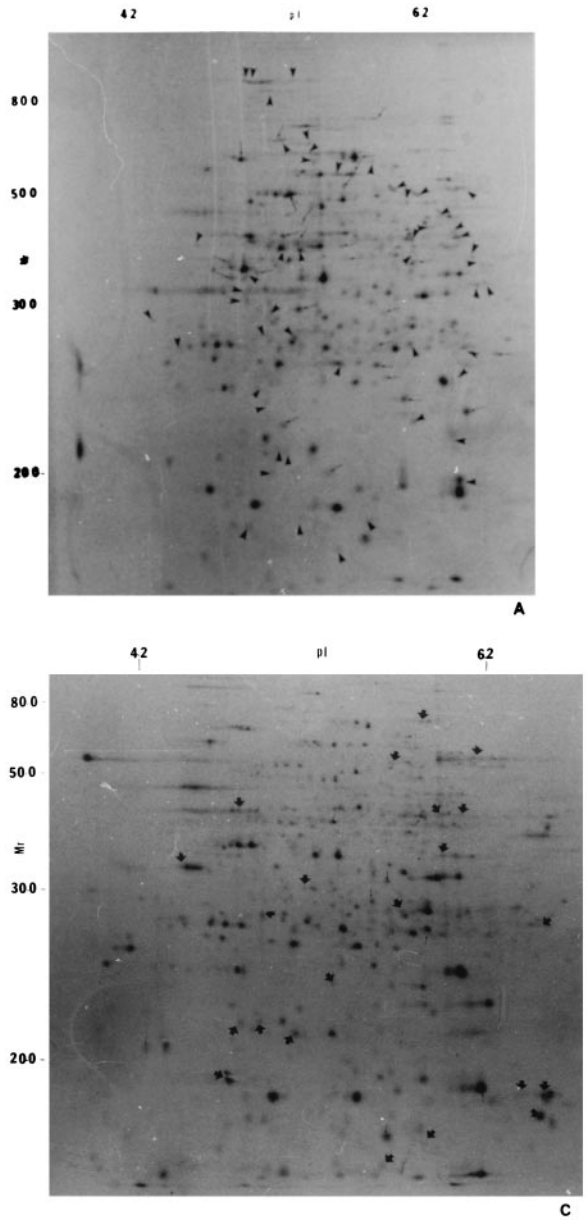
FIG. 3. Two-dimensional profile of *B. abortus* protein synthesis in response to an increase in temperature. (A) *B. abortus* labeled in minimal medium at 37°C for 60 min. Proteins whose expression has decreased or that have not been expressed in response to increased temperature after 20 min or 60 min have been marked with → or ➤, respectively. (B and C) *B. abortus* labeled in minimal medium at 42°C for 20 and 60 min, respectively. Induced proteins and proteins whose expression has increased during the upshift of the temperature have been marked with ➧ and ⇀, respectively. Proteins whose expression has been increased or induced both during intracellular survival and during survival at 42°C have been marked with letters.

Western blotting (immunoblotting). *Brucella* cells were grown on tryptose plates and harvested after 72 h. Cells were washed twice with saline and were transferred to RPMI 1640 medium prewarmed to 37°C containing 15% serum and 50 μCi of ^{35}S protein labeling mix per ml. *B. abortus* was labeled for 4 h at 37°C and 5% CO_2. The cells were then washed, and labeled proteins were solubilized as described above. Two concentrations of labeled proteins (36 and 27 μg) were separated by two-dimensional PAGE as described above. Proteins were transferred to a polyvinylidene difluoride membrane (Micron Separation, Inc.) with a Polyblot transfer system (American Bionetics, Inc.) according to the instructions recommended by the manufacturer. The blot was air dried overnight and then was dried under a heat lamp for 5 min. An X-ray film was exposed to this blot for 30 days. The membrane was blocked in TBS (10 mM Tris [pH 7.4], 150 mM NaCl)–5% skim milk for 1 h at room temperature. The primary antibodies were polyclonal and were raised against *E. coli* GroEL, *B. abortus* SodC and HtrA, and *E. coli* DnaK in rabbits. The antibodies against GroEL, SodC, HtrA were generously provided by R. W. Hendrix, F. Tatum, and M. Roop II, respectively. GroEL, SodC, HtrA, and DnaK antibodies were used at 1:3,000, 1:500, 1:5, and 1:50 dilutions, respectively. These antibodies were reacted with the blot overnight at 4°C. The blot was washed once in TBS, twice in TBS–0.5% Tween 20, and once in TBS–5% skim milk for 5 min each at room temperature. It was then incubated with a 1:30,000 dilution of alkaline phosphatase conjugated with anti-rabbit immunoglobulin G (Sigma) containing TBS–0.125% skim milk for 2 h at room temperature. The membrane was washed once in TBS, twice in TBS–0.5% Tween 20, and twice in TBS for 5 min each at room temperature. Detection was accomplished by addition of 45 ml of 100 mM Tris-HCl, (pH 9.5)–100 mM NaCl–100 mM $MgCl_2$ containing 300 μg of nitro blue tetrazolium (Sigma) and 150 μg of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) per ml at 35°C.

RESULTS

Identification of macrophage-induced proteins in *B. abortus*. In order to characterize the response of *B. abortus* during macrophage infection, *B. abortus* was labeled with ^{35}S protein labeling mix after phagocytosis by bovine macrophages. The protein synthesis pattern of *B. abortus* in response to macrophages was compared with that of cells grown under identical culture conditions in the absence of macrophages. The analysis of protein synthesis patterns of macrophages labeled in the presence of cycloheximide by SDS-PAGE indicated that macrophage protein synthesis was inhibited by cycloheximide. The growth and protein synthesis of extracellular bacteria were inhibited by streptomycin. Comparison by two-dimensional PAGE of proteins synthesized during intracellular growth with those synthesized in the absence of macrophages revealed differences in the protein synthesis patterns between the two conditions. These differences include both induction and repression of several proteins (Fig. 1). The expression of 43 spots was induced during intracellular growth. Of these, 24 appear to be uniquely expressed in the macrophage environment (Fig. 1B and C); these proteins are hereafter called macrophage-induced proteins. In contrast, the synthesis of 73 spots was decreased after engulfment by macrophages (Fig. 1A).

Protein synthesis and response of *B. abortus* to heat. The appropriate temperature for heat shock experiments and phys-



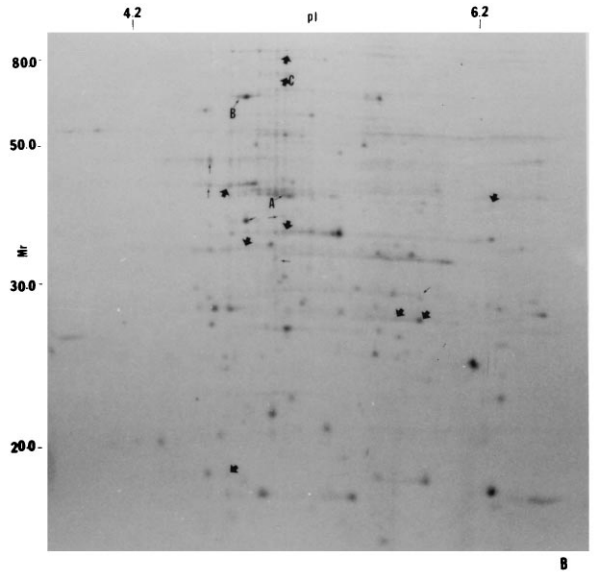
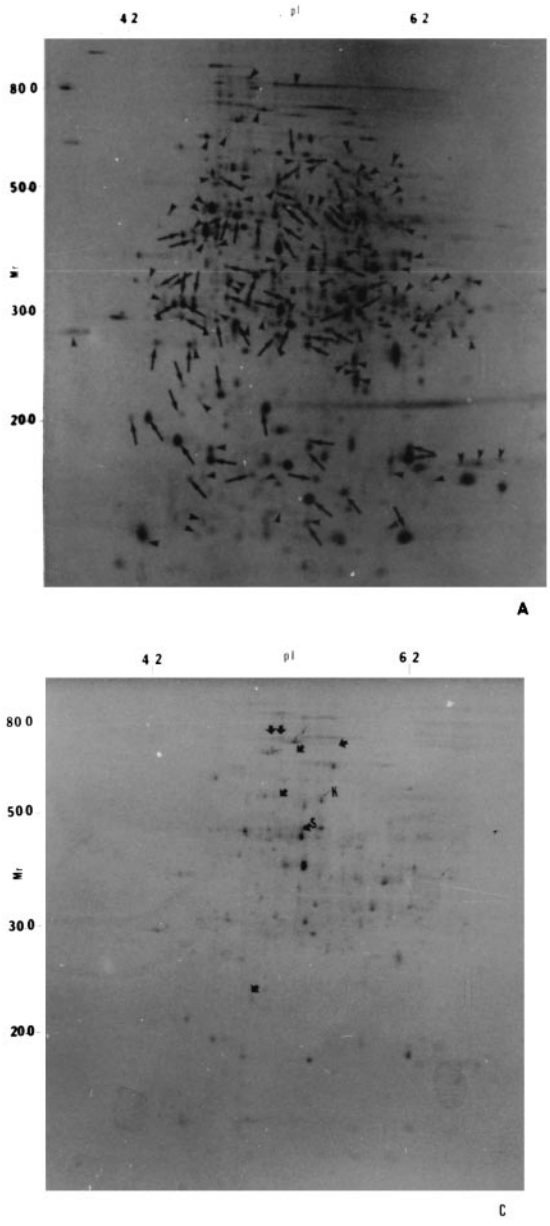


FIG. 4. Two-dimensional protein synthesis pattern of *B. abortus* in response to H_2O_2. (A) *B. abortus* labeled in saline in the absence of H_2O_2 for 60 min at 37°C. Proteins whose expression has decreased after 20 or 60 min of exposure to H_2O_2 have been marked with ➞. Proteins that have been repressed after 20 or 60 min of exposure to H_2O_2 have been marked with ➤. (B and C) *B. abortus* labeled in saline in the presence of 0.005% H_2O_2 for 20 and 60 min, respectively. Proteins whose expression has increased or has been induced in response to H_2O_2 have been marked with → and ➧, respectively. Proteins whose expression has been increased or induced both during intracellular survival and in the presence of H_2O_2 have been marked with letters.

iological response of *B. abortus* to an upshift of temperature was determined by subjecting *B. abortus* to temperature shifts from 37 to 42 or 45°C (Fig. 2a). The survival plot indicated that the growth pattern of *Brucella* cells was not greatly changed when the temperature was increased from 37°C to 42°C. However, an increase from 37°C to 45°C was lethal to the bacteria within 4 h. The temperature that was not lethal to *Brucella* cells but still high enough to lead to a response was chosen for labeling experiments (23, 24). This comparison has shown that a large number of proteins are repressed after the upshift of temperature (Fig. 3A). Furthermore, 35 new spots were synthesized after exposure of *Brucella* cells to a higher temperature (Fig. 3B and C). The expression of about 70 spots increased after exposure to increased temperature. The major proteins expressed during temperature upshift in *Brucella* cells fall into groups with approximate molecular masses of about 20, 40, 60, and 70 kDa.

Protein synthesis and response of *B. abortus* to H_2O_2. The response of *B. abortus* to H_2O_2 was studied by challenging cells with 0.005% H_2O_2. Figure 2b indicates that although exposure to this level of H_2O_2 is lethal to *B. abortus*, the killing occurs slowly: 40% of the cells survived after 6 h. Comparison of the two-dimensional protein profiles of *B. abortus* in the presence and absence of H_2O_2 indicated that the expression of about 101 proteins was repressed. Out of these 101 proteins, 19 showed a decrease in their expression level after 20 and 60 min of exposure to H_2O_2, and the rest of these proteins were completely repressed in the presence of 0.005% H_2O_2 (Fig. 4A). This comparison has also revealed that 21 spots were detected only in response to H_2O_2. The molecular masses of the major proteins that are expressed only in response to H_2O_2 fall in the range of 15, 20, 30, 40, and 70 kDa (Fig. 4B and C). In addition, the expression of 16 spots increased in *B. abortus* in the presence of H_2O_2. The molecular masses of these proteins are between 10 and 45 kDa (Fig. 4B and C).

Protein synthesis and response of *B. abortus* to menadione. Various concentrations of menadione were used to determine the effect of superoxide (19) on the growth of *B. abortus*. Our results indicated that high (50 to 150 mM) concentrations of menadione had immediate effects on *B. abortus*. These concentrations were able to decrease the percentage of survival to approximately 10% within 6 h. In contrast, more than 50% of the cells survived the presence of 10 mM menadione for 6 h (Fig. 2c). The protein synthesis patterns of *B. abortus* in the presence of 25, 50, and 75 mM menadione were investigated. Menadione concentrations of 25 mM and above resulted in very little labeling of proteins. Therefore, a 10 mM concentration of menadione was used. Comparison of the two-dimensional protein synthesis patterns of *B. abortus* in the presence and absence of 10 mM menadione indicated that a large num-



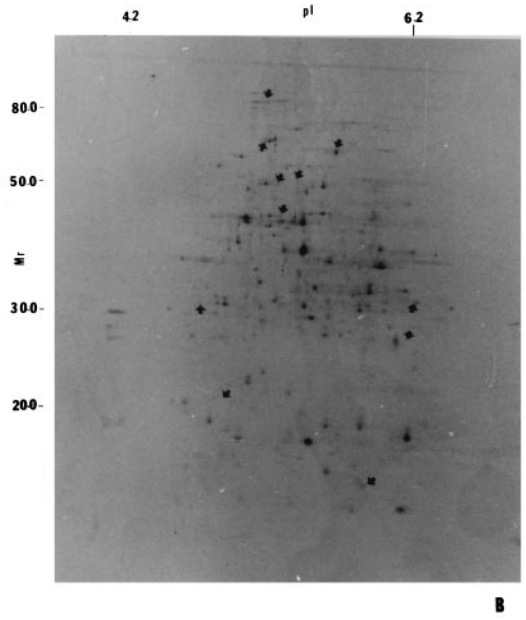
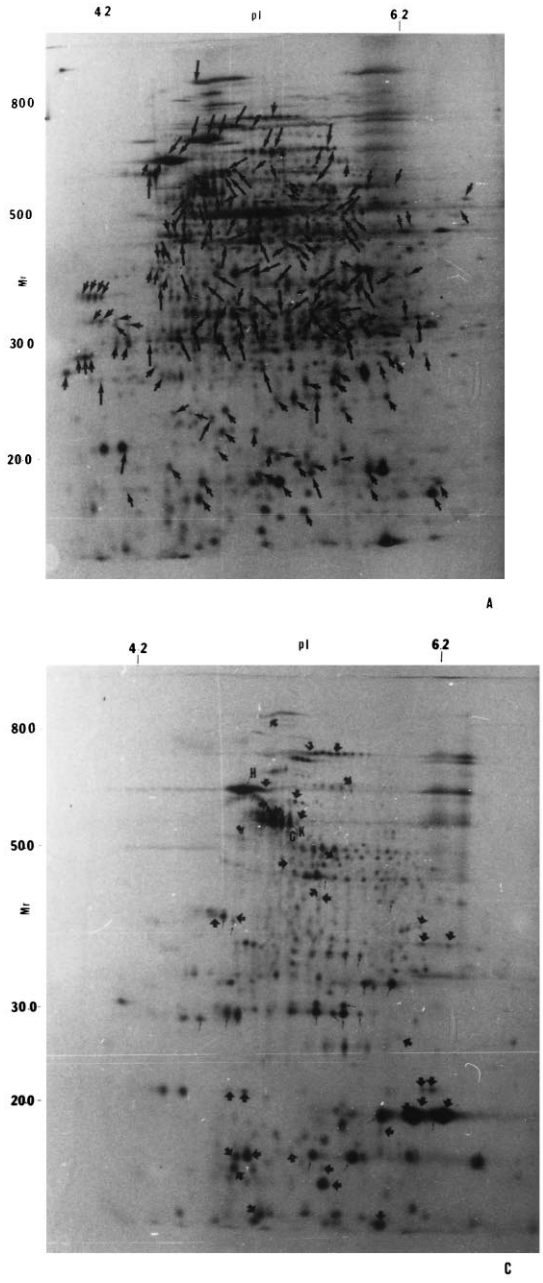


FIG. 5. Two-dimensional protein synthesis pattern of *B. abortus* in the presence of 10 mM menadione. (A) *B. abortus* labeled in saline in the absence of menadione for 60 min at 37°C. Proteins whose expression has decreased or has been repressed in response to menadione after 20 or 60 min have been marked with → and ➤, respectively. (B and C) *B. abortus* labeled in the presence of 10 mM menadione at 37°C for 20 and 60 min, respectively. Proteins with induced or increased expression in response to menadione have been marked with ➧ and →, respectively. Proteins whose expression has increased or has been induced both during intracellular survival and in the presence of menadione have been marked with letters.

ber of spots disappeared in response to menadione. In contrast, 17 new spots and 10 spots with increased expression could be detected in response to menadione (Fig. 5B and C). The molecular masses of most of the proteins induced in response to menadione were above 35 kDa.

Protein synthesis and response of *B. abortus* to reduced pH. In order to determine the physiological response of *B. abortus* to reduced pH, survival of bacteria at pH 5.5 and 4.5 in minimal medium was monitored for several hours. Citrate was added to the minimal medium for buffering. Figure 2d indicates that at pH 4.5, *B. abortus* rapidly loses viability, but at pH 5.5, viability is still 40% after 8 h. The protein synthesis pattern of *B. abortus* was determined in response to growth at pH 5.5 and was compared with that obtained at pH 7.0 by two-dimensional PAGE. This comparison indicated that there were 48 new spots that were only expressed after exposure of *B. abortus* to pH 5.5. In addition, expression of 30 spots increased in response to reduced pH in *B. abortus* (Fig. 6B and C). The molecular masses of the major proteins induced in response to reduced pH were in the range of 15 to 20 and 60 kDa. Finally, expression of more than 100 spots was found to be repressed in *B. abortus* as a response to pH 5.5 (Fig. 6A).

Protein synthesis and response of *B. abortus* to nutritional stress. The response of *B. abortus* to nutritional stress was studied by transferring cells from tryptose plates to RPMI 1640 medium containing 15% serum, saline, minimal medium (17), and tryptose medium. The response of the cells was examined by determining the number of viable cells at various time points. Figure 7 indicates that transfer of the cells to each medium is followed by an initial drop in the number of viable cells, a recovery phase, and a log period in the case of RPMI medium and tryptose medium. With these two media, the number of viable cells starts to increase after 1.5 h of exposure to each medium. Exposure to minimal medium resulted in a very slow increase in the number of viable cells. Finally, transfer to saline was followed by a slow decrease in the number of viable cells, which continued for 5 h. To determine the protein synthesis pattern during nutritional deprivation, *B. abortus* was labeled with ^{35}S protein labeling mix after a shift from tryptose medium to RPMI 1640 medium containing 15% serum, minimal medium, or saline. Comparison of two-dimensional profiles of the protein synthesis pattern of cells exposed to RPMI 1640 medium with those exposed to minimal medium or saline indicated that 14 new spots were induced when *B. abortus* was labeled in minimal medium (Fig. 8B), with one major protein with a molecular mass of 34 kDa. Eight new spots were induced when *B. abortus* was labeled in saline (Fig. 8C). The molecular masses of major proteins were about 67, 51, 42, and 14 kDa. In contrast, over 70 new spots were detected when *B.*



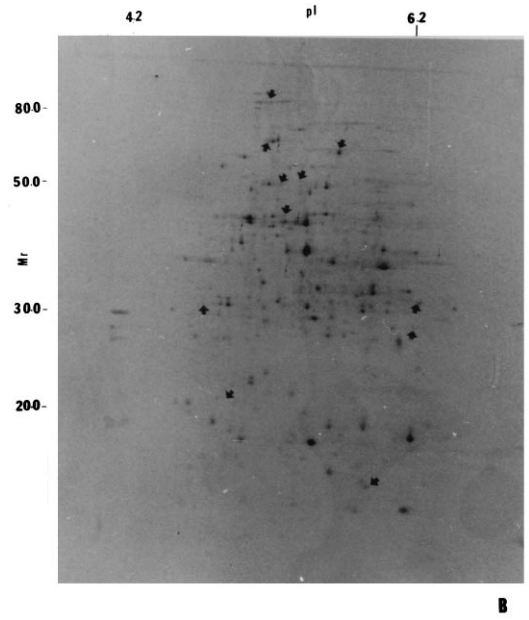


FIG. 6. Two-dimensional protein synthesis pattern of *B. abortus* in response to reduced pH. (A) *B. abortus* labeled in minimal medium containing citrate at pH 7.0 for 60 min at 37°C. Proteins whose expression has decreased or has been repressed after 20 or 60 min of survival at reduced pH have been marked with → and ◗, respectively. (B and C) *B. abortus* labeled in minimal medium at pH 5.5 for 20 and 60 min at 37°C, respectively. Proteins whose expression has increased or has been induced in response to low pH have been marked with → and ◗, respectively. Proteins whose expression has increased or has been induced during intracellular survival and survival at reduced pH have been designated with letters.

abortus was labeled in RPMI 1640 medium (Fig. 8A), but not when *B. abortus* was labeled in minimal medium or saline.

Western blot analysis. Western blot analysis was used to find out whether DnaK, GroEL, SodC, and HtrA were among the proteins induced during intracellular survival and other stress conditions. Several proteins with the same molecular mass as DnaK and GroEL cross-reacted with the antibodies specific for these proteins. The protein spots with the strongest reaction with the antibodies were accepted as the actual DnaK and GroEL. These studies have indicated that DnaK and GroEL were induced during intracellular survival (Table 1 and Fig. 1). In contrast, the expression of SodC and HtrA was repressed during intracellular growth. Table 1 indicates whether the expression of any of these proteins is induced under the various stress conditions used in this study. DnaK, GroEL, and HtrA were induced during heat and reduced pH. GroEL was induced in response to nutritional stress. SodC was induced in response to H_2O_2 and reduced pH but not in response to menadione.

Comparison of intracellular protein synthesis pattern with that of various stress conditions. In order to characterize the response of *B. abortus* to intracellular survival, the protein synthesis pattern during intracellular growth was compared with those of various stress conditions that were thought to resemble the intracellular conditions. A summary of these com-

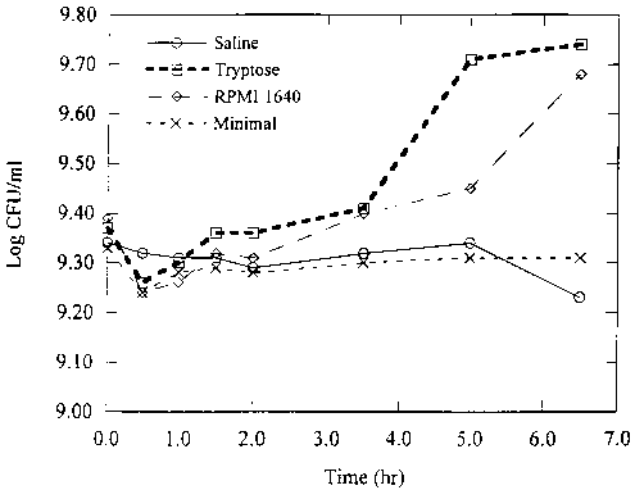
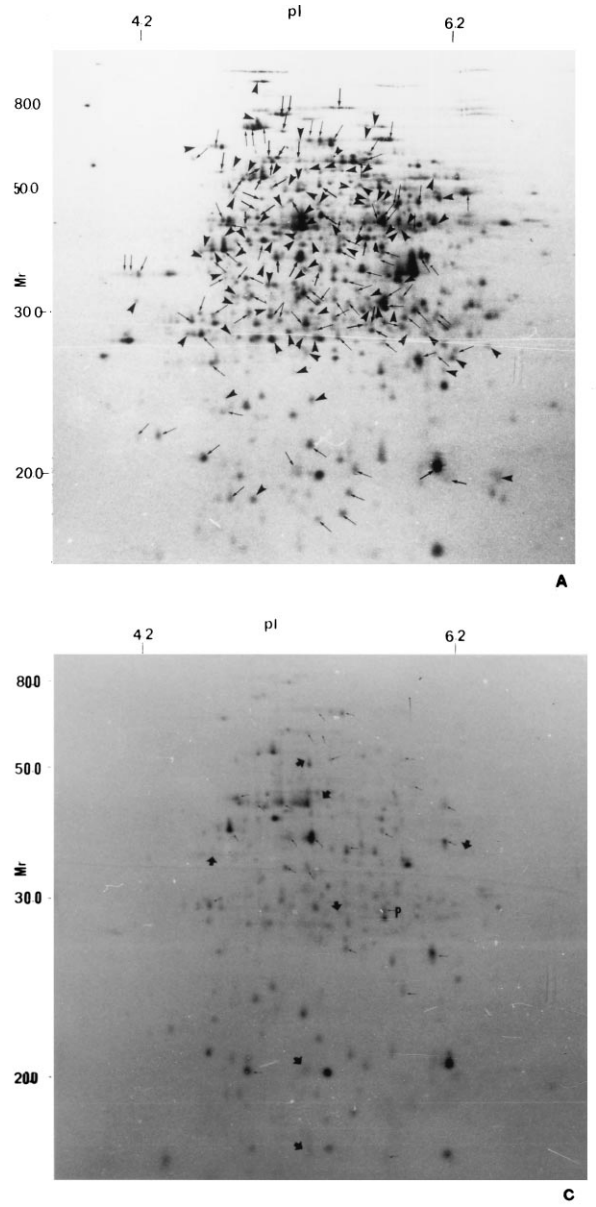


FIG. 7. Survival of *B. abortus* in various media. Minimal, minimal medium.



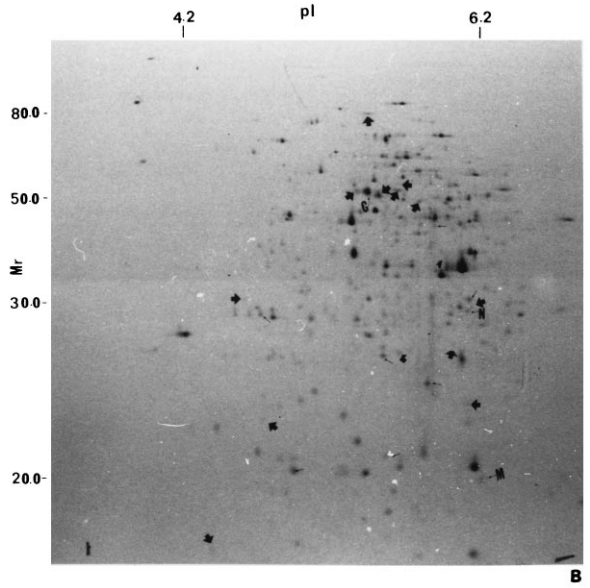


FIG. 8. Two-dimensional profile of *B. abortus* protein synthesis in response to nutritional stress. (A) *B. abortus* labeled in RPMI 1640 medium containing 15% serum at 37°C. Proteins whose expression has decreased or repressed have been marked with → and ➤, respectively, (B) *B. abortus* labeled in minimal medium (pH 7.0) at 37°C. Proteins whose expression has been induced or increased in minimal medium in comparison with RPMI 1640 have been marked with ◗ and →, respectively. (C) *B. abortus* labeled in saline at 37°C. Proteins whose expression has been induced or increased in saline but not in RPMI medium have been marked with ◗ and →, respectively. Proteins whose expression has been induced or increased during intracellular survival and nutritional stress have been marked with letters.

parisons is presented in Table 2. The comparisons indicated that there are very few proteins induced both during intracellular survival and during any of the specific environmental conditions studied.

DISCUSSION

Study of the intracellular survival of *B. abortus* suggested that this organism achieves survival within macrophages through a major change in its protein synthesis and gene expression. These findings are in agreement with those shown by Lin and Ficht (24), who have shown, by SDS-PAGE, that the pattern of gene expression of *B. abortus* changes during intracellular survival in macrophage-like cells. However, our studies extend these results by using a comparison of the two-dimensional protein synthesis pattern of *B. abortus* grown in tissue culture medium with that of cells grown in bovine macrophages. Our results, like those obtained by Lin and Ficht (24), indicate that there is an increase in the expression of GroEL and DnaK during intracellular survival.

A second unique aspect of our studies is the comparison of the protein synthesis pattern of *B. abortus* during survival in macrophages with the patterns induced under other environmental conditions. These conditions included reduced pH, oxidative stress, starvation, and heat. The first three conditions are thought to be those that *B. abortus* may have to cope with during intracellular survival. The shift to the higher temperature was chosen because the response is a well-characterized one. In addition, it is thought that heat shock proteins are involved in and are required for a successful adaptation by the intracellular pathogens because of the temperature differences between the environment and the host cells. This hypothesis is based on the observation that the level of expression of DnaK and GroEL of intracellular pathogens such as *Salmonella typhimurium* (7) and *Legionella pneumophila* (2) increases during growth in macrophages in vitro.

Comparison of the protein synthesis pattern of *B. abortus* during intracellular survival with those obtained under the different stress conditions used in these studies indicates that the response to intracellular growth is a unique response and is not simply the sum of the responses to various stress conditions. There is very little known about adhesion, entry, and survival of *B. abortus* in macrophages. Jiang et al. (20) have shown that macrophages infected with *B. abortus* are able to kill intracellular bacteria over the first 12 to 24 h after infection. Thereafter, surviving bacteria replicate, and the number of cells increases. It is possible that longer labeling of *B. abortus* during survival in macrophages would reveal induction of a larger number of proteins that are induced by other stress conditions. Prolonged labeling of *S. typhimurium*, which has a

TABLE 1. *B. abortus* stress proteins whose expression was altered by various stress conditions and macrophage infection

Stress condition	Expression of stress protein			
	SodC	DnaK	GroEL	HtrA
Intracellular growth				
60 min	Decrease	No change	Decrease	Decrease
120 min	Decrease	Increase	Increase	Decrease
H_2O_2				
20 min	No change	Decrease	Decrease	No change
60 min	Increase	Decrease	Decrease	No change
Menadione				
20 min	Decrease	Decrease	No change	No change
60 min	Decrease	Decrease	Decrease	No change
Starvation				
Saline	Decrease	Decrease	Decrease	Decrease
Minimal medium	Decrease	Decrease	Increase	Decrease
Heat				
20 min	No change	Increase	Increase	Increase
60 min	No change	No change	Increase	No change
Low pH				
20 min	Decrease	Decrease	Increase	Decrease
60 min	Increase	Increase	Increase	Increase

TABLE 2. *B. abortus* proteins induced by stress conditions and by growth within bovine macrophages

Stress condition	No. of proteins induced			Proteins induced under stress conditions and during intracellular growth
	Total	Short exposure to stress[a]	Long exposure to stress[b]	
Intracellular growth	39	26	17	
Heat	117	53	74	G, B, H, F, E
H_2O_2	35	18	19	A, B, C
Menadione	26	16	12	S, K
Reduced pH	68	17	63	G, K, H
Starvation	54	25	33	G, N, P, M

[a] For starvation stress, the number of proteins induced during short exposure corresponds to the number of proteins induced in *Brucella* cells in minimal medium.

[b] For starvation stress, the number of proteins induced during long exposure corresponds to the number of proteins induced in *Brucella* cells in saline.

similar survival pattern in macrophages (7), has revealed that the response to intracellular survival is more complex than a summation of various stress conditions (1).

In vivo studies of *htrA* null mutants indicated that HtrA protein contributes to the virulence of *B. abortus* (13). Western blot analysis and two-dimensional protein synthesis patterns of *Brucella* cells during intracellular survival indicated that HtrA is expressed both within macrophages and in the tissue culture medium in the absence of macrophages, although there is less expression in macrophages. Expression of HtrA during intracellular survival may indicate that this protein plays a role during survival within macrophages. Furthermore, Western blot analysis and comparison of two-dimensional protein profiles of *B. abortus* protein synthesis during intracellular survival and under stress conditions indicated that the expression of proteins of the same molecular masses as those stained with antibody specific for DnaK or GroEL were increased during intracellular survival and under all stress conditions except starvation. These proteins may represent different charge forms of DnaK and GroEL.

Foster and coworkers (15, 25) have shown that the response to low pH depends on the composition of the media. Cells survive reduced pH better in rich medium than in minimal medium. Furthermore, this group has shown that the presence of glutamate or arginine provides protection from acid shock in *E. coli* and *Shigella flexneri*. Our studies conducted with minimal medium (17) containing glutamate and the studies by Lin and Ficht conducted with RPMI 1640 medium (24) indicated that the survival response of *B. abortus* to reduced pH depends on the composition of the medium. Comparison of the SDS-PAGE protein synthesis pattern of *B. abortus* in RPMI 1640 medium under reduced pH with that at pH 7.3 has identified proteins with relative molecular masses of 60 and 20 kDa to be induced during acid shock (24). Proteins with similar molecular masses were induced in our comparisons, but at a higher pH, so the response may just be shifted by the medium.

The role played by superoxide dismutase (Sod) in protecting intracellular pathogens against the oxidative antimicrobial mechanism of phagocytic cells is unclear. Papp-Szabò and coworkers (28) indicated that a mutation in the *sodB* gene of *E. coli* did not change the resistance of these bacteria to the killing by human polymorphonuclear leukocytes. Furthermore, it has been shown that *sodA* mutants of *S. typhimurium* were only slightly attenuated in the mouse model of infection (33). In contrast, studies of survival of *katFG* and *sodB* mutants of *S. flexneri* have indicated that the protective mechanism used by this bacteria for survival within phagocytic cells involves Sod, with catalase activity participating to a lesser extent (16). In vivo studies of Cu-Zn Sod null mutants of *B. abortus* have shown conflicting results. One study reported that SodC mutants are fully virulent and are able to colonize the spleens of BALB/c mice (22). Studies conducted by a second group indicated that SodC plays a role in survival and pathogenicity of *B. abortus* during splenic infection of BALB/c mice (32). Therefore, SodC expression may not increase in *B. abortus* because the oxidative antimicrobial mechanism used by macrophages may not play an important role in the killing of these bacteria. Canning et al. (8) have identified GMP and adenine synthesized by *Brucella* cells during intracellular survival as inhibitors of the oxidative antimicrobial mechanism of bovine polymorphonuclear leukocytes by inhibition of the myeloperoxidase-H_2O_2-halide system. Jiang et al. (20) have shown that enhancement of oxidative killing by the addition of electron carriers would increase the initial killing of intracellular *B. abortus*, indicating the susceptibility of these bacteria to reactive oxygen intermediates. Bounous et al. (6) have observed that *B. abortus* cells opsonized with sera containing complement are more readily phagocytosed by bovine macrophages than are *B. abortus* cells opsonized with sera lacking complement. Thus, *B. abortus* cells, like *Legionella* (29), *Mycobacterium* (31), and *Salmonella* (33) cells, may enter phagocytic cells via complement receptors, a mechanism which is thought not to trigger the release of oxygen intermediates by macrophages and thus would allow the pathogen to avoid the antimicrobial effects of the oxidative burst.

The primary objective of this work was to determine the gene expression and protein synthesis of *B. abortus* in response to intracellular growth within macrophages and to characterize this response by comparison of the protein synthesis pattern with those obtained under defined environmental stress conditions. Our data represent the first comparison of the responses of *B. abortus* proteins on a whole-cell level to various

environmental conditions. We also provide a picture of the *B. abortus* response to an early stage of infection.

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REFERENCES

1. **Abshire, K. Z., and F. C. Neidhardt.** 1993. Analysis of proteins synthesized by *Salmonella typhimurium* during growth within a host macrophage. J. Bacteriol. **175:**3734–3743.
2. **Abu Kwaik, Y., B. I. Eisenstein, and N. C. Engleberg.** 1993. Phenotypic modulation of *Legionella pneumophila* upon infection of macrophages. Infect. Immun. **61:**1320–1329.
3. **Ames, G. F., and K. Nikaido.** 1976. Two-dimensional gel electrophoresis of membrane proteins. Biochemistry **15:**616–623.
4. **Bendixen, P. H.** 1981. Reversible detachment of blood derived bovine macrophages by replacement of culture medium with phosphate-buffered saline solution. Am. J. Vet. Res. **42:**687–688.
5. **Birmingham, J. R., and E. L. Jeska.** 1980. The isolation, long-term cultivation and characterization of bovine peripheral blood monocytes. Immunology **41:**807–814.
6. **Bounous, D. I., F. M. Enright, K. A. Gossett, and C. M. Berry.** 1993. Phagocytosis, killing, and oxidant production by bovine monocyte-derived macrophages upon exposure to *Brucella abortus* strain 2308. Vet. Immunol. Immunopathol. **37:**243–256.
7. **Buchmeier, N. A., and F. Heffron.** 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science **248:**730–732.
8. **Canning, P. C., J. A. Roth, and B. L. Deyoe.** 1986. Release of 5′-guanosine monophosphate and adenine by *Brucella abortus* and their role in the intracellular survival of the bacteria. J. Infect. Dis. **154:**464–470.
9. **Canning, P. C., J. A. Roth, L. B. Tabatabai, and B. L. Deyoe.** 1985. Isolation of components of *Brucella abortus* responsible for inhibition of function of bovine neutrophils. J. Infect. Dis. **152:**913–921.
10. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41:**753–762.
11. **Coleman, R. M., M. F. Lombard, and R. E. Sicard.** 1989. Effectors of nonspecific immunity. *In* C. H. Wheatley (ed.), Fundamental immunology, 2nd ed. W. C. Brown Publishers, Dubuque, Iowa.
12. **Corbeil, L. B., K. Blau, T. J. Inzana, K. H. Nielsen, R. H. Jacobson, R. R. Corbeil, and A. J. Winter.** 1988. Killing of *Brucella abortus* by bovine serum. Infect. Immun. **56:**3251–3261.
13. **Elzer, P. H., R. W. Phillips, M. E. Kovach, K. M. Peterson, and R. M. Roop II.** 1994. Characterization and genetic complementation of a *Brucella abortus* high-temperature-requirement A (*htrA*) deletion mutant. Infect. Immun. **62:**4135–4139.
14. **FitzGeorge, R. B., J. Keppie, and H. Smith.** 1965. The relation between resistance to hydrogen peroxide and virulence in brucellae. J. Pathol. Bacteriol. **89:**745–746.
15. **Foster, J. W.** 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J. Bacteriol. **173:**6896–6902.
16. **Franzon, V. L., J. Arondel, and P. J. Sansonetti.** 1990. Contribution of superoxide dismutase and catalase activities to *Shigella flexneri* pathogenesis. Infect. Immun. **58:**529–535.
17. **Gerhardt, P., and J. B. Wilson.** 1948. The nutrition of brucellae: growth in simple chemically defined media. J. Bacteriol. **56:**17–24.
18. **Goddeeris, B. M., C. L. Baldwin, O. Ole-Moi Yoi, and W. I. Morrison.** 1986. Improved methods for purification and depletion of monocytes from bovine peripheral blood mononuclear cells. J. Immunol. Methods **89:**165–173.
19. **Greenberg, J. T., and B. Demple.** 1989. A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. J. Bacteriol. **171:**3933–3939.
20. **Jiang, X., B. Leonard, R. Benson, and C. L. Baldwin.** 1993. Macrophage control of *Brucella abortus*: role of reactive oxygen intermediates and nitric oxide. Cell. Immunol. **151:**309–319.
21. **Kreutzer, D. L., L. A. Dreyfus, and D. C. Robertson.** 1979. Interaction of polymorphonuclear leukocytes with smooth and rough strains of *Brucella abortus*. Infect. Immun. **23:**737–742.
22. **Latimer, E., J. Simmers, N. Sriranganathan, R. M. Roop II, G. G. Schurig, and S. M. Boyle.** 1992. *Brucella abortus* deficient in copper/zinc superoxide dismutase is virulent in BALB/c mice. Microb. Pathog. **12:**105–113.
23. **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 GroEL heat shock proteins. Infect. Immun. **60:**2425–2431.
24. **Lin, J., and T. A. Ficht.** 1995. Protein synthesis in *Brucella abortus* induced during macrophage infection. Infect. Immun. **63:**1409–1414.
25. **Lin, J., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster.** 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. J. Bacteriol. **177:**4097–4104.
26. **Moulder, J. W.** 1985. Comparative biology of intracellular parasitism. Microbiol. Rev. **49:**298–337.
27. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
28. **Papp-Szabò, E., L. Sutherland, and P. D. Josephy.** 1993. Superoxide dismutase and the resistance of *Escherichia coli* to phagocytic killing by human neutrophils. Infect. Immun. **61:**1442–1446.
29. **Payne, N. R., and M. A. Horwitz.** 1987. Phagocytosis of *Legionella pneumophila* is mediated by human monocytes complement receptors. J. Exp. Med. **166:**1377–1389.
30. **Riley, L. K., and D. C. Robertson.** 1984. Ingestion and intracellular survival of *Brucella abortus* in human and bovine polymorphonuclear leukocytes. Infect. Immun. **46:**224–230.
31. **Schlesinger, L. S., and M. A. Horwitz.** 1991. Phagocytosis of *Mycobacterium leprae* by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) and IFN-γ activation inhibits complement receptor function and phagocytosis of this bacterium. J. Immunol. **147:**1983–1994.
32. **Tatum, F. M., P. G. Detilleux, J. M. Sacks, and S. M. Halling.** 1992. Construction of Cu-Zn superoxide dismutase deletion mutants of *Brucella abortus*: analysis of survival in vitro in epithelial and phagocytic cells and in vivo in mice. Infect. Immun. **60:**2863–2869.
33. **Tsolis, R. M., A. J. Bäumler, and F. Heffron.** 1995. Role of *Salmonella typhimurium* Mn-superoxide dismutase (SodA) in protection against early killing by J774 macrophages. Infect. Immun. **63:**1739–1744.
34. **Yamamori, T., K. Ito, Y. Nakamura, and T. Yura.** 1978. Transient regulation of protein synthesis in *Escherichia coli* upon shift-up of growth temperature. J. Bacteriol. **134:**1133–1140.

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